European Cooperation in the Field of Scientific and Technical Research

COST 843 ACTION:
QUALITY ENHANCEMENT OF PLANT PRODUCTION THROUGH TISSUE CULTURE

COST 851 ACTION:
GAMETIC CELLS AND MOLECULAR BREEDING FOR CROPS IMPROVEMENT

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The generation of genetic novelties for the improvement of diet, feed, timber and ornamentals in Europe requires the availability of strategies for the control and modulation of the creation of plants with an improved physiology, metabolism and productivity. In this respect, our COST Action 843 has addressed several *in vitro* approaches to achieve these aims.

With the recent advent in times of new knowledge on the proteomics, genomics and molecular biology of plants in general, and added to the relentless advances in regeneration of plants *in vitro* for an ever wider range of species, the analysis of the produced plants and their eventual modifications as compared to mother plant genotypes now appears feasible. This will help us understand the difficulties that still exist for the reliable production of *in vitro* plants for some species and will provide new insight on how to resolve them.

In this final meeting of our Action, when we are also joined by some participants from Action 851, the impressive and wide array of research results to be presented gives a clear indication of the state-of-the-art in the domain of plant biotechnology in Europe at present. Indeed, around 100 colleagues are convening on this occasion and nearly 130 articles are going to be presented, including over 50 oral presentations and around 80 posters. We are also honoured by the participation of six distinguished keynote lecturers who have agreed to share their knowledge with us.

The COST office (Drs. Bouktje Stol and Christophe Peeters) and ESF have been instrumental for this meeting and we also gratefully acknowledge their constant support, not only for the previous meetings of the three working groups, but also for the funding of a rather large number of Short Term Scientific Missions from which many members of our Action have benefited.

We are also indebted to the local organizers of this meeting, Dr. Alena Gajdosova and her team, who have invested lots of time and efforts in making of this a successful event.

Our Cost Action 843 now draws to its end and, as chairman, I would like to thank you all for your participation and for our mutual scientific enrichment over these last few years. More importantly, aside from new and old friendships made, initiatives like this give us also the opportunity to tie links which have (and will) crystallized in new joint research endeavours.

So, I bid you farewell (maybe until a future Action for which a new proposal is presently being written up by several of us) with my best regards to you all.

Sergio J. Ochatt
COST Action 843, chairman

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SESSION I:
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GENERATION AND REGENERATION: PLANT BIOLOGY AND THE ORIGIN OF LIFE

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Introduction

The theory of panspermia suggests that life can travel through space. To survive space travel, life requires encapsulation in a vehicle as well as a source of propulsion. Proof of transmission of life through space is evident in Man's recent explorations of the solar system. We propose considering plant seeds as terrestrial models for a vectored life form. They resist deleterious conditions found in space: ultra low vacuum, extreme temperatures and intense UV light, which is largely due to flavonoids in the seed coat that are uniquely suited to protecting DNA in the UVB, UVC and VUV.

Plant tissue culture shows us that plant cells can survive and replicate in a sterile medium, and that in many cases whole plants can regenerate from isolated cells or tissues. In an axenic, prebiotic environment, seeds could liberate a viable embryo, viable higher cells or a viable free-living organism (*e.g.*, an endosymbiont or endophyte). Even if the viability of the cells and organelles carried by seeds were completely lost, seeds could still liberate functional macro and small molecules, *e.g.* DNA, RNA, proteins, amino acids, lipids, etc. that could provide the chemical basis for life. The possible release of viable eucaryotic-like cells implies that the three domains of life, defined in DNA sequence phylogenies, could have been imported simultaneously.

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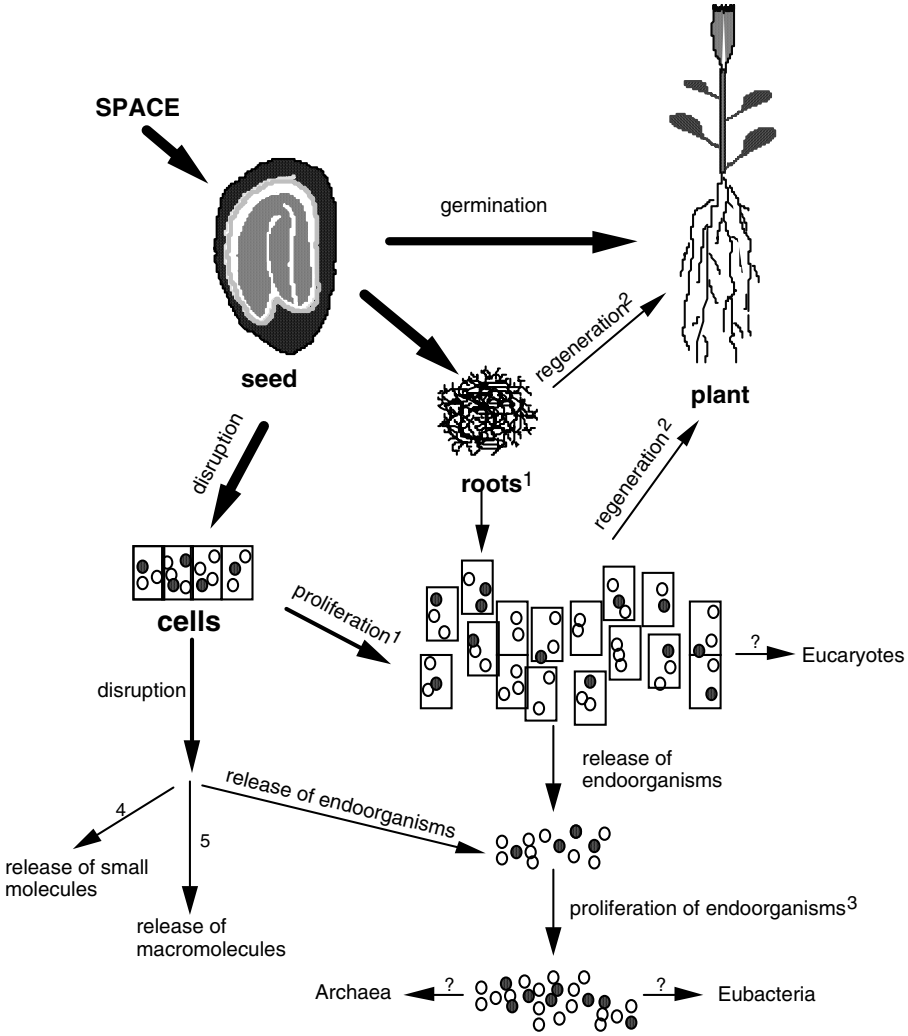

Figure 1

Degrees of survival

[1] Some plant cells and organs (*e.g.*, roots) grow autonomously in chemically defined medium consisting of minerals, vitamins and a carbon source, which can be photosynthesis.

[2]Many plant cells and roots can regenerate a whole plant.

[3]Eucaryotic cells within seeds contain endosymbionts (mitochondria and chloroplasts), ancient bacteria no longer capable of living free of the host cell. Seeds also sometimes contain endophytic bacteria and fungi, capable of living outside the host. They will be released upon disruption of the seed.

[4]Disruption of cells will release small molecules: e.g., nucleotides, amino acids, lipids, co-factors, etc. that could provide the chemical basis for life.

[5]Cell breakage will also release macromolecules: *e.g.* DNA, RNA, proteins (including enzymes and enzyme complexes with nucleic acids), complex polymers (including cellulose and lignin), as well as membranes, cell walls, ribosomes, chromatin, viruses, viroids, etc. These could provide the structural basis for a reorganization of life.

APPLICATIONS OF CRYOPRESERVATION IN BANANA

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Introduction

Bananas and plantains (*Musa* spp), with an annual production of about 100 million tons, are important for developing countries of the tropics and subtropics both as a subsistence and export crop. Banana plantations are extremely vulnerable to pests and diseases due to clonal propagation and monocropping. Besides biotic stresses, bananas are also in need for improvement for tolerance to wind, salinity, cold and water stress, improved yield and shelf life and fruit quality. Germplasm collections can provide a source of such 'superior' varieties. Moreover, the availability of a wide variety of germplasm is essential for classical and modern breeding. Cryopreservation or freeze-preservation at ultra-low temperatures (-196 °C) is the method of choice for the long term preservation of the banana biodiversity since at these conditions biochemical and most physical processes are arrested. As such, plant material can be stored for unlimited periods. The availability of embryogenic cell suspensions of banana is an essential requirement for genetic engineering of *Musa* spp. (Sagi *et al*., 1995). However, the initiation of transformation competent banana embryogenic suspensions is still a difficult and time-consuming endeavour. Their safe, long-term storage through cryopreservation is therefore also recommended. Recently, we observed that cryopreservation is not only applicable as a conservation tool for banana tissues but can also be applied to preserve banana nematode cultures and to eradicate plant viruses such as CMV and BSV (Helliot *et al*., 2002).

Materials and methods

Cryopreservation of meristem cultures

Three cryopreservation protocols are available for shoot-meristematic tissues of banana; (i) simple freezing of proliferating meristem culture using a sucrose preculture (Panis *et al*., 1996) (ii) vitrification of apical meristems (Panis *et al*., 2005) and (iii) vitrification of sucrose precultured meristem cultures (Panis and Thinh, 2001).

Cryopreservation of embryogenic cell suspensions

Embryogenic cell suspensions are cryopreserved using a classical freezing protocol that involves slow freezing (1 °C.min^{-1}) in the presence of 7.5% dimethylsulfoxide (DMSO) and 140 g.l^{-1} sucrose (Panis and Thinh, 2001).

Virus eradication

From the CMV and BSV infected banana material, meristem clumps were prepared and cryopreserved using vitrification of sucrose precultured meristem cultures (Panis and Thinh, 2001). Virus detection was performed by Enzyme Linked ImmunoSorbent Assay (ELISA) with the Loewe kit (Biochemica GmbH) (for CMV) and TAS-ELISA (for BSV) (Helliot *et al*., 2002).

Results and discussion

Cryopreservation of meristem cultures

Two methods use highly proliferating meristem clusters that are pre-cultured on sucrose (0.4 M) enriched medium during 2 weeks. Groups of pre-cultured meristem clumps are either directly frozen (simple freezing method), or subjected to a vitrification treatment

(vitrification method) prior to freezing. It was observed that post-cryopreservation viability rates for the simple freezing method depend on the genotype and vary from 0% for *Musa acuminata* and Ensete over 19-25% for AAA, AAB dessert bananas and AAB plantains to 53% for ABB cooking bananas. Viability rates for the vitrification method appeared to be relatively high compared to those of the first method. Post-thaw recovery rates for ABB clones remains between 50% whereas increased recovery growth rates are obtained for AAB dessert bananas, AAB plantains and AAA dessert bananas (41-51%). Viability rates for Ensete, AAA-highland bananas and *Musa acuminata* accessions, however remain very low (0-30%). The third method involves vitrification of tiny individual meristems. When this protocol was applied to 56 accessions belonging to 8 different genomic groups of *Musa* spp. and one *Ensete* spp., an average of 52.9% post-thaw regeneration was obtained. These results were relatively genotype independent. Only wild diploid *Musa acuminata* accessions proved to be somewhat more recalcitrant towards cryopreservation though an acceptable average regeneration rate of 39% was still obtained (Panis *et al*., 2005), Figure1. This method, although more than 2 times more laborious compared to the previous ones, offers a good alternative for those cultivars responding unfavorably towards the freezing of highly proliferating meristem cluster. The combination of the three techniques is now being used on a routine basis to cryopreserve the whole collection and resulted in the safe storage of 330 banana accessions belonging to the various genomic groups so far.

Cryopreservation of embryogenic cell suspensions

The total number of 2 ml cryotubes stored in liquid nitrogen containing transformation competent banana cell lines is 2140. These represent 13 cultivars and 96 independent cell lines (Table 1). After cryopreservation, suspensions need to be (i) viable, (ii) able to produce high quality cell suspensions, (iii) true-to-type and (iv) transformation competent. We have proven that all these requirements are met for the stored suspensions (Panis *et al*., 2004).

Virus eradication

The virus eradication frequency for CMV and BSV was 30% and 90% respectively after cryopreservation. To understand the mode of action of cryopreservation for the eradication of viral particles, we examined the structure of meristem-tips by light microscopy. We demonstrated that the cryopreservation method used only allowed survival of small areas of cells in the meristematic dome and at the base of the primordia.

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Figure 1 Average post-thaw regeneration rates of banana accessions grouped according their genomic constitution after the application of three different cryopreservation protocols

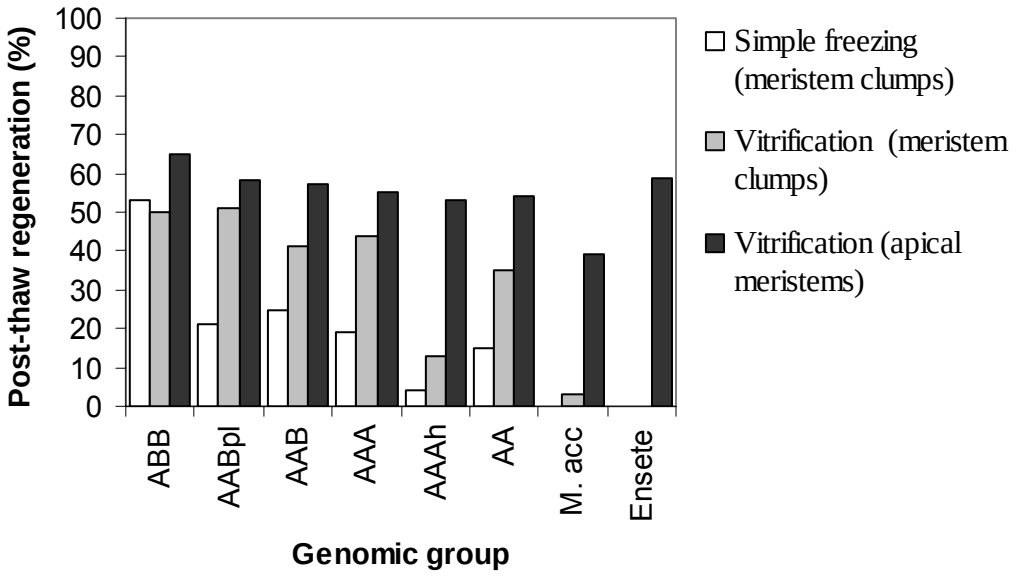

Table 1 Independent cell lines stored in liquid nitrogen at the Laboratory for Tropical Crop Improvement, Leuven, Belgium

Banana cultivar	Genomic group	Number of independent cell lines stored in LN
Agbagba	AAB plantain	4
Three Hand Planty	AAB plantain	6
Orishele	AAB plantain	10
Obino L'Ewai	AAB plantain	1
Dominico	AAB plantain	2
Bisé Egomé	AAB plantain	2
Bluggoe	ABB	6
Cacambou	ABB	16
Cachaco	ABB	5
Dole	ABB	8
Grande Naine	AAA	11
Gros Michel	AAA	1
Williams	AAA	24

ANALYSIS OF REGENERATION CAPABILITIES IN FRUIT TREE SPECIES AS A FUNCTION OF THE EXPLANT ORIGIN

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Adventitious budding of fruit trees cultivated *in vitro* is a tissue culture technology that started about 20 years ago. Except a few very reactive plant model, low regeneration efficiency and recalcitrance remain limiting factors when genetic transformation and/or somaclonal variation are concerned.
In the frame of the creation of new fruit tree genotypes, the CRA-W Department Biotechnology investigated for a better knowledge of the plant origin and a better regulation of the regeneration procedures.
The multiapexing technique firstly set up from apple meristems (Druart, 1997) has been successfully extended to pear, cherry rootstocks, plums and peach as well. Leaflets from such regenerants were so reactive that buds appear spontaneously from epidermal and sub-epidermal cells with M26 apple rootstock or after a few days of subculture with other apple cultivars. The use of apple cytochimaeras as plant material (Druart, 2004) led us to some considerations; as well expected as not expected. Adventitious shoots and roots are originally issued from several cells. Different cell layers would be involved in the regeneration processes mainly according to the cytokinin.
Roots, mainly of *Prunus* species, regenerate buds through suckering. *P. incisa x serrula* is one of the only species that also form somatic embryos from different parts of the roots including the apical cells of secondary roots. The inheritance of such property has been confirmed (Druart and Laimer, 2003).
The interest of *in vitro* culture techniques for fruit tree improvement will be illustrated with the variability of the fruit colour induced in Jonagold and the fruiting of single aneuploid genotypes of apple.

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IN VITRO PROPAGATION OF *Fraxinus excelsior* L. BY EPICOTYLS

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Summary

Common ash (*Fraxinus excelsior* L.) cuttings are considered difficult to root and it has been found possible only for juvenile material. The seeds of the common ash generally present dormancy up to 6 years and it could be overcome by 32 weeks of stratification. *In vitro* propagation allows for the large-scale production of plants and may provide rejuvenated plants with a high rooting capacity.

Epycotyl segments harvested from *in vitro* obtained 42-days old seedlings were used as explants. For inducing of shoot formation were used Murashige and Skoog and Woody Plant media supplement with thidiazuron (TDZ, 0.1, 0.5 or 1.0 $mg.l^{-1}$), zeatin (2.0 or 5.0 $mg.l^{-1}$), or 6-Benzylamino purin (BAP 3.0 or 4.0 $mg.l^{-1}$). Shoot proliferation occurred on all media. Increasing of TDZ concentration promoted multiple shoot formation but shoots were short and often vitrified Addition of IBA (0.01 or 0.1 $mg.l^{-1}$) or GA_3 (1.0 $mg.l^{-1}$) promoted shoot elongation. On Woody Plant media (WPM) with BAP (3.0 or 4.0 $mg.l^{-1}$) and Indole-3-Butyric Acid (IBA 0.1 $mg.1^{-1}$) cultures showed a high multiplication rate and shoots were long and suitable for rooting.

The structure of normal and fasciated stems, that have developed (during the multiplication *in vitro*), was studied in semi-thin sections of paraffin-embedded samples by bright field, polarized light, and fluorescence microscopy. The vascular cylinder in fasciated stems was not circular but elliptical in cross sections and the volume of fasciated stems was considerably larger that that of normal stems. The vascular cylinder in normal stems was rounded in cross sections with small wedges of pith parenchyma and vascular cells that projected outwards into the cortex. Such wedges appeared to be points of intensive divisions of perimedullar parenchyma and cambial cells that might result in lateral growth and fasciation of the stem. Confirmation of this would require further observations. In addition, in our samples, large intercellular spaces were always seen in the cortex of normal stems but not in fasciated stems.

Adventitious root formation was studied on half strength WPM, supplemented with IBA (0.5 or 1.0 $mg.l^{-1}$), α-Naphthalene acetic acid (NAA 0.5 or 1.0 $mg.l^{-1}$) or in combination (0.5 $mg.l^{-1}$ IBA and 0.5 $mg.l^{-1}$ NAA). After 7, 14 or 21 days on the above inductive media the cultures were transferred on half strength, hormone-free WPM (expressive medium). The results were compared with half strength WPM without or with the above mentioned auxin concentrations on which the shoots were cultivated without transfer. The best percentage of rooting (96.67 ± 3.33 %) was achieved on media with 0.5 $mg.l^{-1}$ IBA and 0.5 $mg.l^{-1}$ NAA for 14 days.

ROLE OF ENDOGENOUS HORMONAL LEVELS IN ORGANOGENIC ABILITY OF ELM MULTIPLICATED SHOOTS

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Abstract

The aim of this work was to study the possibility to increase the elm explant multiplication rate by cutting the multiplicated shoots to apical and basal parts. The study presents the comparative analyses of endogenous levels of auxin (IAA), cytokinins (CKs), polyamines (PAs), and phenolic acids (PhAs) in both types of explants with regard to the regeneration of shoots and roots. The shoot forming capacity was higher in the apical part. However, the timing of root formation was in this type of explant significantly delayed (compared with the organogenic potential of basal part). Significantly higher contents of free bases, ribosides and ribotides of isopentenyl adenine, zeatin and dihydrozeatin that were found in the elm apical segments, might be considered as the most important factor affecting *in vitro* shoot formation. The content of endogenous free IAA was approximately three times higher in the basal shoot parts in comparison with the content determined in the apical parts. The amounts of putrescine and spermidine were higher in the apical part, which generally contains less differentiated tissues than the basal parts of shoots. The predominant phenolic acid in both types of explants was caffeic acid, and concentrations of other PhAs decreased in the following order: p-coumaric, ferulic, sinapic, vanillic, chlorogenic, p-hydroxybenzoic and gallic acids. The contents of all determined PhAs in their free forms were higher in the basal parts. With respect to the role of PhAs in auxin catabolism, the increased amounts of free caffeic, ferulic and chlorogenic acids should be emphasised. The higher contents of glycoside-bound p-coumaric, ferulic and sinapic acids, precursors for lignin biosynthesis, that were found in basal parts, might be prerequisite for restart of lignification which coincides with rooting induction.

Additional key words

Auxin; Cytokinins; Multiplication of elm; Phenolic substances; Polyamines

EFFECTS OF PACLOBUTRAZOL ON *Salvia greggii variegata IN VITRO*

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Introduction

Salvia greggii is a biennial plant originating from both Mexico and Texas and is commonly known as "Autumn Sage" (Kawahara *et al*., 2003). The variegated form, 'Desert Blaze', has attractive leaves and flowers over a long period. *Salvia* is required in large numbers by the nursery trade and plants produced must meet the market specifications. Micropropagation techniques are being developed to rapidly multiply and yield large numbers of commercially viable uniform plants of *Salvia greggii*, which retain their variegation. The objectives of this study were: 1) to investigate the use of micropropagation techniques for establishing viable shoot cultures *in vitro* and 2) to determine if paclobutrazol (PAC) treated plants propagated *in vitro* exhibit any dwarfing effects.

Materials and methods

Initiation. Shoots, from nursery stock plants were collected and sterilised by immersion in 7% calcium hypochlorite for 15 minutes, followed by three five minute washes in sterile water. Explants were cultured in vitro and maintained on MS modified medium (Murashige and Skoog, 1962 macronutrients and micronutrients) with B5 vitamins (Gamborg *et al*., 1968) to which: 30 $g.l^{-1}$ glucose, 3.3 $g.l^{-1}$ phytagel, 0.1 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ IBA were added. The pH was adjusted to 5.8 and medium was autoclaved in jars containing 30 ml aliquots for 20 minutes at 121 ºC and 15 lb/in². All cultures were maintained in a growth room under a 16h photoperiod regime at 22ºC under Philips cool white fluorescent tubes (58.6 µmol m^{-2} s^{-1} at bench level).

Paclobutrazol treatment *in vitro*

As micropropagated plants of *Salvia greggii* tend to grow tall with a resulting irregular canopy, the dwarfing effect of Paclobutrazol (PAC), an anti-gibberellin, on both shoot and internode length was tested on 2-node explants cultured on MS supplemented with PAC. The explants were examined under five different treatments: F1 = MS, F2 = MS+1.0 $mg.l^{-1}$ PAC, F3 = MS+2.0 $mg.l^{-1}$ PAC, F4 = MS+4.0 $mg.l^{-1}$ PAC, and F5 = MS+8.0 $mg.l^{-1}$ PAC for four weeks. The media composition and culture room conditions are the same as described above. Data was analysed with the statistical package, Statistica 6.0 using one-way ANOVA to determine differences in shoot and internode length and rooting rates under PAC concentrations.

Results and discussion

Salvia greggii variegata was easily established in the MS modified medium and an average of 7.1±1.35 explants were obtained per shoot per sub-culture period.

Elevated concentrations of PAC applied *in vitro* significantly reduced both shoot length ($p \leq 0.00670$) (Table 1) and internode length ($p \leq 0.00001$) (Fig. 1). Explants treated with high levels of PAC *in vitro* showed a significant difference in the number of nodes pro-

duced compared with that of the untreated controls ($p \leq 0.00656$) (Table 1). This correlates with the findings of Smith *et al.* (1990). High levels of PAC stimulated rooting ($p \leq 0.0000$), with 4 and 8 mg.l^{-1} PAC achieving 100% (Table 1). No loss in variegation was observed in plants treated with PAC. Results indicate that the application of PAC *in vitro* may have a role in the production of compact plants with a reduced shoot and internode length.

Figure 1 Effects of PAC on internode length of *Salvia greggii variegata in vitro*. PAC levels (F1-F5) as described in Materials and Methods

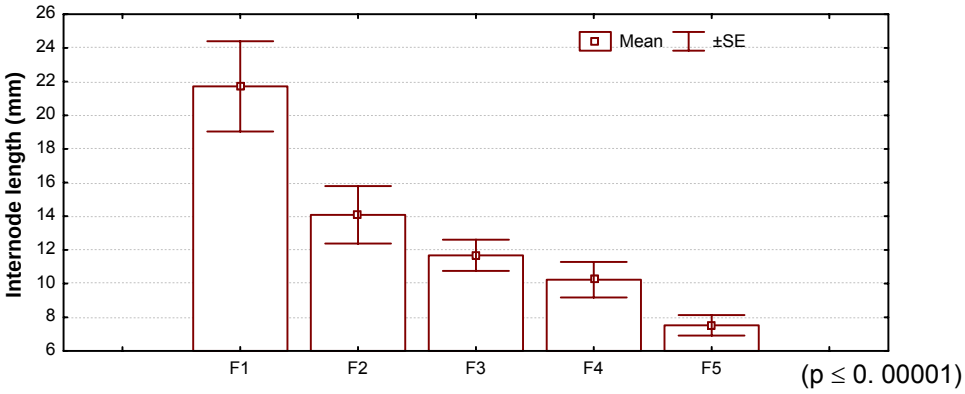

Table 1 Effects of PAC applied *in vitro* on shoot length, rooting and the number of nodes produced. Values in columns with different superscripts differ significantly

PGR	Shoot Length (mm)	No. Nodes per shoot	Mean No. of roots
0 mg.l^{-1} PAC	98.53±9.7^{a}	4.00±0.42^{a}	1.06±0.15^{a}
1 mg.l^{-1} PAC	76.06±6.7^{b}	4.08±0.51^{a}	1.47±0.25^{ab}
2 mg.l^{-1} PAC	75.56±9.5^{b}	5.33±0.44^{ab}	1.75±0.32^{bc}
4 mg.l^{-1} PAC	64.20±7.4^{b}	4.94±0.50^{ab}	5.03±0.03^{d}
8 mg.l^{-1} PAC	54.06±4.9^{b}	6.22±0.53^{b}	5.00±0.00^{d}

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EFFECT OF PLANT GROWTH REGULATORS ON ADVENTITIOUS ROOTS OF *Panax ginseng*

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Introduction

Panax ginseng is very important tonic medicinal plant, highly valuated for anti-stress, adaptogenic and geriatric properties. Biologically active compound - ginsenosides are extracted from roots. The alternative source of ginsenosides might be reached by mass cultivation in *in vitro* cultures. The *in vitro* synthesis of secondary compounds from plants is one source of scarce and valuable phytopharmaceuticals and offers unique advantages. Often, some level of cellular or tissue differentiation is needed for the biosyntheses of many of these important compounds (Ellis *et al*., 1996). We found differentiated tissue in *Panax ginseng* cultures very positive for ginsenoside production. Adventitious root cultures initiated from plantlets regenerated from somatic embryos, contrary to callus cultures (undifferentiated tissue), provide full range of ginsenosides distributed analogous as in roots of native plants (Langhansova *et al*., 2003). The aim of this study was to increase saponin production and biomass growth by stimulation the adventitious roots with different plant growth regulators and their combinations.

Materials and Methods

Initiation of adventitious root culture

Roots were isolated from plantlets regenerated from somatic embryos and cultivated separately in liquid media. Formation of adventitious roots was reached on liquid SH (medium according to Schenk and Hildebrandt, 1972) containing 3 % sucrose, 0.1 % myo-inositol and 24.6 µM IBA (Choi *et al*., 2000). Roots were cultivated in 500 ml Erlenmeyer flasks on rotatory shaker at 125 rpm in dark at 24 ± 1 °C.

Regulation of tissue differentiation by plant growth regulators

Adventitious roots of *Panax ginseng* were transverse cut to Thin Cell Layers (tTLCs) and placed on Petri dishes (Ø 90 mm, 10 explants in each). Five auxins (IBA, BSAA, 2,4-D, NAA and IAA) and four cytokinins (TDZ, Kin, 2iP and BAP) and all auxin/cytokinin combinations were applied. Plant growth regulators were added to basal SH medium. The concentration of auxin was 3 $mg.l^{-1}$, cytokinins 2iP and BAP was 0.2 $mg.l^{-1}$ and Kin and TDZ was 0.02 $mg.l^{-1}$ according to the activity. Callus formation was evaluated after 4 and root multiplication after 12 weeks of cultivation.

Cultivation in Temporary Immersion System

Adventitious roots were inoculated into a RITA® (Vitropic s.a., France) bioreactor. The inoculum was 2.50 g of fresh weight (0.28 g of dry weight) for 100 ml medium. Flooding was set to 5 min/1 hour Cultivation was held in the dark conditions at 24 ± 1 °C. Cultivation period was 8 weeks.

Conditions of HPLC analysis

HPLC analyses were performed on the system consisting of two high pressure pumps (DeltaChrom, SDS 020 a SDS 030) with a mixer (SunChrom GmbH) and PDA detector (JASCO, MD 1510); the stainless steel column (250 x 4 mm) packed by reverse phase Si-C18, 7 µm (Biospher); flow-rate 1 $ml.min^{-1}$. The injection volume was set up at 20 µl in the autosampler (TSP, AS300). Eluents: (A) 15 % acetonitrile and water, (B) 100 % acetonitrile; Gradient elution profile: 0 - 40 min, 0 - 35 % B; 40 - 45 min, 35 % B. The

peaks were monitored by UV detection at 203 nm. Ginsenoside content was expressed in $mg.g^{-1}$ of dry weight.

Results and discussion

We focused on effect of plant growth regulators and their combinations in order to regulate a level of tissue differentiation and to increase biomass production. Our result shoved that cytokinins Kinetin and 2iP induce root formation and multiplication but only in combination with auxins, namely BSAA, NAA and IBA. 2,4-D inhibits root formation and induces only callus formation. BSAA increase significantly biomass growth (see Figure 1).

In our previous work we tested different bioreactor systems to find optimal conditions for large-scale cultivation of ginseng adventitious roots. The best conditions for growth and ginsenoside production were provided by temporary immersion system RITA. Therefore we tested optimal combination of growth regulators in this system. Cultivation of adventitious roots in RITA bioreactor on media containing BSAA auxin showed best results comparing to IBA and NAA, mainly in high biomass growth (see Figure 2). In conclusion we found auxin benzo[b]selenienyl acetic acid (BSAA) to be most suitable for biomass production and following root multiplication particularly in combination with kinetin.

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Figure 1 Effect of plant growth regulators and their combinations on callus formation and root multiplication on tTLCs of ginseng adventitious roots

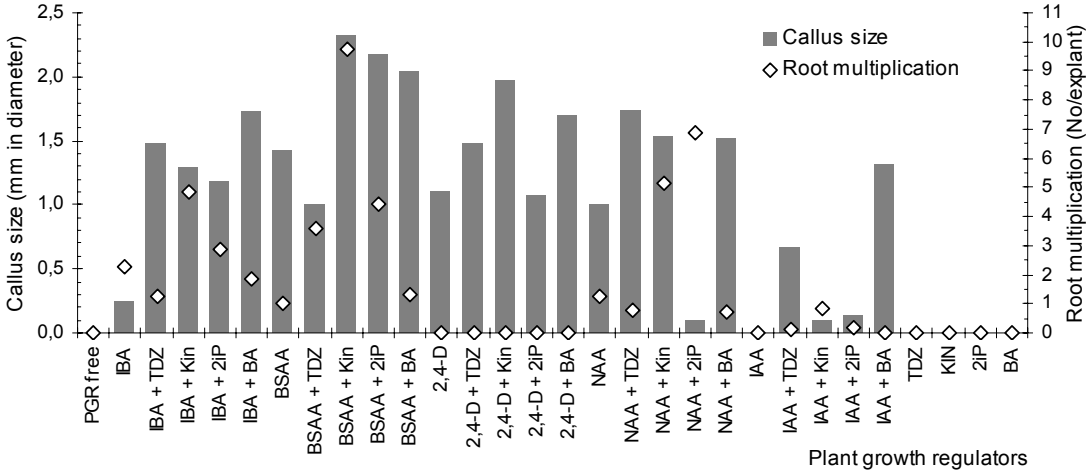

Figure 2 Effect of auxins on ginsenosides production of ginseng adventitious roots cultivated in RITA bioreactor

PGR	Inoculum	Growth value	Harwested biomass (g dw)	Saponin production (mg/g dw)	Total saponin yield (mg)
NAA + KIN		**2,1491**	3,1491	**12,1633**	**38,30**
BSAA + KIN	1	**5,6181**	6,6181	**15,9410**	**105,50**
IBA + KIN		**1,8978**	2,8978	**13,6540**	**39,57**

IN VITRO MORPHOGENIC RESPONSE TO CPPU IN KIWIFRUIT AND PEAR CULTIVARS AND RELATIONSHIP WITH THE PLOIDY LEVEL

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Introduction

In vitro techniques, and in particular those concerning regeneration of whole plants from adult tissues, permitting the alteration of few characters through exploitation of somaclonal variation or application of gene transfer, can accelerate the obtaining of improved genotypes in perennial fruit species. The availability of efficient adventitious shoot regeneration systems is related to various factors acting on the morphogenic response of the explants. Type and concentration of cytokinins play a critical role in the shoot regeneration induction. BA, TDZ and zeatine are the most common cytokinins used in the regeneration protocols for woody species and in particular for fruit trees such as *Pyrus communis*, *Actinidia deliciosa* (Caboni *et al*., 1999; Kumar, Sharma, 2002; and references within included).

The growth regulator N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) is widely used to increase yield and fruit size (Famiani *et al*., 1998; Cruz-Castillo *et al*., 1999), but there are only few reports concerning the *in vitro* morphogenic response in woody fruit trees (Kadota and Niimi, 2003; and references within included)

The aim of this work was to study the effect of various cytokinines, included CPPU, on shoot regeneration of *Actinidia chinensis*, *Actinidia deliciosa* and *Pyrus communis*. The relationship of citokinin induction effect with the ploidy levels of the genotypes was also evaluated in *Actinidia deliciosa.*

Materials and Methods

Materials: one genotype of *Actinidia chinensis*, Planch, genotypes of *Actinidia deliciosa* A. Chev. (female cultivar Hayward, male cultivar Tomuri and male clones with different level of ploidy) and some cultivars of *Pyrus communis* (William, "Decana del Comizio" and Conference) were used. *A. deliciosa* clones with different ploidy levels (diploid, triploid and tetraploid) have been obtained in the laboratories of "Dipartimento di Produzione Vegetale dell'Università di Viterbo".

In kiwifruit the leaf explants were prepared from *in vitro* grown shoots and cultured according to Caboni *et al*. (2003). The cytokinins tested were the following: 1) *Actinidia chinensis* genotype and A*ctinidia deliciosa* cultivars, were treated with benziladenine (BA), zeatin (ZEA) or N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) at two concentrations (1 = 10 μM; 2 = 16 μM). 2) The clones with different ploidy levels were treated with 16μM BA, ZEA or CPPU.

In pear the effect of various cytokinins was evaluated both in the multiplication phase (MP), on the proliferation of axillary buds, and on the induction of adventitious shoots (ASI) from adult explants. BA, KIN (kinetin), CPPU, 2iP (isopentenyladenin), TDZ (Thidiazuron) or ZEA, were used, 1.7 μM and 10μM, respectively for the MP and ASI. For the ASI the effect of the combination with 5μM IBA (indolebutiyric acid) or NAA (naphtalenacetic acid) was also evaluated. The other culture conditions have been previously reported (Caboni *et.al*., 1999).

Results and discussion

Kiwifruit*:* from the results reported in table 1, it is evident that BA gave the lowest adventitious shoot induction, while CPPU e ZEA highly promoted the morphogenic response in all the genotypes. The results concerning the clones with different ploidy are detailed in Table 2. Also in these clones the explants treated with CPPU and ZEA induced higher morphogenic ability than those treated with BA. The ploidy level affected the morphogenic response and this effect was strongly evident in the case of the BA treatment.

Pear: from the data reported in table 3 on axillary bud proliferation, it is evident that TDZ gave the highest multiplication rates. Unfortunately, most of the shoots were completely hyperidric. The best proliferation response, in terms of multiplication rates and quality of the explants, was obtained with BA in Conference and "Decana del Comizio" and with CPPU in William. CPPU induced adventitious shoot formation also in all the genotypes tested of this species. The highest regeneration responses with CPPU were obtained in combination with IBA. Conversely to the results reported on *P. pyrifolia* (Kadota and Niimi, 2003), CPPU neither induce hyperdricity in *P. communis* cultivars or in *Actinidia spp*.

Conclusions

The type of cytokinin highly affect the morphogenic response in these 2 species and the effect is strongly genotype dependent in pear. The results of CPPU application allow to propose a wider use of this growth regulator in tissue culture. The level of ploidy seems to influence the morphogenic response.

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Table 1 Adventitious shoot regeneration (%) in *A. deliciosa*, (cv. Hayward, cv. Tomuri) and *A.chinensis* treated with BA, ZEA or CPPU at 2 concentrations (1 = 10 μM; 2 = 16 μM)

Adventitious shoot regeneration (%)						
	BA1	BA2	CPPU1	CPPU2	ZEA1	ZEA2
A. *del.* Cv. Hayward	10	5	70	65	35	60
A. *del*. cv Tomuri	17	3	53	77	27	57
A. chinensis	47	30	73	93	97	90

Table 2 Adventitious shoot regeneration (%) in *A. deliciosa* male clones with different ploidy treated with 16μM BA, ZEA or CPPU

Cytokinins	**CLONES**		
	diploid	**triploid**	**tetraploid**
ZEA	40.74	74.36	72.22
BA	2.78	46.43	33.33
CPPU	48.65	33.33	64.86

Table 3 Multiplication rates in *Pyrus communis* cultivars with various cytokinins

Multiplication rates			
Cytokinins	**Conference**	**Dec.del Comizio**	**William**
BA	5.09	2.9	2.92
CPPU	4.83	2.3	4.47
2iP	2.07	2.0	2.07
KIN	2.07	2.0	2.06
TDZ	6.01	5.2	11.5
ZEA	2.64	2.0	2.61

REGENERATION TECHNIQUES UTILISED FOR GENETIC TRANSFORMATION OF PEAS (*Pisum sativum* L.)

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Introduction

Efficient regeneration system belongs to one of the most important prerequisites for successful genetic transformation. The regeneration systems used with the objective to develop genotype-independent genetic transformation of peas were two tissue culture methods based on (1) embryogenesis, (2) organogenesis, and "non-tissue" culture of (3) imbibed trimmed seeds (*in vivo*). Generally, majority of leguminous plants are considered as difficult to genetic transformation. In case of peas, all three techniques were applied for *Agrobacterium*-mediated transformation with plasmid containing reporter gene *uidA* and selection *nptII* or *bar* gene.

Material and Methods

Cvs. Lantra, Komet, Olivín and Tyrkys were used in optimisation experiments for *Agrobacterium*-mediated (EHA 105 with plasmid pBIN19) transformation of peas. The explant preparation, transformation, selection and regeneration were done according to Švábová *et al.* (2005). The embryogenic culture (EC) was derived from apical meristems on MSB medium with picloram, organogenic multiple shoot culture (MSC) was initiated from axillary meristems of cotyledonary nodes on MSB medium with BAP and NAA. The *in vivo* system (IVS) was based on the growth of imbibed seeds after cutting off one cotyledon and culture in perlite with ½ MSB medium (Tab. 1). GUS histochemical assay was carried out based on Potrykus and Spangenberg. (1995). To reduce natural endogenous GUS activity, pH of staining solution was increased to 7.0-7.2 and 20 % methanol was added into extraction buffer. GUS-positive plants were then tested with PCR and Southern blot for the presence of *uidA* and *nptII.*

Results and Discussion

Tissue and genotype specific reactions to selective agents, sonication and/or vacuum infiltration treatments were recorded during the optimisation of transformation protocol. Specific selective concentrations were determined separately for each regeneration system: 20 $mg.l^{-1}$ kanamycin for apical meristems, 100 $mg.l^{-1}$ for cotyledonary nodes and 220 $mg.l^{-1}$ for trimmed seeds. The side effects of phosphinotricin (inhibition of embryogenesis, decrease of shoot induction) led us to delay the time of its application to later developmental phases, i.e. rooting. Another possibility is to spray unselected regenerated plantlets with Basta solution.Vacuum infiltration had generally more destructive effect on explants as compared to sonication. Nevertheless, longer time of sonication may damage constitution of plasmid (Švábová & Smýkal – unpublished results). This problem can be overcome with the application of *Agrobacterium* suspension immediately after sonication – distinctively from generally used protocol of Santarém *et al.* (1998). EC regeneration system has the lowest initial regeneration capacity (about 20% as compared to MSC and IVS, which offer in control variants nearly 100% regeneration). We reached only sporadic regenerants, but none of them was PCR positive. All plants from MSC and IVS systems after transformation with plasmid pBIN19 were histochemically tested for the presence of *uidA* gene. About 20% of positives were false due to the strong endogenous *uidA* gene activity in pea (Hodal *et al*., 1992). PCR analy-

ses of T_2 plants detected the integration of complete gene cassette, but in 5% of individuals partial integration of the introduced genes was recorded (*uidA* or *nptII* was deleted from the genome). The Southern detection proved 1 to 4 T-DNA cassettes per genome, with one copy as the most frequent number (Švábová *et al.,* 2005).
One important criterion of successful transformation of plants is competent target tissue for regeneration and propagation (Hansen & Wright 1999). The main focus of this study was to elaborate efficient and genotype-independent protocol of genetic transformation for peas. However, in peas, there is a limited *de novo* regeneration potential *in vitro*. Up to date, there are only limited data on reproducible *de novo* regeneration from callus and namely protoplasts of pea (Puonti-Kaerlas & Eriksson 1988; Lehminger-Mertens & Jacobsen 1989; Ochatt *et al*., 2000). Regeneration via somatic embryogenesis is highly genotype-dependent; some genotypes are completely non-embryogenic (Griga, 1998). On the contrary, organogenesis from axillary meristems of cotyledonary nodes is very easy to accomplish and it is genotype-independent; thus, it is often used in legume transformation systems. Most transformation studies on peas utilised exactly this regeneration system, but with various results. Transformation efficiency did not exceed 15% (e.g. Bean *et al*., 1997 (1.1%); Davies *et al*., 1993 (1.44%); De Kathen & Jacobsen, 1990 (1%), Chowrira *et al.,* 1996 (14.73%); Nadolska-Orczyk & Orczyk, 2000 (4.2%)). Transformation methods *in planta* or *in vivo* were applied on several legume species. These methods circumvent regeneration problems of *in vitro* cultures, but the inheritance of introduced genes must be verified in consequential generations (Chowrira *et al*., 1996; Švábová *et al*., 2005; Trieu *et al*., 2000). In our experiments, the *in vivo* system seemed to be genotype-independent, resulting in efficient, time-saving (one month shorter than MSC), and reliable transformation system for pea.

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Table 1 Optimised protocols of *Agrobacterium*-mediated transformation of peas

Culture	Media	Conditions	Duration
Agrobacterium			
maintenance	LK, rifampicin, kanamycin	-80°C	long-term maintenance
revitalisation	LK, kanamycin	shaker, 28°C centrifuge shaker, 28°C	24 hod 20 min 60 min
Apical meristems – whole apical parts of germs are used for cocultivation with *Agrobacterium,* the meristems are isolated after cocultivation, washing and drying			
germination	water, cellulose wadding	laboratory, darkness	4 days
cocultivation in liquid media	MS , acetosyringone , *Agrobacterium* suspension	sonication, vacuum, shaker *Note: Agrobacterium add after sonication!*	30 sec 5 min 60 min
cocultivation on solid media	MS, acetosyringone, glutathion	culture room, day / night	24-48 hours 16 / 8 hours
washing	MS, antibiotics	shaker	20, 40, 60 min
drying	filter paper	flow-box	30 min
selection	MS, B5 vitamines, PIC, antibiotics, selective agent	culture room 20-22 °C	4 weeks
rooting	MS, B5 vitamins, NAA, antibiotics, selective agent	culture room 20-22 °C	3 weeks
regeneration	perlite, 1/2 MS, antibiotics	culture room, 20-22 °C	4 weeks
seed setting	clay-sandy growing substrate	culture room, 20-22 °C	2-3 months
Multiple shoot culture			
germination	water, cellulose wadding	laboratory, darkness	4 days
cocultivation in liquid media	MS , acetosyringone , *Agrobacterium* suspension	sonication, vacuum, shaker *Note: Agrobacterium add after sonication!*	30 sec 5 min 60 min
cocultivation on solid media	MS, acetosyringone, glutathion	culture room, day / night	24-48 hours 16 / 8 hours
washing	MS, antibiotics	shaker	20, 40, 60 min
drying	filter paper	flow-box	30 min
selection	MS, B5 vitamins, NAA, BAP antibiotics, selective agent	culture room 20-22 °C	4 weeks
rooting	MS, B5 vitamins, NAA, antibiotics, selective agent	culture room 20-22 °C	3 weeks
regeneration	perlite, MS, antibiotics	culture room, 20-22 °C	4 weeks
seed setting	clay-sandy growing substrate	culture room, 20-22 °C	2-3 months
In vivo			
germination	water, cellulose wadding	laboratory, darkness	4 days
cocultivation in liquid media	MS , acetosyringone , *Agrobacterium* suspension	sonication, vacuum, shaker *Note: Agrobacterium add after sonication!*	30 sec 5 min 60 min
cocultivation on solid media	MS, acetosyringone, glutathion	culture room, day / night	24-48 hours 16 / 8 hours
washing	MS, antibiotics	shaker	20, 40, 60 min
drying	filter paper	flow-box	30 min
selection and regeneration	1/2 MS, antibiotics, selective agent, perlite	culture room 20-22 °C	4 weeks
seed setting	clay-sandy growing substrate	culture room, 20-22 °C	2-3 months

GENETIC VARIABILITY OF ROSE IN RESPONSE TO *IN VITRO* FACTORS

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Introduction

Even a spectacular discovery in tissue culture cannot be applicable to the broad spectrum of genotypes of the same species, which causes a lack of universal protocols. Some factors which are beneficial for the regeneration process in one genotype cause diverse effects in others. The majority of published achievements were obtained on "model" genera, species or even cultivars. If, in a preliminary screening, a genotype is classified as a recalcitrant, it is very difficult to change its competence. We went through this problem while working on our project concerning adventitious regeneration of rose. In this paper, we present a possibility of increasing regeneration potential by pre-conditioning donor shoot cultures on thidiazuron (TDZ)-containing medium and extending in time the regeneration potential by adding 2,3,5-triiodobenzoic acid (TIBA) to the regeneration medium. We show our results against a background of micropropagation potential.

Materials and methods

A two-step adventitious regeneration procedure, based on the one described by Dubois and de Vries (1995), was used. The main differences were that regeneration was induced in leaves obtained from *in vitro* shoot cultures and not from greenhouse-grown plants, and that regeneration was continued for 2-4 months and not 1.5 months. We tried to improve the capacity for regeneration by pre-culturing donor shoots for 15 and 30 days on a medium containing TDZ, and extending the regeneration period by adding 2.5 or 5 $mg.l^{-1}$ TIBA to the regeneration medium. The basic multiplication medium contained Murashige and Skoog salts (MS, 1962), WPM vitamins (Lloyd and McCown, 1982), 30 $g.l^{-1}$ sucrose, 2 $mg.l^{-1}$ BAP, 0.1 $mg.l^{-1}$ IAA, 1 $mg.l^{-1}$ GA_3 and 7 $g.l^{-1}$ Phyto agar. The rooting medium contained ½ MS salts, WPM vitamins and 1 $mg.l^{-1}$ IBA. The basic induction medium contained ½ MS salts, WPM vitamins, 30 $g.l^{-1}$ sucrose, 0.75 $mg.l^{-1}$ TDZ, 0.1 $mg.l^{-1}$ IAA and 3.2 $g.l^{-1}$ Gelrite, while the basic regeneration medium contained 2 $mg.l^{-1}$ BAP, 0.01 $mg.l^{-1}$ NAA and 8 $g.l^{-1}$ Bacto agar. The youngest, but already unfolded, detached leaves were used. They were placed on the induction medium in Petri dishes. The leaves spent the first 5 days in the dark and then under white fluorescent light providing 20 $\mu mol.m^{-2}sec$. The temperature was 23°C. After 14 days on the induction medium, the leaves were transferred onto the regeneration medium. The first data for the regeneration step were collected after 4 weeks. The shoots which were able to survive were cut out and the rest of the leaves were transferred to a fresh regeneration medium for the next 4 weeks so that the procedure could be repeated.

Results

Micropropagation (Table 1)

The numbers of shoots produced by the 5 genotypes tested were found to differ significantly. The cultivars 'Frisco', 'White Gem' and 'Major' produced almost twice as many axillary shoots as 'Arianne' and 'Bonica'. The genotypes also differed in their rooting capacity, with 'White Gem' having the lowest score.

Effect of pre-conditioning on TDZ medium (Fig. 1)

Pre-conditioning increased the number of adventitious shoots in all four genotypes tested. The smallest effect was noted for 'White Gem', which possessed the highest regeneration potential. The increase was greater when pre-culture on the medium with 1 $mg.l^{-1}$ TDZ lasted 30 days. Cv. 'Frisco' produced significantly more shoots on the leaves pre-cultured on 0.05 $mg.l^{-1}$ TDZ, but the longer pre-culture period was more beneficial at both TDZ concentrations, causing the increase in the number of shoots to double. The genotype *Rosa indica* 'Major' produced significantly more shoots during preculture on 1 $mg.l^{-1}$ TDZ, especially when the longer period of 30 days was applied. Cv. 'Bonica', which was very recalcitrant to regenerate adventitious shoots, also reacted positively during pre-culture, more so at 1 $mg.l^{-1}$ TDZ for 30 days, although its potential was very low and hopeless for practical use in breeding.

Effect of TIBA additions to regeneration medium (Fig. 2)

A valuable effect - an extension of the regeneration period, was caused in some cultivars by TIBA. Regeneration on the control medium was usually highest in the first passage, evaluated after 6 weeks, and then the explants became gradually dark and stopped yielding shoots. The leaves on TIBA showed a specific morphogenesis by becoming green and hard, with many meristems visible during anatomical analyses. The cvs 'White Gem', 'Mistral' and 'Charming' regenerated shoots on the TIBA-containing medium in four passages. The TIBA concentration of 2.5 $mg.l^{-1}$ was more effective than 5 $mg.l^{-1}$. A detrimental effect of TIBA - a decrease in the number of adventitious shoots, was noticed in 'Florentina', 'Arianne' and 'Sissel'. No effect was observed in the cultivars 'Compassion' and *R. indica* 'Major'. The huge number of shoots had been induced inside the structures under the influence of TIBA, but some of them continued to grow only when TIBA was excluded from the regeneration medium (Table 2).

Discussion

Effective adventitious regeneration could be of help in non-conventional breeding of rose. Some achievements in rose transformation were obtained when somatic embryogenesis was used for regeneration. Meanwhile, the possibility of using shoot regeneration could be more satisfying because of the shorter time needed and higher genetic stability. A serious obstacle in developing transgenic plants of rose is recalcitrance of the majority of rose genotypes. As a general, it is not easy to predict the regeneration potential of a single genotype (Benson, 2000). The only indication of uniqueness of 'White Gem' with respect to its high regeneration potential was its low capacity for rooting. A low potential for adventitious regeneration of 'Arianne' and 'Bonica' was accompanied by low number of axillary shoots. As we have shown in this work, some improvement in regeneration is possible by pre-conditioning donor shoots on TDZ. Usually, adventitious shoots of the first flush are not transformed homogeneously (Orlikowska & Nowak, 1996), so an extension of the capacity for regeneration by employing the TIBA effect seems to be a valuable achievement.

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Table 1 Micropropagation potential of the cultivars used in adventitious regeneration experiments. N for shoot multiplication and rooting was 36 and 24 explants, respectively.

Cultivar	Shoot multiplication		Rooting		
	No. of shoots	% of shoots > 1 cm	% of rooted shoots	No. of roots	Length of roots
Arianne	2.4a	5.1a	63.2ab	3.6ab	0.7ab
Bonica	1.7a	4.3a	84.3a-c	3.1ab	0.6a
Frisco	4.8b	16.6b	96.2bc	10.8c	1.3bc
R. indica 'Major'	4.5b	5.7a	100c	6.6bc	1.6c
White Gem	4.9b	23.4b	37.1a	1.5a	2.0c

Figure 1 Effect of pre-conditioning of donor shoots on TDZ on adventitious regeneration in three passages

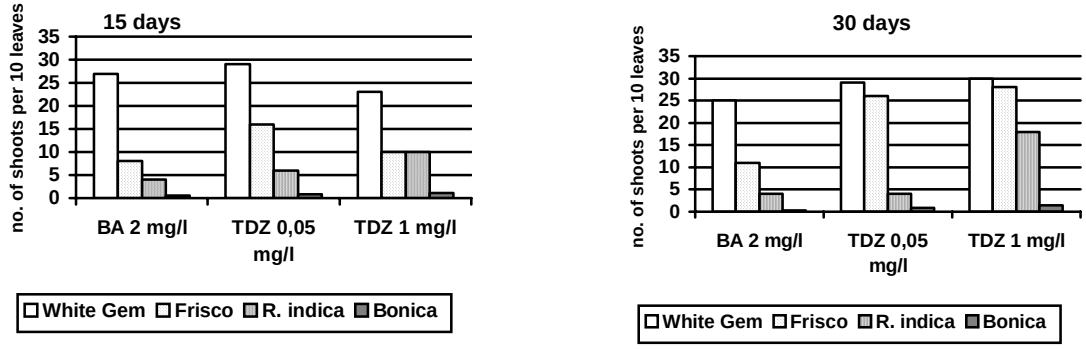

Figure 2 Effect of TIBA on adventitious regeneration of cv. 'White Gem' (left) and 'Arianne' (right) in four and three passages, respectively

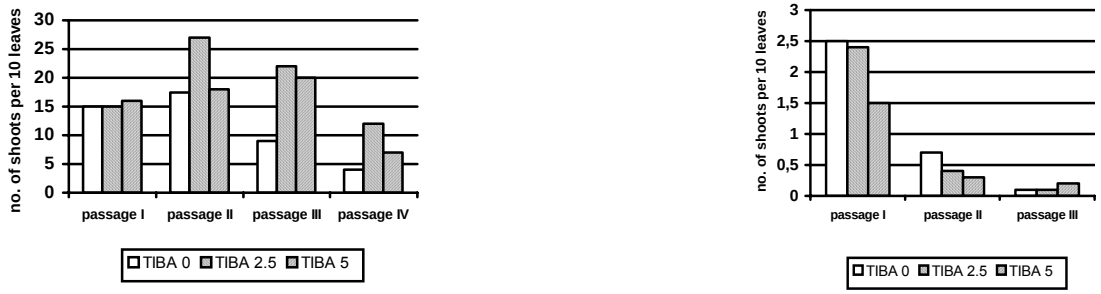

Table 2 The number of adventitious shoots on different media in four passages. Medium A - BAP 2 mg.l^{-1}, NAA 0.01 mg.l^{-1}, TIBA 2.5 mg.l^{-1}; medium B – BAP 2 mg.l^{-1}, NAA 0.01 mg.l^{-1}; medium C – BAP 2 mg.l^{-1}, IAA 0.1 mg.l^{-1}

Passage	No. of adventitious shoots per 10 leaves		
I	17.9 a(medium A)	17.1a (medium A)	17.6a (medium A)
II	19.6a (medium A)	17.9a (medium A)	16.7a (medium A)
III	14.0a (medium A)	14.4a (medium B)	21.0b (medium C)
IV	6.1a (medium A)	9.6a (medium B)	10.4a (medium C)

ENDOPOLYPLOIDY AND ITS CONSEQUENCES FOR *IN VITRO* REGENERATION

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The terms endopolyploidy and polysomaty refer to a common, well-studied phenomenon in plants, characterized by multiplication of nuclear DNA amounts. Endopolyploid tissues such as cotyledons, radicles, hypocotyls, leaf stalks, or flowers, might contain cells with 8C, 16C, 32C, 64C or higher nuclear content. This trait is mainly associated with taxonomic position of the plant species (Barow and Meister, 2003). Cytogenetically phenomenon was extensively studied since 1930ies (Geitler 1953; Tschermak-Woess, 1973). From the tissue culture point of view relatively few studies were focused on the correlation of organogenic/embryogenic potential and endoreduplicated status of target tissues. The existing references quote that regeneration from tissues of higher nuclear content was difficult to achieve (Coutos-Thevenot, 1990) and often expressing morphological abnormalities (Colijn-Hooymans, 1994). Our studies of endoreduplicated nuclei (Šesek *et al*, submitted) were focused on determination of chromosomal status within endoreduplicated nuclei. Based on FISH staining of diploid and tetraploid cabbage endoreduplicated nuclei within root tips, we proposed, that chromosomes in such nuclei are rather bind together and not separated as in true polyploids. This findings actually support previous assumptions, based on microscopic observations of nuclei as for instance reviewed by Therman (1995). Author explains that endoreduplication leads to the formation of bundles of multiple chromatids. Regarding this view, a general conception that endoreduplicated nuclei are true polyploids is wrong – possibly because of terminological missunderstanding. Endoreduplicated nuclei can be viewed as polyploids only in a broad sense but actual status of chromosomes in a true polyploid or within endoreduplicated cell is completely different – individually separated or bound. Since this two options are not generally understood and often termed the same – polyploid – quotes that regenerants from tissues possessing endoreduplicated nuclei would lead to regeneration of polyploids often occurs (Sliwinska and Lukaszewska, 2005). In fact published reports of ploidy of regenerants from endoreduplicated tissues of various species actually support bound nature of chromatids. In most cases where endoreduplicated tissues were cultured mainly diploid regenerants were induced. To some extend tetraploid regenerants might occur but we found no reports of regenerants of higher ploidy, although would be expected according to the structure of inoculated tissues (Jacq *et al*., 1992; Jacq *et al*., 1993; Iantscheva, 2001; Ellul, 2003). Therefore the most likely explanation is, that only the cells possessing a basic number of chromosomes divide and regenerate while those of higher endoploidy levels are possibly recalcitrant to dedifferentiation. It should be mentioned, that the occurrence of tetraploid regenerants needs further attention to elucidate the mechanism of their formation. At least theoretically it is clear that such plants can be either induced by disruption of mitotic spindle of diploid nuclei during mitosis or by chromatide separation of endoreduplicated nuclei during amitotic cell division.

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ENHANCED ACTIVITY OF PHOSPHOLIPIDS HYDROPEROXIDE GLUTATHIONE PEROXIDASE INTERFERES WITH PLANT REGENERATION IN TRANSGENIC *Tobacco, Tomato* AND *Potato* BUT NOT IN *Arabidopsis* PLANTS

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Introduction

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique plant glutathione peroxidase-like protein. This PHGPx is over-expressed under abiotic and biotic stress conditions which mediate oxidative stress. It is likely that by reducing of phospolipid (PL) hydroperoxide, PHGPx protect the cell membrane from oxidative damage. To establish its biological role and its potential to confer stress resistance in plants, we tried to obtain over-expression of citrus PHGPx in transgenic plants. Early attempts to regenerate plants following agrobacterium-mediated transformation in tomato, potato and tobacco have failed. Characterization of the factors which hamper such transformation, in order to produce PHGPx transgenic plants, provided useful information and shed light on the role and significance of the level of PHGPx enzymatic activity during plant regeneration.

Results

Transformation of enzymatically active and inactive PHGPx

We assumed that the enzymatic activity of PHGPx is correlated with the failure to produce transgenic plant over-expression PHGPx. We produce an enzymatically inactive enzyme by substitution of the catalytic residue Cys^{41} into Ser by point mutation, which eliminate the enzymatic activity of the citrus PHGPx. No regenerated plants were obtained following PHGPx transformation using the active enzyme. However, transformed plants over-expressing the enzymatically inactive [Ser^{41}]PHGPx were successfully obtained. Western blots confirmed these results.

Transformation of PHGPx under non-regenerative conditions

In order to understand if over-expression of PHGPx blocks cell division or the regeneration process, we expressed citrus PHGPx in cell suspensions of potato, tobacco (BY2 cells) and in tobacco leaf discs that following transformation were not transferred to regeneration media but into callus induction media, leading subsequently to the establishment of cell suspensions derived from callus cell surrounding the transformation site. Each transformation was performed using the constitutive 35S promoter and the inducible RD29A promoter (activated by ABA, NaCl, cold and drought). PHGPx did not inhibit cell divisions and transformed cell suspensions were obtained using the constitutive or inducible promoter as indicated by Western blot analysis.

In-planta transformation by the infiltration method of *Arabidopsis thaliana* with 35S-Cit-PHGPx.

Overexpression of the citrus enzymatically active PHGPx was obtained in 90% of the tested plants as verified by Western blotting on germinated plants. It may indicate that PHGPx under the constitutive expression of the 35S promoter does not interfere with the normal process of seed embryogesis following fertilization.

Transformation of an active PHGPx under the control of a strictly inducible promoter

Tobacco plants were transformed with PHGPX under the control of the estradiol regulated inducible XVE vector. The time window during regeneration in which PHGPx activity inhibit regeneration was characterised using regeneration media supplemented with estradiol. Results will be described in the presentation.

Discussion

In an attempt to understand why over-expression of plant glutathione peroxidase, following *Agrobacterium*-mediated constitutive transformation of citrus-PHGPx, was unsucessful, several manipulations have been employed to obtain such plants. We have found that efficient PHGPx expression was obtained in tobacco and potato cell lines and leaves only when cultured, following transformation, on non-regenerative callus- induction media. Moreover, mature transgenic plants were obtained either by:

1. Performing *in-planta* transformation
2. Using strictly, non-leaky promoter
3. Abolishing the enzymatic activity via point mutation of the catalytic residue codon.

These results clearly demonstrate the importance of regulating PHGPx enzymatic activity during plantlets regeneration and that uncontrolled PHGPx overexpression interferes with such regeneration, probably during differentiation.

ECTOPIC SHOOTS ON LEAVES OF *KNAP1*-TRANSFORMED *Kohleria* RESEMBLE AXILLARY RATHER THAN ADVENTITIOUS SHOOTS

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Introduction

Knox (*knotted*-like homeobox) genes encode transcription factors and are involved in the regulation of cell fate determination in plants (Reiser *et al.*, 2000). *Knox* genes comprise two classes of which class I genes are expressed in the shoot apical meristem, but usually not in lateral organ primordia. Overexpression of both endogenous and heterologous class I *knox* genes in transgenic plants cause dramatic changes in leaf shape and eventual formation of ectopic shoots, as observed *e.g.* in tobacco (Sinha *et al.*, 1993), tomato (Janssen *et al.*, 1998) and *Arabidopsis* (Chuck *et al.*, 1996). There is indication that these changes in part are mediated by alterations in endogenous hormones (*e.g.* Tamaoki *et al.*, 1997; Hay *et al.*, 2002). In the present study it was examined, whether ectopic shoot formation caused by *knox* overexpression and hormone-induced adventitious shoot regeneration in tissue explants represent equivalent or different morphogenic pathways. Both types of shoots were compared histologically in non-transgenic and transgenic *Kohleria* overexpressing *KNAP1*, a *knox* gene from apple (Watillon *et al.*, 1998). *Kohleria* was chosen as a model, because hormone-induced adventitious shoots are of unicellular origin and arise from one particular cell type, *viz* the basal cell of certain glandular trichomes (Geier and Sangwan, 1996).

Material and Methods

Microshoots of a *Kohleria*-hybrid were maintained by cultivating node segments on Nitsch (= N69) medium. Adventitious shoots were induced from internode and leaf explants on SIM (= N69 + 2.5µM IAA + 2.5µM BA). For transformation, internodes were co-cultivated with *Agrobacterium tumefaciens* LBA 4404 harbouring p35S KNAP1 which contains the *KNAP1* coding sequence under the control of the CaMV 35S promotor (Watillon *et al.*, 1998). Putative transgenics obtained on SIM plus 50 mg. l^{-1} kanamycin and 250 mg.l^{-1} cefotaxime were examined by PCR for the presence of *NPTII* and *KNAP1* sequences, and by Southern blotting. Histological studies of shoot regeneration were performed using light microscopy (LM) of fresh and semi-thin plastic sections as well as scanning electron microscopy (SEM).

Results

A wide range of leaf morphological changes was observed in greenhouse-grown *KNAP1*-transformants of *Kohleria*, ranging from almost normal (simple with serrate margin) to strongly lobed and transitions to palmately compound leaves with continuous production of new lobes from the leaf base. Ectopic shoot buds were found in only one of 41 clones (clone 'T5-121') and only on three occasions, pointing to the possibility of environmental influences on expression. In total, about 50 such buds were observed. Southern blotting indicates the presence of at least two *KNAP1* inserts in this clone. The buds were always produced on the adaxial leaf surface at the margin between lobes. Examination of young buds by SEM strongly suggests a multicellular origin, as indicated by the smooth transition from the surrounding tissue towards the bud with con-

tinuous decrease in cell size from normal epidermal cells to cells of the meristem. LM of sections further support the multicellular origin of buds and demonstrate the participation of subepidermal tissues. When leaf segments of non-transgenic control, clone ‘T5-121‘ and other *KNAP1*-transformed lines were cultivated on SIM, adventitious shoot regeneration first appeared close to wounded sites and eventually spread over the entire adaxial and abaxial surfaces of explants. Both LM of sections and SEM confirmed that adventitious shoots originate from the basal cell of short-stalked glandular trichomes, as reported for internode segments (Geier and Sangwan, 1996); advanced bud stages remain sharply delimited from surrounding tissues.

Discussion

Ectopic shoots formed *in planta* on leaves of a *KNAP1* overexpressing transgenic *Kohleria*-clone and adventitious shoots induced by application of hormones to leaf segments *in vitro* differ with regard to their cellular origin and development. Ectopic shoots have a multicellular origin comprising epidermal and subepidermal layers, and arise at the leaf margin between lobes, which parallels *KNAT1*-transformed *Arabidopsis* (Chuck *et al*., 1996). This mode of origin is morpho-anatomically similar to axillary bud formation. On the contrary, adventitious shoot regeneration occurs from single cells of a particular epidermal cell type. Overexpression of *knox* genes was found to cause increased cytokinin and reduced gibberellin content, *e.g.* in tobacco overexpressing *NTH15* (Tamaoki *et al.,* 1997). In transgenic *Arabidopsis*, *KNAT1* misexpression was suppressed by gibberellin, but enhanced through inhibition of gibberellin signalling or synthesis (Hay *et al.,* 2002). The promotive effect of cytokinin (Skoog and Miller, 1957) and inhibitory effect of gibberellins (Murashige, 1961) on shoot formation are long known. Thus, ectopic shoot formation in *knox* overexpressing transgenic plants might seem to be caused by changed endogenous hormone levels. The fact, however, that ectopic shoots occurred in only one out of 41 transgenic *Kohleria*-lines and only on few occasions suggests that induction of those shoots may require a highly sensitive balance of conditions, while adventitious shoots are produced under a wide range of hormonal regimes. It is concluded, therefore, that ectopic shoot formation due to *KNAP1* overexpression and hormone-induced adventitious shoot regeneration represent distinct morphogenetic pathways, involving cells of different competence.

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OLIGOGALACTURONIDES PROMOTE CAULOGENESIS NEGATIVELY INTERFERING WITH POLYAMINES AND AUXIN

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Introduction

Alpha-1,4-linked oligogalacturonides (OGs) when released from plant primary cell walls by partial hydrolysis with endopolygalacturonase function as elicitors of defense responses by activating the transcription of defense-related genes (Messiaen and Van Cutsem, 1993). In addition, OGs exhibit a morphogenic role on cell elongation (Branca *et al*., 1988), flower formation (Marfà *et al*., 1991), and rhizogenesis (Bellincampi *et al*., 1993). To date, there is no information regarding their effect on caulogenesis (i.e. adventitious shoot formation).

OGs require a degree of polymerisation (DP) ranging from 10 to 15 in order to exert their biological activity (Bellincampi *et al*., 1994), and their mechanism(s) of action remain(s) to be elucidated. Unravelling this mechanism is complicated by the multiple interrelationships between OGs and other plant growth regulators and/or signalling molecules (Ridley *et al*., 2001). In particular, OG effects on plant development appear to be associated with auxin action (Branca *et al*., 1988; Bellincampi *et al*., 1993, 1995). In every case reported to date their effect is the opposite of that of exogenous auxin.

Millimolar concentrations of Ca^{2+} are required for biological activity of OGs in carrot and tobacco cell suspensions (Ridley *et al*., 2001, and references therein); the formation of a Ca^{2+}–dependent conformation, known as the "egg-box" conformation, is formed by OGs with DPs equal to or greater than 10 (Messiaen and Van Cutsem, 1994). Physiological concentrations of the polyamines (PAs), spermidine (Spd) and spermine, inhibit the biological activity of OGs, possibly because they selectively prevent OGs from adopting this conformation (Messaien and Van Cutsem, 1999).

In the light of these interactions between OGs, auxin, calcium and PAs on plant development, the present study was undertaken, using the tobacco leaf explant model system, to examine the morphogenic effects of OGs on adventitious caulogenesis, and the way in which these effects are modulated by the presence/absence of auxin and PAs.

Materials and Methods

Leaf explants (5x15 mm containing the midrib) were excised from vegetative plants of *Nicotiana tabacum* cv. Samsun and cultured for 30 days on Murashige and Skoog (1962) medium in the presence or absence of $CaCl_2$ (3 mM). The medium was supplemented with 10 µM BA plus 1 µM IAA (Altamura *et al*., 1998), and/or 2 $\mu g.ml^{-1}$ OGs (DP 9-18), and/or with 1 mM Pu, and /or 0.4 mM Spd. In another set of experiments the same compounds were added to the medium without IAA. Putrescine (Pu) and Spd were applied with or without their corresponding biosynthetic inhibitors, i.e. DFMO (1 mM) for Pu, and CHA (10 mM) for Spd. The caulogenic response was histologically evaluated during the culture period. Free and conjugated PA titers were also evaluated during culture according to Biondi *et al*. (2003).

Results

Caulogenesis was enhanced in the presence of exogenous Calcium and PAs. In particular, Spd stimulated the process, affecting the phase of meristemoid formation. Poly-

amine inhibitors strongly inhibited caulogenesis, and the inhibition was reverted by the application of the inhibitor with the corresponding PA. Also OGs stimulated caulogenesis, and even in the absence of exogenous Calcium. They positively affected the same phase of PAs (i.e. meristemoid formation). In the absence of auxin, but in the presence of exogenous Calcium, both Spd and OGs caused an increase in the number of shoots per explant, and OGs were able to further increase this number when also Calcium ions were excluded from the medium. Xylogenesis, i.e. the formation of vascular nodules is a morphogenic programme stimulated by auxin. In the medium containing IAA and BA, PAs caused an increased xylogenesis. By contrast, OGs inhibited this response. Early in culture (day 4) no significant change in free Pu and Spd was caused by OGs, both in the presence of exogenous calcium and in its absence (media with and without IAA). Instead, OGs caused an increase in the levels of the conjugated insoluble PAs, Pu in particular, when applied to the medium containing exogenous Calcium and IAA+BA. In the absence of exogenous Calcium and IAA, not only Pu level was increased by OGs, but also that of Spd. In the conjugated soluble fraction, OGs also caused rises in Pu and Spd titers, and mainly in the presence of auxin in the medium.

Discussion

In tobacco leaf explants cultured under caulogenic conditions (i.e. MS medium with IAA+BA), PAs stimulate meristem formation, but their action depends on exogenous Calcium. By contrast, OGs with DP from 9 to 18 promote per se shoot meristemoid formation, in that their promotive action does not need exogenous Calcium. No promotion of shoot formation is caused by the combined presence of PAs and OGs in the medium. Instead, OGs cause rises in endogenous PAs levels. Since OGs inhibit auxin-induced root formation (Bellincampi *et al*., 1996) and xylogenesis (present paper), and promote BA-induced caulogenesis, a specific role of these compounds in enhancing shoot formation through a modulation of PA levels, may be suggested.

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ALMOND SHOOT REGENERATION IN *Prunus dulcis* – A MOLECULAR APPROACH TO THE REGENERATION PROCESS

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Introduction

The fact that developmental processes occur in specialized tissues or cell types, makes gene expression studies very appellative. Till very recently, adventitious organogenesis was mostly studied through a histological or physiological perspective, observing morphological changes in the tissues or obtaining empirical data based on trial and error experiments. However, puzzling such a developmental process demands an extensive dissection at the transcriptome level, to gain insight into molecular mechanisms determining "when" and "where" organogenesis is to happen.

To approach the molecular mechanisms underlying almond shoot neo-formation, we have developed two main strategies, a candidate gene approach targeting almond Knotted-1 [1] and CDKA:1 [2] (described as putative markers for organogenesis events) and a transcriptomic approach and micro-array technology, using two suppression subtractive hybridisation libraries constructed from two defined time frames of organogenesis, to screen and discover novel markers.

These studies were conducted on an indirect shoot induction system in a woody fruit species of *Rosaceae*, the almond tree.

Material and Methods

***The candidate gene approach*:** Total RNA was extracted from apical and axillary meristems, and leaves of *in vitro* micropropagated almond shoots. The three samples were used for RT-PCR using degenerated primers for the conserved domains of Knotted-1 (maize), and CDKA:1 (*Arabidopsis*) genes. An STM (Knotted-like) and a CDKA:1 almond sequences were obtained, and the 5' and 3' primed ends were extended by RACE-PCR using SMART and Marathon uncloned cDNA libraries of meristem tissues. Total RNA was extracted from leaves from each single day of adventitious shoot induction (21 days) and STM expression was followed throughout organogenesis using Real-time PCR.

Northern blot analysis was performed with total RNA (50 μg) isolated from meristem tissues and leaves, separated by electrophoresis, blotted onto nylon membrane and hybridised with almond CDKA:1 and STM internal probes under high stringency conditions. For the Southern blot analysis 10 μg of genomic DNA were used in each single digestion and the membrane was hybridised with the same probes used for the northern blot analysis.

***The transcriptomic approach*:** RNA, isolated from leaves of *in* vitro shoots and from leaves induced for regeneration, was used to produce cDNA clones using the "BD PCR cDNA Synthesis Kit". Two cDNA subtractive libraries, corresponding to early and late stages of organogenesis, were cloned using the "BD PCR-Select cDNA Subtraction Kit". To build the almond micro-array, the cDNA clones from both libraries were PCR amplified, products were dried out and ressuspended in printing buffer, transferred to

384 microtiter plates, and printed in slides using an array printing tool (Genetic Microsystems GMS 417 Arrayer).

***Micro-array hybridisation*:** RNA was extracted from regenerating leaves, induced to regenerate, throughout 20 days of induction period, and two RNA bulks (early and late organogenesis) were used to synthesize the dye swap probes. To visualize the differential expression, the two RNA pools were labelled with distinct fluorescent dyes (Cy3 and Cy5) (Genesphere kit cy5 and cy3, Oakland, NJ) and hybridised overnight in an humid dark chamber.

***Sequencing and Real-time PCR*:** Clones with a >2fold expression level differentially expressed either in the early or late organogenesis were sequenced and 8 genes were selected to perform Real-time PCR (using the ABI Prism device and Sybr Green PCR Master mix) to confirm the experimental data obtained from the micro-array.

Results and Discussion

The isolation of the full length mRNA of almond Stm (Knotted-like) was successfully obtained by the extension of 3' and 5' primed ends, by RACE-PCR, using almond STM gene specific primer. By Southern blotting two copies of the almond stm gene and one copy of the CDKA:1 gene were identified. The almond Stm transcript was detected by northern blotting in meristem tissue but not in leaf. The almond CDKA:1 was present in both meristems and leaf samples and apparently also expressed throughout the whole "*de novo*" shoot induction period. Therefore, STM appears to be more useful as shoot induction marker than CDKA:1. The Real-time PCR results obtained for STM throughout regeneration point for a production of the transcript from the very beginning of the induction period. However the expression levels obtained in early stages was very low and a higher transcript accumulation was only detected from day 15 up to day 20.

From the statistical analysis performed using GeneSpring software, of data collected from micro-array hybridisation, a range of clones was identified from both libraries. A total of 58 cDNA clones differentially expressed in the early organogenesis with an expression ratio higher than 2>fold with 95% of confidence were sequenced. A total of 70 cDNA fragments have shown to have a differential 2>fold higher expression rate in the late *versus* early organogenesis. The cDNA clones sequenced represent 92 unique gene fragments. The gene annotation based on their BLAST homology against GeneBank sequences, showed the presence of proteins such as Heat shock proteins, alpha Elongation factors, Beta 1,3-Glucanases, Proline-rich peptides and Sac domain proteins reported for the first time in almond and in their involvement in adventitious developmental processes. Further Real-Time PCR analysis of selected genes confirmed and supported the micro-array results. The disclosure of a variety of known and unknown transcripts directly involved in adventitious regeneration, screened using the micro-array profile, may elucidate about their putative regulatory roles in the totipotency and shoot regeneration.

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HYPOACETTYLATED HISTONE H4 IS ASSOCIATED WITH REPRODUCTIVE COMPETENCE IN *Pinus radiata* D. Don.

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Introduction

DNA methylation is the best-known epigenetic modification during animal (Bird, 2002) and plant (Finnegan *et al.*, 1998) development. DNA methylation is part of a complex molecular machinery that, in collaboration with histone modifications, constitutes a specific language ("epigenetic code") controlling gene expression and chromatin structure (Loidl, 2004). Histone acetylation, especially acetylation at histone H4, is the most widely characterized modification affecting protein components of animal chromatin (Turner, 1998). Several effects of histone acetylation on heterochromatin formation (Richards & Elgin, 2002), gene regulation (Roth *et al.*, 2001) and control of other biological processes, such as DNA replication and DNA repair (Eberharter & Becker, 2002) have been reported. In the case of plants, acetylation of histone H4 has been associated with DNA replication rather than transcription (Jasencakova *et al.,* 2000) in *Vicia faba*, but almost nothing is known about it´s.

Implications on forestry species maturation. Our recent findings about changes in DNA methylation during radiata pine ontogenic development (Fraga *et al.*, 2002) have prompted us to study variation of other global epigenetic markers, such as histone H4 acetylation. In this paper the levels of global acetylated histone H4 in needle primordia of non-flowering and reproductive radiata pine trees in relation with our latest results will be presented.

Material and Methods

Plant material: Analyses were performed in apical (b1a) and basal (b1b) portions from one-month-old needles with exact lengths of 7 and 6 mm, respectively. All needles were obtained from annual growth units of eight-year-old *Pinus radiata* D. Don. Two kinds of mature b1 needles were compared: b1 needles located on non-flowering branches (vegetatively mature) (VM) and b1 needles located on flowering verticila (reproductively mature) (RM).

Histological and immunofluorescence analyses: The assays were carried out as described in González-Melendi and Shaw (2002). Analyses were performed by differential interferential contrast (Nomarsky contrast). Images were captured with software coupled to the confocal microscope and then processed with Adobe Photoshop 7.0 image software. Using the images obtained by immunofluorescence, a global histone acetylation index (AI) was developed. The relative degree of histone acetylation of each b1 sample was measured as the percentage of histone-acetylated nuclei, given by the number of nuclei labeled with acetylated histone H4 x 100 / total number of nuclei present in the image.

Protein extraction, electrophoresis and western blotting: *Pinus* b1 needles (500 mg) were frozen in liquid nitrogen, powdered with a mortar and pestle, and further homogenized in 3 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 10% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol and the protease inhibitor 1mM phenylmethyl sulfonyl fluoride (PMSF). Total amounts of protein were estimated (Bradford, 1976). Protein samples and prestained standard proteins sepa-

rated by electrophoresis were transferred to Immobilon membranes (Millipore Corporation; Bedford, MA, USA). Before immunodetection, an equal amount of total protein was confirmed with Ponceau S (Sigma; St Louis, MO, USA) staining. Membranes were incubated incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (Boehringer Mannheim; Mannheim, Germany) diluted 1:1000 in the blocking buffer. Proteins recognized by the antibody were revealed. Relative intensity of spots was measured with Labmage v2.62a (© 1999-2003 Kapelan GmbH).

Results and Discussion

Immunofluorescence experiments with an antibody against histone H4 acetylated at all the possible lysines in the histone tail revealed differential patterns of modification that were dependent on tissue-type and reproductive/vegetative states of the radiata pine needles. Interestingly, the b1 needles from vegetative branches showed significantly less staining than did those from reproductive branches. Differences between needles from vegetative and reproductive branches were much greater in the basal portions (48% vs 8% of the nuclei stained, respectively) than in the apical portions (60% vs 50%). With respect to the apical/basal distribution of histone H4 acetylation, needles from both vegetative and reproductive branches stained more strongly in the apical portions containing the shoot apical meristem (SAM) than in the basal segments of the organs. It is important to note that nuclei labelled for global-acetylated histone H4 were homogeneously associated throughout the tissue. The smaller amount of global histone H4 acetylation in basal portions of reproductive needles than in their vegetative counterparts was corroborated by western blotting. Densitometric quantification of the immunoblots revealed twice as much acetylated H4 in vegetative needles as in reproductive needles.

Very little is known about the role of histone modifications in plant ontogenic development. No previously published work has associated patterns of histone acetylation with specific phases of development in forestry species, making the present work a pioneering study in this area. However, epigenetic variations linked to maturation of other plant species have been reported (Kinoshita *et al.*, 2001). Wagner (2003) proposed that transition from embryogenesis to the vegetative and reproductive phases of Arabidopsis thaliana is controlled by chromatin-remodeling factors.

As it is widely accepted that most of the genome in differentiated higher eukaryotic cells. Consists of hypoacetylated inactive chromatin (Eberharter & Becker, 2002), reproductive b1 needles must be hypoacetylated relative to vegetative b1 tissues. Our results lead to the conclusion that patterns of global acetylated histone H4 are specific markers of the vegetative/reproductive phases in radiata pine, and that these maturation-related epigenetic variations could be influenced, at least in part, by the level of tissue differentiation.

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PROPAGATION TECHNIQUES OF HORTICULTURAL CROPS IN CYPRUS

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The broad agricultural sector, despite the reduction of its contribution to the Gross Domestic Product and total employment, continues to be a fundamental sector of the Cyprus economy, both with respect to the production of essential food items for the population and exports and with respect to the employment of thousands of rural residents and the containment of the depopulation of the villages. The broad agricultural sector contributed some C£ 211.8 mln or about 3.7% to GDP and employed approximately 23.0 thousand persons or 6.9% of the total economically active population. Agricultural exports (raw and processed) reached C£ 36.9 mln, constituting 16.6% of total domestic exports. Cyprus agriculture may be divided into two major sub-sectors, namely crop production and livestock production, which, contributed 62.1% and 31.4% respectively to the value, added of the broad agricultural sector. The most important crops were potatoes, vines and citrus, which were also major export commodities, and cereals. Horticultural production in Cyprus is considered as a major part of the agricultural system, as the government gave high priority to high income crops. Its share to the total value of crop production stood at 76.7% in 2002 (Anonymous, 2003).

Plant Propagation is a major component of horticulture. Availability of quality planting materials is a salient factor in promoting a fruit industry and in fact, it is one of the main factors that contribute to the development of fruits in any country. Different types of greenhouse covered with white plastic polyethylene sheet are the basis of most facilities used in a nursery today for successful vegetative and seed propagation in Cyprus. The two main types of greenhouses used a) the single-span, round-arched type and b) the multi-span, round-arched type greenhouse. Both types are of low cost structure using G.I. pipes and wooden beams. The multi-span greenhouse apart of fruit tree propagation can be also used as mist propagation units, for acclimatization of plants produced from the Tissue Culture Lab, as well as for seedlings of vegetables and ornamentals. In the multi-span greenhouses artificial ventilation and heating-cooling systems are installed using air-heaters and a complete fan and pad cooling system. Modern propagation techniques and equipments and shade net-houses for hardening (weaning) of grafted/budded plants and cuttings are used. There are suitable institutional arrangements (Quality Standards, Legislations and Regulations) for registration of nurseries and quality control for assuring quality standards for production and marketing of healthy high quality plant multiplication material.

Propagation from seed is particularly used for vegetables, ornamental and tree seedlings where small differences in genetical variation are not important. Vegetative propagation is essentially for the reproduction of fruit trees where the plant material should be true to type with the exact characteristics of the parent material with regard to genotype and health status. The grafting and budding techniques are mainly used for fruit trees. (Gregoriou, 1984) Mist propagation is used for rooting cuttings of olives, clonal rootstocks and ornamentals. Fogging is used for difficulty to root species. The maintenance the stock (mother) plants can be divided into three categories: a)Permanent stock plants in the open ground without protection b) Permanent stock plants in the open ground

with overhead protection and c) Stock plants grown in specific containers (50 litres) under protection in screen houses (pre and basic materials).
For rapid multiplication and breeding purposes, micro propagation techniques *in vitro* and semi *in vivo* have been initiated which guarantee healthy plant material, multiplied at a fast rate. During 2003, a long term programme was initiated for the local production of seed potatoes through the production of micro-plants and mini-tubers. Projects are under way for the cleaning of clonal propagation stocks and the development of mass micro-propagation techniques for the production of pathogen-free propagation stocks of table and wine grape clonal cultivars and rootstocks. Another programme has initiated aiming in the production, maintenance and distribution of healthy citrus planting material. The technique of shoot-tip grafting *in vitro* was used for elimination of viruses and viroids in twelve isolates of the local mandarin variety and three isolates of lemon. All citrus virus-free material either imported or produced locally so far by micrografting, is maintained in a prebasic plantation under double insect-proof screen. Also, the virus-free nuclear stocks of vines, introduced from abroad or produced locally, are maintained in a prebasic plantation under double insect-proof screen, and presently comprise of 119 selection lines of 72 genotypes (Gregoriou, 2004; Iannou *et al*., 2004; Minas, 2004).

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IN VITRO PRODUCTION OF "CUT" AND "POT" FLOWERS IN SERBIA

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Introduction

In this report, we will discuss the improvement which has been achieved during the last decade in developing plant tissue culture methods, techniques and potential products obtained by the use of plant biotechnology.

Materials and methods

In our laboratory of plant tissue culture (1995-2005), rapid micropropagation methods (by androgenesis, somatic embryogenesis, organogenesis) have been developed for several species important in horticulture, namely some "cut"-flowers (CF): spp. *Allium*, cvs. *Chrisanthemum morifolium,* spp. *Fritillaria,* cvs*. Dianthus caryophyllus,* cvs. *Gerbera jamessonii,* spp. *Iris*) and "pot" flowers (PF): sp. *Dianthus petreus*, ornamental cvs. *Nicotiana tabacum*, cvs. *Pelargonium hortorum*, cvs. *Saintpaulia ionantha*.

Results

"Cut-flowers" *ALLIUM* spp. Plant regeneration (PR) from mature zygotic embryos (ZEs) culture of *Allium aflatunense* and *A. fistulosum* was achieved by somatic embryogenesis (SEm). Mature ZEs were cultured on BDS or MS mineral solution, 30.0 $g.l^{-1}$ sucrose, solidified by 0.7 $mg.l^{-1}$ agar and with (in $mg.l^{-1}$): inositol 100.0, nicotinic acid 10.0, casein hydrolysate (CH) 100.0, L-proline (Pro) 500.0, glycine 2.0, vitamin B_6 1.0, 2,4- dichlorophenoxyacetic acid (2,4-D, 9.0 μm), or thidiazuron (TDZ, 9.0 μM). Somatic embryos (SEs) and bulblets (Bs) were formed directly in coleoptile and radicule region of ZEs on BDS medium + TDZ. The presence of 2,4-D in BDS medium provoked SE formation indirectly *via* embryogenic callus (EC). ***FRITILLARIA* spp**. In order to establish an efficient protocol for PR of *Fritillaria imperialis* and *F. meleagris* the kind of optimum culture conditions for induction SEm and organogenesis (Or) was investigated. Mature ZEs were cultured on MS mineral solution containing 30 $g.l^{-1}$ sucrose, solidified by 0.7 $g.l^{-1}$ agar with (in $mg.l^{-1}$): 2,4-D 1.0, CH 250, Pro 250 or TDZ 1.0. After 8 weeks on MS medium + 2,4-D, the EC was obtained. Processes of SEm was asynchronous and on the surface of EC, many SEs at different stages of development are observed. Direct SEm was achieved on ZEs on MS medium + TDZ. During prolonged culture on same medium, multiplication of SEs, Bs and shoots (SHs) formation were achieved. ***CHYSANTHEMUM* cvs.** We investigated *in vitro* PR of 19 *Chrysanthemum* cultivars: "Violet Spider" (VS), "White Spider" (WS), "Yellow Spider" (YS), "Vesuvio" (V), "Copper" Spider" (CS), "Albert Heyn" (AH), "Papagaj" (P), "Reagan White" (RW), "Reagan Sunny" (RS), "Reagan Splendid" (RSp), "June Yellow" (JY), "June Rose" (JR), "Tigerrag" (T), "Yellow Jubile" (YJ), "Bronze Mundial" (BM), "Fanshine Improved" (FI), "Klondike" (K), "Pink Snowdon" (PS) and "Rivarly" (R). Induction of Or is achieved in steam segment culture on MS medium. Shoots multiplication (SHsM) was investigated on six MS media supplemented with 6- benzyl amino purine (BAP), Kin, or TDZ (1 mgL^{-1}, respectively) and naphtalenacetic acid (NAA, 0.1 or 0.5 $mg.l^{-1}$, respectively). The best SHsM index was observed on MS medium + BAP+ NAA for ten cultivars. According these results, annual plant production for cv. "R" could be 1.4×10^{13} shoots, and the lowest for cv."RSp" is 7.8×10^{6} shoots. Rooting of shoots (95-100%) was satisfactory on MS hormone-free medium. Potted plants were acclimatized in arrange 55-84%. ***DIANTHUS*** cvs. Several cultivars of *Dianthus caryophyllus* were subjected to cell suspension, stem segment or meristem tip cultures. **Cell suspension** of seven cultivars calli were established on MS medium with (in $mg.l^{1}$): 2,4-D 5.0 + Kin 1.0 + CH 500. "Arthur Sim"(AS) and "Telstart" (T) formed "micro" calli. Only cv."AS" formed green nodule and PR were achieved. **Stem segments** of "AS", "Lena" (L), "Tangerina" (T) and "T" formed organogenic callus (OC) with adventitious buds (ABs) on MS medium + NAA + Kin (1.0 $mg.l^{-1}$, each). In **meristem tips culture** of cvs. "TAN", "Scania" (S), "Dark Purple" (DP), "AS", "L", "White Sim" (WS), and "T" leaf rosette formation (LR) and axially buds (ABs) induction were achieved on MS medium with (in $mg.l^{-1}$): 2,4-D 0.02 + IBA 0.1 + BAP 2.0 + GA_3 0.1. Cultivars "TAN", "S", "DP", "AS", "L", "WS", and "T" gave 42, 50, 73, 76, 90, 90 and 93% of newly-formed LRs, respectively. MS multiplication medium containing (in $mg.l^{-1}$): NAA 0.2 + IBA 0.02 + Kin or BAP (0.01, 0.1, or 1.0). The choose of cytokinin depended on the cultivar responsiveness. Kin was more favorable for SHsM of "T", "L", and "S", while BAP was better for "AS", "DP", "TAN" and "WS". During shoot elongation GA_3 was omitted.

Shoot rooting achieved on MS medium without or IBA (0.5, 1.0 mg.l^{-1}) + Kin 0.05 mg.l^{-1} varied from 71-100% for different cultivars. ***GERBERA*** cvs. Nutrient media for clonal micropropagation of *Gerbera jamesonii* cvs. "Bingo" (B), "Clemen- tine" (C) and "Marleen" (M) by shoot apex were studied. At the same time optimal medium for Or by tissue culture of young floral and leafstalks segments of gerbera cvs. "Claudia" (Cl) and "Florijn" (F) was establi- shed. Explants were cultured on MS medium supplemented with B_1 vitamin, amino acids, IAA and Kin (0.5, 5.0 mg.l^{-1}, respectively). Different number of LRs was obtained by the apex cultures depending on the culti- vars. The adventitious buds were formed at the floral and leafstalks segments by direct caullogenesis. Number of explants that give a morphogenetic response varied with both explant origin and cultivar. Shoots of all cultivars were multiplied on MS + IAA + Kin (0.5, 5.0, respectively) by ABs and SHsM index varied in rela- tion to the cultivar. Rooting was achieved on MS + IBA 0.2 mg.l^{-1}. Acclimation of plantlets was quit satisfactory for all cultivars (70-100%). ***IRIS*** **spp.** We summarized the best protocols for *in vitro* PR of several irises (*Iris halophila, I. pumila, I reichenbachii, I. sibirica*). The PR of these species is achieved in mature ZEs, leaf base culture of *in vitro* grown shoots and cell suspension culture. Morphogenesis *in vitro* was obtained *via* SEm and Or. The SEm of *I. halo- phila, I. pumila* and *I. sibirica* was induced after seven days by growing ZEs explants on MS medium + 2.4-D 5.0, Kin 1.0, CH 250 and Pro 250.0 (in mg.l^{-1}, each). Further development of EC and differentiation SEs oc- curred on MS + 2,4-D 0.1 mg.l^{-1}.The best morphogenetic potential had *I. pumila*. After acclimation in green- house plantlets had no somaclonal variation. The application of presented biotechnological protocols could be useful for mass production of these species without destroying natural habitat.

"Pot-flowers" ***DIANTHUS*** **sp.** The PR of *Dianthus petreus* subsp. *noenanus* was achieved through micropropagation from meristem culture (clone "DP") and from adventitiou buds regenerated from organogenic calli (OC) in stem segments culture (clone "DPS"). Meristem with two leaf primordia formed numerous LRs on MS_1 medium + IBA + NAA (0.02, 0.2 mg.l^{-1} + Kin 1.0 mg.l^{-1}. Stem segment were cultivated on MS_2 medium + 2,4-D + Kin (1.0, mg.l^{-1}, each) + Pro 250 mg.l^{-1} on which they formed OC. After transfer of OC on MS_1 medium ABs were observed. Multiplication of clone "DP" and "DPS" shoots on MS_1 or MS_3 (MS + IBA 0.02 + NAA 0.2 + BAP 1.0 mg.l^{-1}, each). Rooting of clone "DP" (7 and 27%) and "DPS" (70-91%) depended on the concentration of IBA (0.5-1.0 mg.l^{-1}, respectively). Carnation plantlets were grown in greenhouse until flowering. ***SAINPAULIA*** cvs. We investigated the conditions for *in vitro* vegetative propagation of different cultivars of *Saintapaulia ionantha* focusing our attention on selection of most suitable nutritive medium, origin and age of explants. The PR of African violet developed from petiole and lamina explants were cultivated on MS medium + Kin 1.0 mg.l^{-1}+NAA or IAA (2.5-5.0 mg.l^{-1}). Anther of *S. ionantha* cv "Fan 2, violet group 83 A" and "Fan 4, white group 155 D" with uninuclear microspores were cultured on MS medium + NAA + Kin (2.5 mg.l^{-1}.After 2 -3 weeks of culture, EC was formed. The PR of *Sainpaulia* plantlets followed the process of SEm to the plantlets formation. ***PELARGONIUM*** **cvs.** Plantlets of *P. hortorum* developed only from stem explants were cultured on the LB medium + Kin + IAA (1.0, 2.0 mg.l^{-1}, respectively). With both species (*Saintapulia, Pelargonium*), PR was effected organogenitically, following the pattern: callus ⇒ shoot ⇒ root ⇒ plant. Albino plants were observed in the both case, as well as somatic chimeric mutations of leaves. Mutations of flowers were observed in *Saintpaulia.*

Discussion

The increase interest in application of tissue culture techniques for clonal propagation of different horticultural plants is indicated by the numerous plant species regenerated by *in vitro* approaches (Holdgate, 1977). The methods applied so far are aimed at the production of a large number of normal regenerated plants. Our results show that any of the three approaches (androgenesis, somatic embryogenesis, organogenesis) used throughout this work strongly depend on the culture conditions, primarilly culture medium composition, species or cultivars. In the future, the application of biotechnological techniques, such as genetic engineering coupled with conventional crossing and breeding programs, may be useful alternatives in producing novel, cut and/or potted plant varieties.

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A SURVEY OF EUROPEAN RESEARCH METHODOLOGY AND PROBLEMS IN PLANT TISSUE CULTURE

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Introduction

This work involved the distribution of a detailed questionnaire to tissue culture laboratories around Europe in order to examine the research methodology in use and the problems typically encountered in tissue culture. Individuals were asked a number of questions under the following broad headings;

- Culture conditions (containers and medium type)
- Culture problems (e.g hyperhydricity, contamination)
- Disease and contamination elimination
- Testing methods for contamination problems
- Weaning
- Biotization of plants
- Certification of plant material
- Research – in which field is it most necessary?

Materials and Methods

A total of three hundred and fifteen completed questionnaires were returned. The results of the questionnaire were analysed using SPSS 12.0.1 for windows.

Results and Discussion

Not all respondents answered all of the questions on the questionnaire and not all answered in the format requested. The results for each category varied widely (a full discussion is given in the poster). The majority of respondents listed research into 'faster propagation methods' as the field in which most work is needed. The methodology it seems remains quite traditional with the most popular tissue culture containers used still being glass and plastic and the medium (approximately 63% of respondents) being Murashige and Skoog. Culture contamination appeared to be the most frequent problem encountered in the laboratory (bacterial and fungal contamination had the highest frequency). For the elimination of contaminants meristem culture was the main method used followed by chemotherapy. The majority of respondents (approximately 58%) said that they did not biotize their plants and that there is no need for the certification of material. According to the respondents other areas where future research should be directed included transgenic research, rejuvenation of starting material, secondary metabolite production, somatic embryogenesis and protoplast fusion amongst others.

IN VITRO ESTABLISHMENT OF TISSUE CULTURES FROM A LOCAL AUSTRIAN ACCESSION OF QUINCE SHOWING FIELD RESISTANCE TO FIRE BLIGHT

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Introduction

Old pip fruit cultivars constitute an essential part of the rural landscape of South East Austria. Moreover old varieties of quince (*Cydonia oblonga* Mill.) like "Alexia" represent a valuable raw material for distillation of special brandies with unique flavour and excellent taste. In 2003 an orchard planted with quince "Alexia" was infected by *Erwinia amylovora* (Fire Blight) and had to be rooted out according to national phytosanitary regulations. Since some plants within the infected orchard had shown no visible symptoms of bacterial infection, they were transplanted to pots and transferred to the AGES for further analysis of disease status and *ex situ* conservation of germplasm via micropropagation.

Materials and Methods

The disease status of the mother plants was repeatedly checked by PCR (Stöger *et. al.*, 2005) and found negative for three plants.

Shoot tips of (3-5 mm) of quince "Alexia" were isolated from growing buds of one greenhouse grown plant about three weeks after flowering. After surface sterilisation (Laimer *et. al.*, 1991) explants were kept on ice in MS-liquid medium in the dark. Isolated shoot tips were cultured in Cellstar® TC-plates with 24 wells (Greiner) using three different stage I media (see Table 1) at 23 ± 1 °C and a 16 hour photoperiod with light intensity of approximately 40 µmol $m^{-2}.s^{-1}$. Shoot tips on medium 1 and 2 were kept in the dark for 48 h after isolation. Explants were put on fresh liquid medium after four days, and after 16 days explants were transferred to semisolid Medium 2 into GA7 magenta vessels. For shoot multiplication regenerated plants were transferred to micro boxes (vessel height 800 mm) with hermetic covers and breathing strips (Duchefa) filled with 60 ml MS-medium C1 3%(w/v) sucrose or 3% (w/v) sorbitol, IBA 0.1 mg l^{-1}; BAP 0.4 mg l^{-1}; pH 6.2 after eight weeks. Regeneration and development of plants was scored during a period of three months after isolation (compare Table 1).

Results and Discussion

All types of media used were suitable for culture initiation of quince "Alexia". Regeneration on medium 2 was most efficient with a regeneration rate of 82% compared to 41% on medium 1 and 37% on medium 3. Plants regenerating from medium 2 were developing faster than from media containing mannitol. Since reduced nitrogen content of medium 2 resulted in yellowing of leaves and insufficient shoot elongation, shoot multiplication C1 medium was used for further multiplication of plants. Replacing sucrose by sorbitol in medium C1 had an advantageous effect on multiplication rate of quince "Alexia". Nodal cuttings in microboxes obtained a plant height of 5-8 cm within four weeks. On the contrary, growth of microcuttings on medium B1 (Brisset *et al.*, 1988) with the same growth regulator combination as C1 was severely impaired. This can be explained by the higher pH in medium C1 as well as by different composition of macroelements in the media (Fisichella *et al.*, 2000). These results indicate that the combination of increased pH, liquid medium and reduction of nitrogen during the start-

ing phase of shoot formation is essential for the successful establishment of quince "Alexia."

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Figure 1 Shoot regeneration response of quince "Alexia" using different sugars and growth regulator combinations

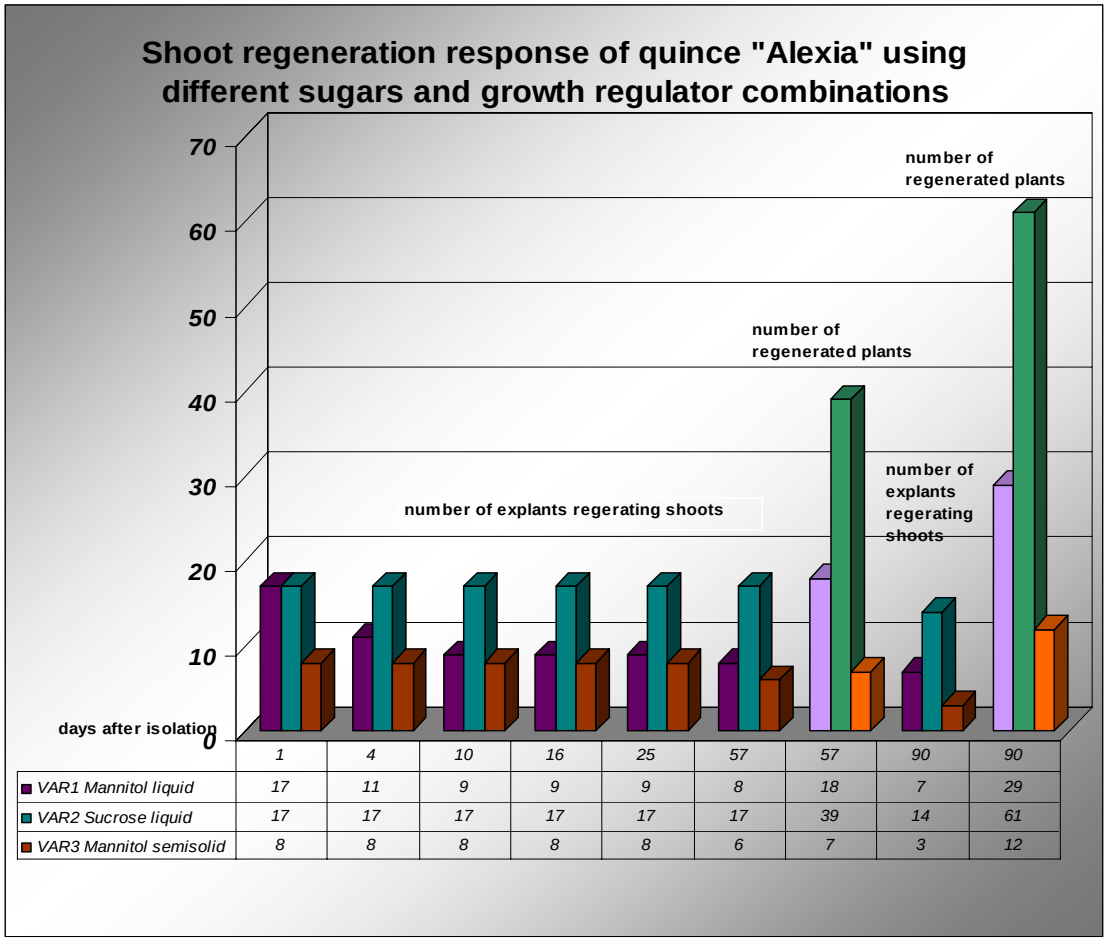

Table 1 Composition of stage I and II culture media

	Medium 1	**Medium 2**	**Medium 3**
Macroelements	MS	COST 5[1]	MS
Microelements	MS	MS	MS
Vitamines	B1[2] + folic acid 0.5 $mg.l^{-1}$	B1 + folic acid 0.5 $mg.l^{-1}$	B1 + folic acid 0.5 $mg.l^{-1}$
Sugar	Mannitol 3% (w/v)	Sucrose 3% (w/v)	Mannitol 3% (w/v)
Agar	liquid	liquid and Daishin 0,6%	Daishin 0,6%
Growth Regulators ($mg.l^{-1}$)	IBA 0.1; BAP 1; GA3 0.1	IBA 0.03; BAP 0.	IBA 0.1; BAP 1; GA3 0.1
PPM®	0.25%	0.25 %	0.25 %
pH	6.4	6.4	6.1

[1] COST 5 with reduced macro elements ($mg.l^{-1}$):
KH_2PO_4 (125)**;** KNO_3 (1250); Mg SO_4 x 7 H_2O (185); NH_4NO_3 (825); CaCl2 x 2 H2O (220)

[2] Brisset, M., Paulin, J. & Duron, M., 1988. Feasibility of rating fire blight susceptibility of pear cultivars (*Pyrus communis*) on *in vitro* microcuttings. Agronomie 8, 49-52.

BENEFICIAL USE OF LIGNOSULFONATES IN *IN VITRO* PLANT CULTURES

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In order to improve rooting and vigour for acclimatization, two critical stages in micropropagation, lignosulfonates (LIGNs), low-cost waste effluents from the paper industry, have been tested. These complex polymers result from the solubilization of lignin in alkaline conditions and, according to the nature of the base used during this chemical process, various chelated types were distinguished such as Fe-, Ca- or K-chelated lignosulfonates (Fe-LIGN, Ca-LIGN, K-LIGN).

LIGNs are commercialized as dispersal or binding agents for several industrial purposes. For agriculture, experiments in laboratory, glasshouse and field had showed their stimulating effects on both vegetative and reproductive growths; their favourable effect on the development of the root system was particularly noticed (Soteras, 1994; Telysheva *et al*., 1992, 1997). Quite similar results were obtained in several *in vitro* culture systems such as callus growth, shoot multiplication and rooting (Kevers *et al*., 1999).

In this context, our aim was to extend the *in vitro* application of LIGNs at different plant types, looking at their effects at the successive developmental processes involved in micropropagation.

The present results show the beneficial effects of various LIGN applications along growth of a tropical orchid, *Phalaenopsis*, multiplication of *Saintpaulia ionantha* and rooting of poplar and S*equoiadendron sempervirens* shoot cuttings. The most interesting observations were the stimulations of growth of *Phalaenopsis* and of rooting of *Sequoiadendron* by Ca-LIGN. The significant and reproducible effect of this LIGN on S*equoiadendron* rooting offers an ideal model to further investigate the mechanism of action of LIGNs in plant cells.

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POTENTIAL REGENERATION CAPACITY OF ROOTSTOCK *Prunus incisa x serulla*

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Introduction

In vitro clonal propagation methods are reported for commercial applications in the tree fruit production. Last decades the exploitation of the regeneration capacity of woody perennial species is one of the more important aim of many researcher. The development of micro propagation technologies were applying for the production of new rootstocks and varieties cultivated on their own roots (Druart, 1980a, 1992; Parfitt and Almehdi, 1986). More later the studies were focused on development the regeneration systems such as adventive budding (Druart, 1990a; Mante *et al*., 1989; Miguel *et al*., 1996), protoplasts cultures (Ochatt, 1991; Ochatt and Power, 1992; Kondakova & Druart, 1997)/ and somatic embryogenesis (Thorpe, 1995 Druart , 1978, 1980b, 1981, 1984).

Irrespective of difficulties the regeneration of rootstocks from protoplasts, leaves and roots, is great interests to develop regeneration system from this sources. From the breeding point of view the main benefit is the recover of potential ability and produce a new clones which often have new nature.

This is the first report for a general investigation on whole plant regeneration from mesophyll protoplasts, leaves and roots of rootstocks *P.incisa x serulla*.

Materals and methods

Our study was directed to revealed of potential regeneration capacity of rootstock *P.incisa x serulla* and define a key factors in the application of tissue culture techniques for the regeneration of woody perennials species. Different plant sources were study - leaves, roots, mesophyll protoplasts and induction of somatic embryogenesis from leaves and roots. Shoots of *P.incisa x serulla* were monthly sub cultivated *in vitro* on 699 medium (Druart, 1988). Different kind of hormones, concentration and basal medium were study.

Results

The leaves regeneration was accomplish by cultivated of young leaves on MS basal medium with the addition of 0.01 $mg.l^{-1}$ NAA, 2 $mg.l^{-1}$ BAP (MSR_2 medium). The single regenerated shoots were observed on middle part of leaves. Roots separated from one month old *in vitro* plants were plated on ½ QL basal medium supplemented with 5 $mg.l^{-1}$ BAP, 0.5 $mg.l^{-1}$ GA and double quantity vit.MS (B_5b medium). In two cases the medium was prepare with 20 $g.l^{-1}$ sucrose, 6 $g.l^{-1}$ agar and pH 5.7.

The recovering of whole plants whom mesophyll protoplasts isolated from *in vitro* growing leaf tissues were obtained. The protoplasts yield (19.8×10^6 protoplasts/g fresh weight and viability 92%) were significantly enhanced after a seven-day pre-treatment.

The initial plating density of 2.5 x 10^5 protoplasts/ml culture medium was required for successful cell division. The regeneration capacity was assessed after buds formation as result of different hormonal effect. Several regenerants from different protoclones have been *in vitro* propagated and *ex vitro* established.
The study of embriogenic competence in *P.incisa x serulla* reacted from leaves and roots is described.

Discussion

The main key factors influencing on regeneration ability were determination - genotypes, age of the *in vitro* shoots and toots; hormonal balance, cultural conditions. The regeneration response has to be optimized according to these factors.

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EFFECT OF ALIEN CHROMOSOMES ON THE *IN VITRO* MORPHOGENIC RESPONSE IN WHEAT-ALIEN CHROMOSOME ADDITION LINES

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Introduction

Wheat - alien chromosome addition lines proved to be a suitable system in studies on effect of alien chromosomes on genetic control of callus culture differentiation ability. In such lines the contribution of each alien chromosome pair to *in vitro* response can be easily evaluated. That is why it is important to investigate the effect on the *in vitro* response of alien chromosomes of species related to wheat for which appropriate marker lines are available. The effect of different rye and barley chromosomes on the *in vitro* response on immature wheat embryos was investigated in wheat-rye and wheat-barley addition lines (Lazar *et al*., 1987, Hay and Pienaar, 1990; Molnar-Lang *et al*., 1996).

The aim of present investigation was to study the influence of each *Aegilops longissima* and *Agropyron elongatum* chromosome pair on morphogenic ability of *in vitro* callus culture in wheat - *Aegilops longissima* and wheat - *Agropyron elongatum* addition lines. Identification of chromosomes containing genes that control tissue culture response would be of great importance for efficient *in vitro* manipulations of wheat.

Material and Methods

An euploid form of cultivar Chinese Spring (2n= 42, AABBDD), seven disomic addition lines (2n=44) of Chinese Spring with chromosomes of *Aegilops longissima* and the amphidiploid Chinese Spring/*Aegilops longissima* (2n=56, AABBDDS^{l}S^{l}), seven disomic addition lines (2n=44) of Ch. Spring whith chromosomes of *Agropyron elongatum* as well as the amphidiploid Chinese Spring/ *Agropyron elongatum* (AABBDDEE) were used as donor plants.

The isolated immature 14 days old embryos from the addition lines and amphidiploids were cultivated for callus induction and than for regeneration on MS (Murashige and Skoog, 1962) nutrient medium, modified by us (Zagorska *et al*., 1991). The data were subjected to ANOVA followed by Student`s t-test.

Results

The data from the Table 1 showed that all addition lines involved in this study were of high callogenesis (about 80%). Substantial decrease in number of initiated calli was observed in cultures of line D which contained long arm of chromosomes 7S^{l} and 4S^{l} from *Aegilops longissima,* as well as of the addition line containing 5E chromosome from *Agropyron elongatum.*

Significant differences among the lines were observed in their regeneration ability. There exists difference in the mean values of both parameters - frequency of regenerative calli and coefficient of propagation. Much higher percentage of morphogenic calli and more regenerants were obtained in lines A,B,E and to some extent - in line G, containing 2S^{l}, 6S^{l} and partially 3S^{l} chromosomes of *Aegilops longissima*. The chromosome 1S^{l} imposes a negative effect on the number and pecentage of morphogenic calli, and the 5S^{l} chromosome affects negatively the propagation coefficient. No differences from the control was observed in the amphidipliod AABBDDS^{l}S^{l} containing all chromosomes from Chinese

Spring and *Ae. longissima*. Significant differences in the morphogenic ability of the varions lines was established in Chinese Spring / *Agropyron elongatum* addition lines, as well. The highest percentage of morphogenic calli and more regenerants were observed in 1E addition line, following by the 6E line. The strongest suppression of morphogenesis was observed in the line carrying 4E chromosome. The influence of 3E and 5E chromosomes is weaker. No difference from the control was observed in the 2E and 7E addition lines and in the amphidiploid (AABBDDEE).

Discussion

As can be seen from the results, *Ae. longissima* chromosomes $2S^l$, $6S^l$ and partially $3S^l$ exert a positive effect on the morphogenic potential of the callus cultures. The same is for chromosomes 1E and 6E from *Agr. elongatum*. The influence could be a result of genes localized of these chromosomes which affect positively the morphogenic response of the embryo cultures or modify the negative effect of genes from the wheat genome (Lazar *et al.*,1987).

The strongest suppresion of organogenesis in the line carrying 4E chromosome may be due to the genetic effect of this chromosome wich is supposed to carry a factor affecting negatively the organogenesis. As it was already mentioned, no difference from the control was observed in the amphidiploids containing all chromosomes of Ch. Spring and *Ae. longissima* or Ch. Spring and *Arg. elongatum*. In our opinion that might be due to the balanced chromosome number in the amphidiploids. Similar data has been reported by Lazar *et al*. (1987) in a hybrid between Ch. Spring and the rye cultivar Imperial.

The variation in the *in vitro* response observed in our study clearly demonstrates that alien chromosomes can improve *in vitro* wheat differentiation and plant regeneration. This finding provides new alternatives for improving plant regeneration of genotypes and species which currently exibit relatively poor response.

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Table 1 Morphogenic response of *in vitro* cultivated immature embryos of wheat (cv. Chinese Spring) with disomic addition of *Aegilops longissima or Arg. elongatum* chromosomes

Li-nes	Added chromosomes	Tested embryos	Induced calli		Morphogenic calli		Fully develop plant-lets	No of regenerated plants per morphogen. callus
		No	No	%	No	%	No	
CS	control	242	213	88.06	102	47.86	51	0.5
A	$2S^I$	161	137	85.09	94	68.61 **	100	1.06 ***
B	$6S^I$(-6B) +$2S^IL/1S^IL$	166	138	83.31	76	55.38 *	82	1.08 ***
C	$1S^I$	171	145	84.53	63	43.38 *	48	0.76 *
D	$7S^IL/4S^IL$	152	104	68.16 *	53	51.12	30	0.57
E	$2S^I$+$6S^I$ (-6B)	180	141	78.33	103	73.05 **	139	1.35 ***
F	$5S^I$	152	132	86.58	62	46.77	20	0.33 *
G	$3S^I$	188	160	85.32	87	54.66 *	44	0.51
TL$^-$ AD	CS/*Ae. longissima*AD	123	94	76.10	46	49.13	24	0.52
CS		214	181	84.6	52	28.7	41	0.78
1E		186	172	92.5 *	73	42.4 **	147	2.01 ***
2E		178	159	89.3	50	31.4	51	1.02
3E		157	130	82.8	28	21.5 *	11	0.39 *
4E		180	162	90.0	18	11.1 **	8	0.44 *
5E		168	127	75.6*	38	29.9	95	0.40 *
6E		188	167	88.8	68	40.7 **	107	1.57 **
7E		168	148	88.1	51	34.4	58	1.14 *
AABBDDEE		170	146	85.9	48	32.8	56	1.17 *

Legend: *, **, *** - statistically significant at P=0.05; P=0.01; P=0.001

INVESTIGATION ON THE RESPONSE OF STABILIZED INTERGENERIC WEATH HYBRID LINES AND AMPHIDIPLOIDS TO *IN VITRO* CULTIVATION AND *IN VITRO* SELECTION

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Introduction

Plant adaptation to abiotic and biotic stress as well as the development of resistant crop cultivars are the challenges of modern plant science. Wild plant species are a rich source of valuable traits including resistance to stress factors, which may be introduced into crop plants. The methods of tissue culture allow observing directly the *in vitro* plant response to stress as well as to identify tolerant genotypes.

The aim of this study was to investigate the response of intergeneric wheat - wheat grass hybrid lines and wheat amphidiploids to *in vitro* cultivation in control and simulated drought conditions.

Material and Methods

Explants from immature embryos and anthers of 17 wheat-wheat grass hybrid lines obtained through intergeneric hybridization of three *Triticum aestivum* cultivars (Bezostaya, Sadovo and Russalka) with *Thinopyrum intermedium* and the amphidiploids *T.aestivum / Thinopyrum elongatum, T. durum / Th. elongatum* and *T. durum / Haynaldia* villosa were used in the experiments. The immature embryos were cultivated on Murashige and Skoog (MS), 1962, nutrient medium modified by us. AMS3 and ACIM nutrient media were used for callus induction in anthers. To simulate water deficiency polyethylene glycol (PEG) 6000 Sigma was added. For this purpose, at rooting the regenerants were grown for three weeks on the following nutrient media: VK1-control, VK1+5% PEG, VK1+10%PEG. The data were subjected to ANOVA followed by Student`s t-test.

Results

The percentage of the produced calli was high for immature embryo explants from all lines but the genotypes differed in their morphogenic potential (Table 1). Statistically proved higher frequency of the morphogenic calli compared to the control, cv.Sadovo, was observed in five lines: №31, №36, №67, №68 and №69.Four lines were of lower morphogenic calli frequency: №30, №53, №63 and №66.

Relatively lower callus induction was observed in all of three amphidiploids, *T. durum / Th. elongatum* AD being of lowest callus induction.It is also of lower regeneration frequency, unlike the amphidiploid *T. durum / H. villosa* which is of highest regeneration frequency among the three amphidiploids.

The calli from anther culture have much lower embryogenic potential than those from immature embryos (Table 1).Twenty genotypes were investigated. Only the lines in wiich callus induction in anthers was succesfull are presented in the table. The total number of the genotypes produced calli was thirteen.Ten of them produced calli on AMS3 nutriend medium and ten - on ACIM.Eight genotypes produced calli on both nutrient media, two- only on AMS3 and three - only on ACIM. Seven genotypes did not produce calli showing no preference to any nutrient medium examined. The difference in nutrient media was expressed as different percentage of the calli induced in each genotype. The total percentage

of calli obtained from all genotypes on AMS3 medium was 37.63% while that on ACIM medium was 70.66%. The data indicate considerable advantage of ACIM nutrient medium. Best response to *in vitro* cultivation was manifested by lines № 68. It produced comparatively high callus percentage on both nutrient media (AMS3 - 8.6% and ACIM - 15.0%). The lines №28 (11.33) and №36 (10.7%) grown on ACIM nutrient media were of relatively high percentage of callus production.

As it was mentioned above the regenerants produced by embryos were rooted in media, to which 5% or 10% PEG was added. The results indicated that the addition of PEG to the nutrient media resulted into decreased rooting for all genotypes. The percentage of well rooted regenerants varied largely among the genotypes. The wheat-wheat grass hybrid line № 31 and the amphidiploid *T.durum/ H.villosa* showed best rooting. They expressed stability and high values of the examined parameters (mean root length and stem height).

Discussion

As already was mentioned, the percentage of produced calli was high for immature embryo explants from all lines tested without significant variation among the lines. This fact indicates lack of genotype differences at that stage of embryo development and is of agreement with Immonen (1993). The data revealed no correlation between the degree of calli induction and the regeneration capacity of the genotypes studied, suggesting that both phases of the *in vitro* response are controlled independently, as it was found by Henry *et al.* (1994). It is evident from the results presented that callus induction in anther cultures, unlike those in immature embryos, is largely dependent on the genotype. This is in agreement with Agarhe *et al*. (1988; 1989), who found independent genetic control of callus induction from anther cultures and from immature embryos.

It was established in our study that the investigated genotypes expressed stability and high values of the examined parameters on selective media. These genotypes exceeded Sadovo`s tolerance to drought, so it may be concluded that they are significantly drought tolerant, WGH №31 and *T.durum / H. villoza* amphidiploid being of highest adaptability potential. Therefore they may be efficiently involved in various projects of wheat breeding to increase wheat drought resistance

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Table 1 Callus induction and regeneration from in vitro cultivated immature embryos and anthers of different wheat cultivars and stabilized intergeneric hybrid lines

Explants	Immature embryos				Anthers	
Genotypes	**Frequency of callus induction (%)**	**Morphogenic calli (%)**	**Regenera-tion frequency (%)**	**Propaga-tion coefficient**	**Medium**	**Anthers produced callus**
Sadovo	91.0	53.61	51.1	1.97	AMS3 ACIM	1.60 2.80
Bezostaya	88.0	56.38	47.3	1.63	AMS3 ACIM	2.50 3.80
Roussalka	94.0	48.12	59.3 *	1.45	AMS3 ACIM	7.63 6.50
WWGH 14	96.0	51.38	46.4	1.83	AMS3 ACIM	1.0 0.0
WWGH 28	92.0	49.56	47.8	2.91 **	AMS3 ACIM	- 11.33
WWGH 30	92.8	43.92 *	38.6 **	1.98	AMS3 ACIM	- 4.0
WWGH 31	81.9	70.5 **	68.6 **	2.68 *	AMS3 ACIM	2.80 4.60
WWGH 36	97.4	69.81 **	59.4 *	1.62	AMS3 ACIM	- 10.70
WWGH 61	95.7	59.16	57.8	2.08	AMS3 ACIM	3.70 -
WWGH 63	84.7	40.78 *	34.7 ***	1.8	AMS3 ACIM	4.70 -
WWGH 67	96.3	65.11 *	59.8	2.58 *	AMS3 ACIM	3.80 5.0
WWGH 68	91.7	72.81 **	64.7 *	1.36	AMS3 ACIM	8.60 15.0
WWGH 69	97.5	78.54 ***	72.1 **	2.63 *	AMS3 ACIM	1.30 6.93
AABBDDH^{v} H^{v}	71.9	72.9	67.3	4.23	-	-
AABBEE	55.7 *	64.3	54.7 *	2.84 *	-	-
AABBDDEE	69.3	55.7	40.8 **	2.71 *	-	-

*, **, *** -significant at P = 0.05; P =0.01; P = 0.001; respectively

ESTABLISHMENT OF LONG-TERM ORGANOGENIC CULTURES OF PEA (*Pisum sativum*) FOR CROP IMPROVEMENT PROGRAMS

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Introduction

Legumes with their high nutritional value play an important role in human and animal diet, and recently, increasing attention is paid to their use in sustainable agriculture. However, genetic improvement of legumes is accompanied by different problems requiring interdisciplinary studies. In this sense plant biotechnology offers original approaches and methods for crop improvement including grain legume breeding (Griga *et al.,* 2001).

Materials and Methods

Two varieties (Sredetz and Balet) and more than 30 Bulgarian lines created by experimental mutagenesis and carrying different commercially important traits (higher productivity, higher protein content, shorter vegetation period, afila, lodging resistance, higher tolerance to biotic and abiotic stress) were screened for their *in vitro* response. Embryonic axis from immature embryos and cotyledonary nodes from germinating mature seeds were used as explants to study potential of the different genotypes to callogenesis, organogenesis and regeneration of plants (Kosturkova *et al*., 1997; Kosturkova *et al.*, 2003]. The influence on cell development and the interaction of different biological (explant, genotype), chemical (media composition, plant regulators) and physical (temperature and light parameters) factors were studied following several criteria: initiation of organogenesis, the percentage of explants forming adventitious buds, the number of buds, index of multiplication (ratio of the number of buds on initial medium to the number of buds on medium for maintenance). To study the effect of different substances (o-alyl-thioureido benzoic acid (ATB), "Humostim", "Moldstim", "Sencor", culture filtrate from the pathogenic fungi of *A. pisi* complex) they were included in the media.

Results

The development of embryo axes from immature embryos on MSPE media for bud formation (Kosturkova *et al.*, 1997) was in the range of 55% to 95% for the 10 lines tested (Table 1). The average number of newly formed buds per explant varied between 1.5 and 3.2 and their size was generally from 0.3 to 0.8 cm. However, bigger shoots up to 2 cm could be formed. Transferred to MSPF media for further development the bud clusters continued to generate new buds and shoots with index of multiplication from 1.5 to 3. Organogenesis was genotype dependent and 3 lines (Sredetz, line 155 and line 268) with better organogenic potential could be elected.

When cotyledonary nodes were used as explants, development of newly formed green buds was initiated on the second week and at the 21st day 58% to 100% of the explants have formed buds on OCHM medium (Kosturkova *et al*., 2003), Table 2. The mean number of buds per explant varied between 1.5 and 3. Bud size ranged from 0.1 cm to

2.0 cm. These explants transferred to OCHMG medium for further development continued both, formation of new buds and growing of shoots. The index of multiplication was higher for Balet (3.6) and Sredetz (4.17) compared to the mutant lines (from 2.12 to 3.30). However, as the lines were characterized by different valuable traits most of them could be used in genetic programs. As a result of the experiments suitable explants and media composition, as well as, lines with better *in vitro* response were chosen.

Once initiated, organogenesis in many lines could be maintained when transferring organogenic cultures from media for bud induction to media for shoot growth and again on media for bud induction. Thus a system was created for efficient regeneration and maintaining of organogenic cultures with index of multiplication up to 50 for more than one year. Flowering and setting of pods, though in low rate, was achieved in the test tube in cultures after 1-2 passages, as well as after 2 years of *in vitro* cultivation. In the latter case flowering was stimulated by γ-irradiation with comparatively low dose, as well as, by treatment with "Humostim" - a natural organic fertilizer (data not published). Prolonged cultures and the high multiplication rate made possible using *in vitro* pea cultures to study the effect of new substances (Mehandjiev *et al*., 2002; Kosturkova *et al.,* 2004) and for *in vitro* modeling of biotic and abiotic stress (Kosturkova *et al*., 2001; Rodeva *et al.*, 2004). As a result for the first time was observed the stimulation effect of ATB, "Humostim", "Moldstim" and γ-rays on organogenesis *in vitro*.

Applying step-selection pea cultures with higher tolerance to culture filtrate of the pathogenic fungi were selected. These organogenic cultures retained their tolerance for more than one year being in culture with or without selective pressure (Table 3).

Discussions

These systems for efficient regeneration and prolonged maintaining of organogenic cultures could be further used in other genetic, physiological, biochemical and other studies and in breeding programs for more efficient tests and selection for resistance.

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Table 1 Induction of organogenesis on MSPE medium from embryonic axes of immature embryos of pea

Line/ variety	Organogenesis [%]	Explants with more than one bud [%]	Mean number of buds per explants
Средец (Sredetz)	78.0	93.7	3.2
424	83.3	56.0	3.3
383	95.0	85.0	3.0
350	95.0	70	3.5
337	100	75	3.0
333	87.0	79.8	2.5
268	70.6	83.3	2.9
155	80.0	73.3	3.2

Table 2 Development of explants from cotyledonary nodes plated on OCHM medium for organogenesis induction and further development on OCHMG medium

Variety/ Line	Bud development [%]	Number of buds per explant on OCHM	Number of buds per explant on OCHMG	Index of multi-plication
Sredetz/Средец	100.0	2.62 ± 0.22	9.6 ± 0.27	4.17
Balet/ Балет	92.9	3.00 ± 0.27	10.8 ± 0.25	3.60
52	62.0	2.00 ± 0.10	4.6 ± 0.22	3.30
56	100.0	2.30 ± 0.15	6.8 ± 0.21	2.95
88	92.3	1.51 ± 0.25	3.2 ± 0.15	2.12
177	58.4	2.28 ± 0.25	7.5 ± 0.25	3.29
191	81.0	1.90 ± 0.17	5.2 ± 0.22	2.73
317	80.0	1.87 ± 0.13	4.3 ± 0.23	2.30

Table 3 Development of selected and non selected pea organogenic cultures in normal (culture media without *A. pisi* culture filtrate - CF) and in stress conditions (media containing sublethal concentrations of fungal CF).

N	Variants of culture conditions	Mean number of buds			Size
		Initial	On the 9th day	On the 14th day	[mm]
1	Cultures without selection in normal conditions	14	15	24	around 2
2	Cultures after selection in normal conditions	17	17	32	around 2
3	Cultures without selection in stress conditions	18	10	17	around 1
4	Cultures after selection in stress conditions	27	33	60	up to 2

MICROPROPAGATION *IN VITRO* OF *Rhodiola rosea* L.

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Introduction

Rhodiola rosea L. (*Crassulaceae*) is a perennial grass of quite limited distribution. Over the last years *Rh. rosea* area drastically decreases because of over exploitation. Therefore the species in included in the Red Book of Bulgaria. The plant contains biologically active substances: flavonoids: rosavin (2.1%), salidrosid (0.8%), rhodiolin, antraglikosides, β-sitosterin, monoterpenoids and tanins (16-18%) (Satsiperova *et al.*, 1993). Substances isolated from the roots are highly efficient in therapy of nervous system diseases, during rehabilitation after infections, in cancer prevention (Kelly, 2001), etc.

There exists only quite insufficient reference concerning *R. rosea in vitro* propagation. Kaftanat *et al*. (1988) have elaborated a method to root shoots segments. Kirichenko *et al*. (1994) studied callus induction and regenerating ability in the leaves. Ishmuratova (1998) obtained regenerants from stem segments about 10-95% of them survived after acclimatization in soil.

The purpose of our experiments was to elaborate an efficient method for *in vitro* propagation of *Rhodiola rosea* L. and its application in the practice.

Material and Methods

Rh. rosea germination *in vivo* is low. Seeds of *R. rosea* were collected from natural locations in the Rila Mountain and were used for *in vitro* culture initiation. To stimulate seed germination the seeds were treated with 0.03% gibberellic acid (GA_3) for 24 h. Ten variations of Murashige and Skoog (1962) nutrient medium (MS) containing 25, 50, and 100 $mg.l^{-1}$ gibberellic acid respectively and 0.4-0.6% agar-agar were tested.

The plants obtained *in vitro* were cut into 2-3 explants with a stem and an apical bud. The explants were grown on 10 variants of different amount of nutrient medium containing organic supplements and phytohormons - zeatin, 2-ip, BA, IAA, NAA and caseinhydrolysate. Sucrose and agar-agar content was constant, 3.0% and 0.7%, respectively. Regeneration proceeded under 22 ^{0}C and 2500 lx illumination at 16 h photoperiod.

Results and Discussion

R. rosea seed germination reaches 20% in the control variants. Highest germination was obtained at MS nutrient medium containing 50 $mg.l^{-1}$ gibberellic acid and 0.4% agar-agar - 84.38% on the 20th day. Germination lasted from the 8th until the 40th day.

Our data suggest that nutrient medium composition and particularly phytohormone concentration are very important for *in vitro* induction of organogenesis and regeneration.

In vitro Shoot Proliferation

Ten variants of nutrient medium based on that of Murashige and Skoog (1962) with different phytoregulators were tested. MS-Z medium containing 2 $mg.l^{-1}$ zeatin and 0.2 mg. l^{-1} IAA as well as the MS-B medium containing 2 $mg.l^{-1}$ BA and 0.2 $mg.l^{-1}$ IAA proved to be the most efficient for propagation. The mean number of regenerants per explant on MS-Z medium was 3.78 with mean plant height 3.84 cm. In the second passage to stimulate propagation the concentration of growth regulators was reduced. Certain increase up to

7.65 shoots per explant on the average was induced by nutrient medium MS-Z1 containing 1 $mg.l^{-1}$ zeatin, 0.1 $mg.l^{-1}$ IAA and 500 $mg.l^{-1}$ casein hydrolysate, some explants developing 9 shoots. Induction of bud formation was effective on MS-B obtaining up to 3.6 buds per explant. However, buds were small and they were transferred on nutrient medium of reduced BA concentration (1.0 $mg.l^{-1}$ and 0.5 $mg.l^{-1}$) to accelerate bud development. Thus, 5.7 shoots per explant of average height 2.25 cm could be obtained.

The data presented indicate that the location of a given explant on the donor plant stem largely affects *in vitro* micropropagation. The explants from apical buds initiate low regeneration.

In vitro Rooting

Rooting was performed using 9 diverse nutrient medium. Initiation of rooting occurred 15 day following planting. The nutrient medium containing IBA, IAA and GA_3 in concentrations of 2.0 $mg.l^{-1}$, 0.2 $mg.l^{-1}$ and 0.4 $mg.l^{-1}$, respectively, proved to be the most efficient one allowing 95.62% rooting.

The results of the experiments led to the development of an efficient system for *in vitro* micropropagation of the endangered medical plant species *Rhodiola rosea*. The system provides opportunities to protect the extinction populations and to cultivation plant stuff for the pharmaceutics industry.

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INTRODUCTION OF *Arnica montana* L. TO BULGARIA USING *IN VITRO* TECHNIQUE

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Introduction

Arnica montana L. (*Asteraceae*) is an herbaceous plant spread in the mountains of Europe. In some countries it is law protected. In Bulgaria *A. montana* is not spread as a wild species. The flowers, roots and rhizomes were widely used in traditional medicine. The main active constituents of *Arnica* are sesquiterpene lactones, flavonoids, volatile oil etc. The sesquiterpene lactones of *Arnica* are associated with anti-inflammatory and cytotoxyc effects, the flavonoids possess antimicrobial, antiflogistic and anti-rheumatic properties and the volatile oil is of antiseptic activity (Roki *et al*., 2001).

The first experiments for *A. montana in vitro* propagation were performed in the early nineties (Daniel and Bomme, 1991). The influence of various plant growth regulators on *A. montana in vitro* propagation was also examined. For bud initiation and proliferation different combinations of BA, kinetin, zeatin, 2- ip, thidiazuron, NAA, IAA etc were used (Lê, 1998, 2000; Butic-Keul and Deliu, 2001; Werememczuk-Jeyna and Wysokinska, 2000).

The purpose of the investigation is to introduce and to cultivate *A. montana* in Bulgaria through *in vitro* techniques.

Material and methods

Seeds of *A. montana* from the German Botanic Garden were used for *in vitro* culture initiation. Sterilization was performed with 70% ethanol and 0.1% $HgCl_2$ applied in combination or independently. The seed samples were rinsed three times for 5, 10 and 15 min in sterile distilled water. *A. montana* germinnation *in vivo* is low. To stimulate it the seeds were treated with 0.03% GA_3 for 24 h and 48 h, then the treated seeds were cultivated on five nutrient medium variants containing different amount of GA_3, 2% sucrose and 0.6% agar. After germination the seedlings were cut into fragments with an apical or axillary bud. The fragments were cultivated on ten nutrient media based on that of MS enriched with different growth regulators (BA, zeatin, 2-ip, IAA, NAA).

To induce root formation the regenerants were transferred to half strength MS medium supplemented with 1% sucrose, 0.4% agar and either IAA, IBA or NAA.

Results and Discussion

Shoot tips of *A. montana* are preferable for *in vitro* micropropagation (Lê, 1998, 2000; Wermemczuk-Jeyna and Wysokiñska, 2000). To initiated *in vitro* culture, we used plant material from seeds germinated *in vitro*. Two patterns of seed sterilization were examined. Disinfecting with 70% ethanol for 2 min and 0.1% $HgCl_2$ for 3 min resulted in 100% seed sterilization. Highest germination (62.00%) resulted from the treatment with 0.03% GA_3 for 24 h followed by cultivation on Murashige and Skoog (1962) nutrient medium enriched with 50 mg l^{-1} GA_3.

Regeneration proceeded on all kinds of nutrient media tested including MS medium without growth regulators. Among the tested nutrient media, containing cytokinins (BA, zeatin

or 2-ip) in various combinations and concentration, most efficient providing highest shoot formation, proved to be the medium supplemented with 1 mg.l^{-1} BA which is in agreement with the results of Lê (1998, 2000). The nutrient medium using from us contains 0.1 mg.l^{-1} IAA instead NAA.

On that medium the mean number of regenerants per explant reached 7.46 while some explants gave rise to 16 regenerants. The height of the regenerants varied from 2.25 to 4.90 cm.

Rooting was attained using different variants of nutrient media. The nutrient medium containing 0.5 mg l^{-1} indolebutyric acid proved to be most efficient allowing 98.67% rooting. Initiation of rooting occurred 7 days following planting.

The elaborated system for efficient *in vitro* micropropagation of *A. montana* L. in very important for its introduction to Bulgaria and production of plant stuff for the pharmaceutics and cosmetic industry.

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IN VITRO MORPHOGENETIC RESPONSES OF EPICOTYL AND COTYLEDON EXPLANTS IN BAMBARA GROUNDNUT [*Vigna subterranea* (L.) Verdc]

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Introduction

Bambara groundnut [*Vigna subterranea* (L.) Verdc] is an indigenous grain legume grown mainly by subsistence women farmers in drier parts of sub-Saharan Africa. The crop has several agronomic advantages including high nutritional value, drought tolerance and ability to produce some yield in soils that are too poor for cultivation of over species such as common beans and groundnuts (Anchirinah *et al*., 2001; Azam-Ali *et al*., 2001). It is very adaptable to hot temperatures but also tolerates rainfall (Dakora and Muofhe, 1996). It serves as an important source of protein in the diet of a large percentage of the population in Africa. Nutritionally, it contains 17.4% protein, 53.1% carbohydrate, 6.1% fat, 6.1% fibre, 3.4% ash, 0.098% calcium, 0.007% iron, 1.2% potassium and 0.003% sodium (Rowland, 1993; Amarteifio *et al*., 1997). The gross energy value of Bambara groundnut seed is greater than that of several pulses (Amarteifio *et al*., 2002; Lacroix *et al*., 2003). However, despite the importance of Bambara groundnut as a food legume, limited breeding efforts have been made to improve this crop (Wazael *et al*., 2004). Genetic improvement of Bambara groundnut by conventional breeding is slow and difficult due to the long generation time and preponderant homozygous nature of this crop. Genetic engineering techniques may facilitate the rapid transfer of valuable genes to the genome of well-established landraces, thus resulting in more efficient genetic improvement. A prerequisite for the improvement of Bambara groundnut using genetic transformation techniques is the availability of an efficient *in vitro* regeneration protocol. Grain legumes have been in general, more recalcitrant to *in vitro* manipulation than other species (Christou, 1997). Literature on tissue culture work on Bambara groundnut is scanty with only two reports. Ochatt *et al*. (2002) have reported on the acceleration of generation cycles using an in vivo plus in vitro strategy. Lacroix *et al*. (2003) report direct organogenesis from the existing meristems in embryo axes. But, when using different concentrations and combinations of BAP, TDZ, TIBA and NAA with explants derived from stem, root, leaf and cotyledon, only limited callus growth without shoot formation was observed. In this study, we report for the first time good callus proliferation, root formation and shoot organogenesis from epicotyl and cotyledon explants of Bambara groundnut.

Materials and Methods

Seeds of seven landraces from the Ivory Coast (Ci1, Ci2, Ci3, Ci4), Ghana (GB1, GB2) and Mali (MB) of bambara groundnut were used to investigate their morphogenetic response from *in vitro* culture.

Seeds were surface sterilized for 1 min in 70% (v/v) ethanol, followed by immersion in a 70% (w/v) solution of calcium hypochlorite for 30 min. The seeds were then rinsed 3 times with sterile distilled water and left to imbibe for 24 hours in sterile distilled water.

Two successive experiments were performed.

In *Experiment I*, Bambara groundnut (landraces Ci1, Ci2, Ci3 and Ci4) seed coats were removed and mature cotyledons excised taking care to remove the embryo axes. Small segments of cotyledons were cultured aseptically on various media supplemented with BAP, ANA, TDZ, ABA and Zeatine at different concentrations and combinations.

In *Experiment II*, the embryo axes of all landraces were removed from the cotyledons and were inoculated for germination onto MS (Murashige and Skoog, 1962) medium with sucrose (3%), agar-agar (0,6%) but without any growth regulators. The pH of the medium was adjusted to 5.8. *In vitro* grown seedlings (3-4 weeks) were then used as the source of epicotyl explants. Five

media were tested, all based on MS medium and including 3 mg.l^{-1} BAP alone or in combination with 0.01; 0.05; 0.1 and 0.5 mg.l^{-1} NAA.

In both experiments described above, the pH of media tested was adjusted to 5.5 prior to autoclaving. The cultures were maintained at a temperature of 25±2°C, under a 16/8 h (light /dark) photoperiod of 100 µE.m^{-2}.S^{-1} provided by warm white fluorescent tubes.

Each treatment (medium-explant-genotype) consisted of 5 replicates and each experiment was repeated at least six times. The experimental design was completely randomised (CRD). Percentage responses for callus induction, root or shoot organogenesis were recorded after 4 weeks of culture.

Results and Discussion

Experiment I. Nodular callus formation by cotyledon explants was the highest morphogenetic responses in all media tested with landraces Ci1, Ci2, Ci3 and Ci4. The percentage of callus formation was more important with landraces Ci2 and Ci3 (72%) and was obtained on media containing 3 mg.l^{-1} BAP in combination with NAA (0.01; 0.05; 0.5 mg.l^{-1}) or 5 mg.l^{-1} BAP in combination with NAA (0.1 and 0.5 mg.l^{-1}). Percentage of shoot formation was low and was especially observed with landrace Ci4 on media containing 5 mg.l^{-1} BAP + 0.1 mg.l^{-1} NAA. Landrace Ci2 and medium supplemented with 3 mg.l^{-1} BAP + 0.05 mg.l^{-1} NAA expressed the highest percentage of root formation (24 and 27%). Previous workers have also obtained a low frequency regeneration when using mature cotyledons in *Vigna radiata* (Gulati and Jaiwal, 1990; Chandra and Pal, 1995).

Experiment II. All media and landraces tested in this study led to callus formation. The lowest percentages were observed on medium containing 3 mg.l^{-1} BAP only (88%) and with landrace GB2 (89.50%). Epicotyl explants induced root formation frequently on medium supplemented with 3 mg.l^{-1} BAP + 0.5 mg.l^{-1} NAA (40%) and landrace Ci1 expressed the highest amount (21%). Shoot formation occurred on media containing 3 mg.l^{-1} BAP (7%) or in combination with 0.5 mg.l^{-1} NAA (6%). Epicotyl explants of landraces Ci2 (12%) and Ci4 (10%) induced respectively the highest percentage of shoot formation. Lacroix *et al.* (2003) obtained shoot organogenesis from meristems in the embryo axes of Bambara groundnut, but this is the first report on *de* novo regeneration from meristem-free explants of this species.

Regenerated shoots elongated on hormone-free MS medium and shoots derived from cotyledon explants on media supplemented with 5 mg.l^{-1} BAP + 0.1 mg.l^{-1} ANA rooted and flowered *in vitro*. It is also noteworthy that, from some cotyledon explants and only on medium with 5 mg. l^{-1} BAP and 0.5 mg.l^{-1} NAA, flowers could also be regenerated directly, without any intervening callus phase. Such result had never been observed in legumes before.

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EFFECT OF GROWTH REGULATORS AND TYPE OF EXPLANT ON PROPAGATION *IN VITRO* OF *Thymus mastichina*

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Introduction

Thymus mastichina L. is a perennial aromatic plant that is found in Spain and Portugal (Tutin *et al*., 1972). Aromatic plants are important to the pharmaceutical, cosmetic and food industries (Balandrin and Klocke, 1988). The interest in this group of plants is in the production of essential oils.

The objective of this study was to develop a micropropagation method for *Thymus mastichina* by using shoot tips and nodal cuttings explants combined with different growth regulators.

Materials and Methods

Actively growing vegetative shoots were collected in June from 8-months old *Thymus mastichina* plants and were used for the establishment of culture *in vitro. In vitro* proliferating shoots with 6-7 internodes, maintained on medium free of hormones, were used as explants source. The effects of the combination of auxins with cytokinins on the proliferation of shoots from two types of explants were investigated. Shoot tips, as well as nodal cuttings were excised from *in vitro* grown shoots and were used as explants in combination with various concentrations of NAA, BAP and Kinetin. Different concentrations of NAA or IBA were used for rooting micro cuttings *in vitro*.

Results

The number of proliferating shoots was significantly affected by the kind of the explant used. Shoot tip explants produced a higher number of axillary shoots per culture compared with nodal cuttings. Also there was a significantly difference among the nodal cuttings used, the youngest one giving the highest number of shoots.

The type of growth regulators used in culture affected the number of shoots produced by the shoot tip explants. The combination of BAP-NAA gave better results than the combination of Kinetin-NAA; the best concentration of BAP-NAA was 2 $mg.l^{-1}$ BAP and 0.05 $mg.l^{-1}$ NAA.

Vitrification was a mayor problem during *in vitro* shoot proliferation. Although the BAP was considered better than Kinetin for the higher number of proliferated shoots produced, BAP was responsible for the incidence of a large number of shoots with vitrification symptoms like shorter internodes, thick- brittle and translucent leaves. NAA was more effective than IBA for the rooting of micro cuttings *in vitro*.

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DIFFERENT WAYS OF REGENERATION OF *Spathiphyllum IN VITRO*

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Introduction

Spathiphyllum floribundum is an ornamental potted plant. It has long lasting showy white flowers and bright green leaves. It became a popular ornamental plant from the beginning of 1980's years. At the beginning the plants were propagated by seeds, but later the *in vitro* propagation was preferred, because the demand for greater uniformity and higher quality.

The first report on micropropagation of *Spathiphyllum* was published by Fonnesbech and Fonnesbech (1979) who cultured leaf and stem explants on MS medium (Murashige and Skoog, 1962), supplemented with 2 $mg.l^{-1}$ PBA and regenerated up to 9 shoots per explant. Webrouck *et al*. (1995, 1996) studied the effect of metatopolin (TOP) as an alternative to benziladenine (BA) in tissue culture of *Spathiphillum floribundum* cv. Petite. They compared the effect of these cytokinines during the propagation, rooting and acclimatisation phase. They studied the metabolism of these cytokinines and find, that only the use of metatopolin gave good post effect during the rooting and acclimatisation. Ramirez-Malagon *et al*. (2001) studied the effect of cytokinies and auxines on micropropagation of *Spatiphyllum floribundum*. They started the culture from the central stem of plants. They used MS medium in combination with BA, kinetin (KIN), NAA, IAA and 2,4-D. The best results in shoot number, leaf number/plant, size of plant was observed on medium which contained 2 $mg.l^{-1}$ BA +1 $mg.l^{-1}$ KIN and 1 $mg.l^{-1}$ IAA. The importance of using particular shoot size (20 mm) for better acclimatization was proved.

In Hungary Hegedűs (1993, 2005) published results in micropropagation of *Spathiphyllum*. She started the culture from flowerbud. It was important, that the spathe was closed, and the surface sterilisation happened in this condition. Following, the inflorescence (spadix) was used as explant. The culture was started on MS medium with 4 $mg.l^{-1}$ BA, 0.5 $mg.l^{-1}$ GA_3 and 0.2 $mg.l^{-1}$ IBA. After 8-10 weeks of culture the spadix swollen, and 10-15 mm long plantlets regenerated from the spadix. Then the little plantlets were cultured on propagation medium with similar additives. The successful propagation elongation and rooting was described as well with the *Spathiphyllim wallissii*.

At first the aim of our work was to work out the micropropagation of *S. floribundum* cv. Petite, with the starting of the culture from spadix. During the establishment of the culture we found an interesting way of regeneration similar to the GGB (green globular body) and later on our aim was to study this phenomenon.

Material and Methods

The culture was started from closed flowerbuds with closed spathe, and after the surface sterilisation the pieces of the spadix were used as explants. The surface strilisation started in running top water with the addition of a few drop of Tween 20 for an hour.

Following the buds were rinsed in 70% ethanol for 5 min. and in 0.1% $HgCl_2$ solution for 5 min. The buds were washed in sterile destillated water 3 times. From the spadix the spatha was cut off, and the spadix was cut in 10-20 mm long pieces transversal and longitudinal. The pieces were put on starting media. As basic medium MS medium was used with half concentration of macro-elements. For establishment of the culture BA 1-3 $mg.l^{-1}$ conc. and 0.1 $mg.l^{-1}$ NAA was added. The different media were supplemented with 20 (A4,5,6 media) or 30 $g.l^{-1}$ (A7,8,9 media) sacharose and 10 $g.l^{-1}$ agar-agar. Similarly, TOP 2-3 $mg.l^{-1}$ conc. was used for starting, combined with 0.1 $mg.l^{-1}$ NAA + 30 $g.l^{-1}$ sacharose (A10,11,12 media).

For proliferation of the sprouts BA was used in 1 and 2 $mg.l^{-1}$ conc. with 30 $g.l^{-1}$ sucrose. For proliferation of the GGB-like ones (GGb) were passed on different media, contained 0.5 (A1) 1.5 (S9), 2 (S10) and 2.5 (S11) $mg.l^{-1}$ BA + 0.1 $mg.l^{-1}$ NAA + 20 $g.l^{-1}$ sucrose.

In all cases the pH was adjusted to 5.6 and the culture media were sterilised at 10^5 Pa pressure for 35 minutes. The cultures were incubated at 18-25° C in 8/16 hours dark/light cycles and illuminated with fluorescent lamps with an intensity of 40 $\mu M.m^{-2}.s^{-1}$.

For studying the GGb-s scanning electron microscope was used. After a special preparation of the GGb-s, pictures were taken form the surface of the GGb-s.

Results

With the method of surface sterilisation described above 82% of the explants become sterile. On the media with low concentration of sucrose (20 $mg.l^{-1}$) only a few sprouts was achieved with the conc. of 3 $mg.l^{-1}$ BA. Much better results were given with the use of 30 $g.l^{-1}$ sacharose. The best result was found on A8 medium (2 $mg.l^{-1}$ BA) because the first sprouts appeared in 4-5 weeks (5.6). It was followed by the A9 medium with 6 weeks (4.6) and last was the A7 medium with 8 weeks (3.8). For proliferation the A8 medium gave better results in the No. of sprouts (14.5) than A7 (11.1) but the sprouts were longer (30 mm) than on the A8 (25 mm).

Next to the sprouts we found on the A8 and A9 media an interesting form of regeneration, (paralell with the sprouts) which seemed to be similar to the GGB that was found during the micropropagation of ferns (Jámbor-Benczúr *et al*., 1997). In the case of using TOP next to the sprouts the GGB-like ones (GGb) were found too, at the concentration of 2.5 and 3 $mg.l^{-1}$. The size of the GGb-s were 1-2 mm in diameter, and 6-8 piece of GGb together was one colony at the starting of proliferation. On the S10 medium the average No of GGb was 14 and on the S11 it was 20.5.

Following all the GGb-s were passed S9 and A1 medium with the hope of getting some normal form, elongated sprouts. On the S9 medium the size of GGb-s were 3-3.5 mm and in the colony 21.5-27.2 piece of GGb was found depending on the previous medium. On 10% of the colonies normal sprouts and 3 cm long roots were found. On the A1 medium the size of GGb-s were 3.5 mm and in one colony 28.7 piece of GGb differentiated. On 40% of the colonies normal form of sprouts, and on 70% of colonies 5 cm long roots were found in average. It was the best result for proliferation of GGb-s and for gaining normal form of sprouts as well.

On the picture of No 1 and 2 can be seen the surface of a GGb. It is similar to a stonecrop, with little scale-like leaves. We named it green globular bud, (GGb) because we want to differ the name from the ferns. The propagation by GGb-s could be a quick method for propagation, but it needs further examination to found a better medium for gain normal form of plants at the same time from every GGb.

Acknowledgement
This work was supported by OTKA, project No. T 049642.

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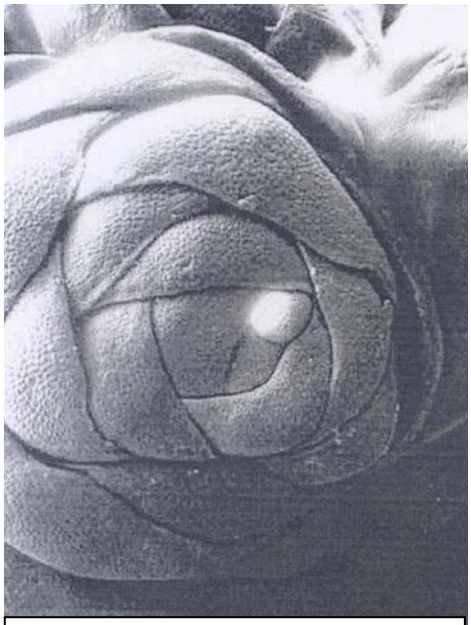

Figure 1 GGb differentiation in the *in vitro* culture of *Spathiphyllum floribundum* cv. Petite, view from above scan. electronmicroscopy 30x

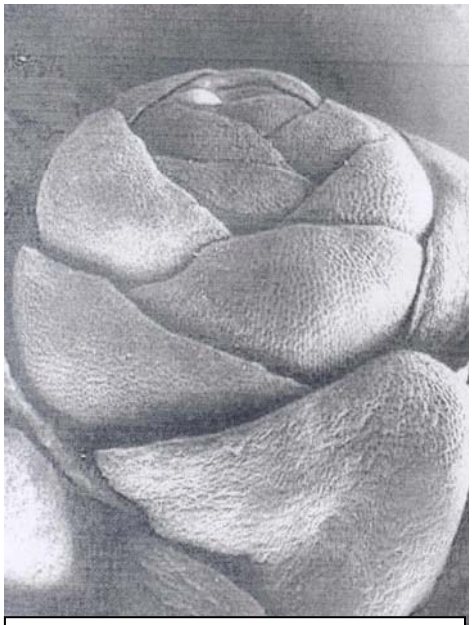

Figure 2 GGb differentiation in the *in vitro* culture of *Spathiphyllum floribundum* cv. Petite, view from the side scan. electronmicroscopy 30x

ROLE OF DIFFERENT CYTOKININS IN THE PROCESS OF SHOOT REGENERATION FROM APPLE LEAVES

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Introduction

Effective regeneration from tissues of in vitro fruit plants is a necessary precondition for the implementation of different biotechnological approaches in plant breeding. (Korban *et al*., 1992). One of the most important factors during regeneration process is the type and concentration of cytokinin applied.

The aim of present work was to evaluate the effects of different types of cytokinins during shoot regeneration from leaf explants of apple scion 'Royal Gala', 'Idared'and apple rootstock 'M.26'.

Materials and Methods

After three-week-long pre-treatment, the upper two, fully-expanded young leaves were used for regeneration as described earlier (Dobránszki *et al*., 2002). Regeneration media consisting of MS salts (Murashige & Skoog, 1962), B_5 vitamins supplemented with 100 $mg.l^{-1}$ myo-inositol, 0.25% gelrite, 3% sucrose, 0.2 $mg.l^{-1}$ NAA and the different types of cytokinins (TDZ, BA, TOP, BAR and TOPR), respectively, in the concentration range 0.5 to 8.0 $mg.l^{-1}$ (Table 1). Explants were incubated in the dark at 24.5 °C for 3 weeks, then in the light at 22 °C with 16 h photoperiod. The light intensity was increased weekly from 35 $\mu mol.s^{-1}.m^{-2}$ up to 105 $\mu mol.s^{-1}.\ m^{-2}$.

After seven weeks the number of explants with regenerated shoot (R %), the number of regenerated shoots per explant (SN), and the number of explants with vitrified shoots (V %) were recorded. The data were analysed statistically by ANOVA followed by Tukey's test by using *SPSS 7.5 for Windows* software. From the data observed 'Organogenetic Index' (OI) was calculated, as follows: OI = (R % - V %) x SN / 100. Experiments were repeated twice in time.

Results and Discussion

The most effective concentration of the different cytokinins could be seen in Table 2 and Table 3 includes the values of SN and OI in all types of cytokinins examined in their most effective concentrations applied. Statistical analysis of SN proved that both the effect of cultivar and that of cytokinin type are significant and there is a strong interaction between cultivar and cytokinin type ($P<0.01$).

The reaction of the three examined cultivars to the TDZ was significantly different. The highest percent of vitrification (V %) was observed when TDZ was applied in the regeneration medium in 'Royal Gala' and 'M.26' but not in 'Idared'. In the case of 'Idared' no any vitrification was detected when TDZ, TOP or TOPR was applied in the regeneration medium and it was under 5% when BA or BAR was applied. In the case of 'Royal Gala', the highest number of regenerated shoots and also the highest OI were detected by the use of TDZ. Its effective concentration was 10-times lower than in the case of other cytokinins similarly to the findings of Huetteman & Preece (1993). In our experiments its optimum level was 2.27 µM similarly to the results of Sarwar & Skirvin

(1997), but contradicting to the results of some other authors (Fasolo *et al*., 1989; Korban *et al.*, 1992; Kubalákova & Strnad, 1992), who found the optimal TDZ concentration between 4-10 µM in other apple cultivars. However, the optimal concentration of TDZ (9.08 µM) was found to be in the above mentioned concentration range in the case of 'M.26' and significantly overshot this range in the case of 'Idared' (29.51 µM). The best regeneration results could be reached in 'M.26' using BAR or TOPR, in 'Idared' using TOP or BA or TDZ considering both SN and OI (Table 3).

Considering the effects of classical cytokinins occurred in the nature, it could be concluded, that the reactions of three tested apple cultivars were different similarly as in the case of TDZ. In 'Royal Gala' the best regeneration (high R%, high SN, low V% and therefore high OI) was achieved if BA, or TOPR was used. The best effects were achieved between 22-27 µM in the case of non-ribosides, but between 14-18 µM in the case of N^9-riboside-forms depending of the type of cytokinins. In the case of 'M.26' the best regeneration answers were observed if ribosides (BAR or TOPR) were used. However, in the case of 'Idared' N^9-ribosides were less effective compared to non-ribosides (Table 3).

Effect of the application of TOP in the regeneration medium depended on cultivars. It was not so effective in the shoot regeneration in 'Royal Gala' and 'M.26' than in the regeneration of other plant species, such as sugar beet (Kubalákova & Strnad, 1992) but it was one of the most effective cytokinins in the case of 'Idared'.

Comparing the organogenetic abilities of the three cultivars, it could be concluded, that 'Royal Gala' was a more responsible cultivar, since 3-3.5-fold higher SN and more than 4-9-fold higher OI was reached with this cultivar than with 'M.26' or 'Idared'.

Acknowledgements

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Table 1 Types and concentrations of cytokinins applied in the regeneration media.

Type of cytokinin	Applied concentrations of cytokinins mg l^{-1} (µM)					
TDZ	0.5 (2.27)	2.0 (9.08)	3.5 (15.89)	5.0 (22.70)	6.5 (29.51)	-
BA	0.5 (2.22)	2.0 (8.88)	3.5 (15.54)	5.0 (22.20)	6.5 (28.86)	-
BAR	0.5 (1.40)	2.0 (5.60)	3.5 (9.80)	5.0 (14.00)	6.5 (18.20)	8.0 (22.40)
TOP	0.5 (2.07)	2.0 (8.28)	3.5 (14.49)	5.0 (20.70)	6.5 (26.91)	-
TOPR	0.5 (1.34)	2.0 (5.36)	3.5 (9.38)	5.0 (13.40)	6.5 (17.42)	8.0 (21.44)

Table 2 The most effective concentration of the applied cytokinins in each cultivar in mg l^{-1} and in brackets in µM

Cytokinins	'Royal Gala'	'M.26'	'Idared'
TDZ	0.5 (2.27)	2.0 (9.08)	6.5 (29.51)
BA	5.0 (22.20)	3.5 (15.54)	3.5 (15.54)
BAR	5.0 (14.00)	6.5 (18.20)	2.0 (5.6)
TOP	6.5 (26.91)	3.5 (14.49)	6.5 (26.91)
TOPR	6.5 (17.42)	8.0 (21.44)	2.0 (5.36)

Table 3 Effects of the examined cytokinins applied in their most effective concentration on the shoot regeneration of 'Royal Gala', 'Idared' and 'M.26' after 7-week-long regeneration.*

Cytokinins	'Royal Gala'		'M.26'		'Idared'	
	SN	OI	SN	OI	SN	OI
TDZ	11.08 c, B	7.05	3.12 b, A	0.63	2.67 ab, A	0.40
BA	4.58 b, B	3.75	2.45 ab, A	1.34	2.57 ab, A	0.77
BAR	3.22 a, B	2.68	3.22 b, B	1.29	1.25 a, A	0.16
TOP	2.70 a, A	2.02	2.26 a, A	1.50	3.67 b, B	0.18
TOPR	3.48 ab, B	2.66	3.18 b, B	1.74	1.0 a, A	0.05

* The statistically homogeneous groups are indicated in each apple cultivar between the types of cytokinins by the same small letter and in every type of cytokinins between the cultivars by the same capital letter.

EFFECTS OF AROMATIC CYTOKININS ON THE ORGANOGENESIS IN APPLE

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Introduction

Effects of different factors influencing the organogenesis were studied mostly during the regeneration phase, however, there are few reports considering the post-effects of culture conditions prior to the regeneration phase (McHughen *et al*., 1989; Swartz *et al*., 1990; Ferradini *et al*., 1996; Sriskandarajah and Goodwin, 1998).

The aim of this study was to test the post-effect of different aromatic cytokinins (TOP, BA, BAR, KIN) on the subsequent shoot regeneration from *in vitro* leaves in apple scions 'McIntosh', 'Freedom' and 'Húsvéti rozmaring'.

Materials and Methods

The effects of 3-week-long pre-treatments of shoots were studied on the regeneration potential of leaves. For pre-treatments 3-week-old shoots having 5-7 leaves with a length of about 35-40 mm were placed on pre-treating media consisting of MS basal medium (Murashige and Skoog, 1962) supplemented with 100 $mg.l^{-1}$ myo-inositol, 0.7% agar-agar, 3% sucrose, 0.3 $mg.l^{-1}$ indole-3-butyric acid (IBA) and 0.2 $mg.l^{-1}$ gibberellic acid (GA_3). and different type of cytokinins as follows: BA (6-benzylaminopurine), its N^9-riboside: BAR and its hydroxylated analogue: TOP (6-(3-hydroxybenzylamino)purine) and KIN (kinetin) in 0, 0.5, 1.0, 1.5 and 2.0 $mg\ l^{-1}$concentrations and TOP or KIN was applied in the above mentioned four concentrations together with 0.5 $mg.l^{-1}$ BA (BA+TOP and BA+KIN treatments). Five shoots were placed vertically on 40 ml of medium in each jar.

After three-week-long pre-treatment the upper two, fully-expanded young leaves were used for regeneration as described earlier (Dobránszki *et al*., 2002) using BA–containing (5 $mg.l^{-1}$ BA) test-regeneration medium. After seven weeks the number of explants with regenerated shoot (R %), the number of regenerated shoots per explant (SN), and the number of explants with vitrified shoots (V %) were recorded. The data were analysed statistically by ANOVA followed by Tukey's test by using *SPSS 7.5 for Windows* software. From the data observed 'organogenetic index' (OI) was calculated, as follows: OI = (R % - V %) x SN / 100. Experiments were repeated twice in time. Our calculation based on the organogenetic index used by Famiani *et al.* (1994) excluded shoot length. Moreover our index includes vitrification percent because of its great importance in shoot regeneration.

Results and Discussion

Figure 1 presents the values of SN and OI in the case of different cytokinins applied in the pre-treatments when they were applied in optimal concentration. Their optimal concentration was genotype-dependent as it can be seen in Figure 1.

Moreover, the following tendency could be obtained: good regeneration (high SN and OI) was detected after pre-treatment with TOP, or BA+TOP, or KIN. The effective con-

centration of these cytokinins showed genotype-dependence. The best pre-treatments were the following:

- in 'McIntosh': 0.5 mg.l^{-1} BA + 0.5 mg.l^{-1} TOP, because significantly the highest SN (5.12) and relative high OI (1.28) was detected,
- in 'Freedom': 0.5 mg.l^{-1} BA + 1.5 mg.l^{-1} TOP (SN: 2.45, OI: 1.88), however, the post-effect of 0.5 mg.l^{-1} KIN was not significantly lower (SN: 2.10, OI: 1.33),
- in 'Húsvéti rozmaring': 1.5 mg.l^{-1} KIN (SN: 3.26, OI: 2.01) or 0.5 mg.l^{-1} TOP (SN: 2.95, OI: 2.21).

Considering these results, it could be seen, that TOP alone or combination with BA was highly effective in pre-treating media, which correspond with our earlier finding, that TOP could improve the physiological status of leaves in apple shoot culture (Dobránszki *et al*., 2000).

These results confirmed that growth regulators applied before regeneration phase could modify the organogenetic potential of leaf tissues, as reported by others (McHughen *et al.*, 1989; Swartz *et al.*, 1990; Ferradini *et al.*, 1996; Sriskandarajah and Goodwin, 1998) and therefore also the regeneration process could be influenced. (Sriskandarajah and Goodwin, 1998; Dobránszki *et al*., 2002). Effectiveness of regeneration could be improved by use of the adequate type of cytokinins in pre-treatment.

Acknowledgements

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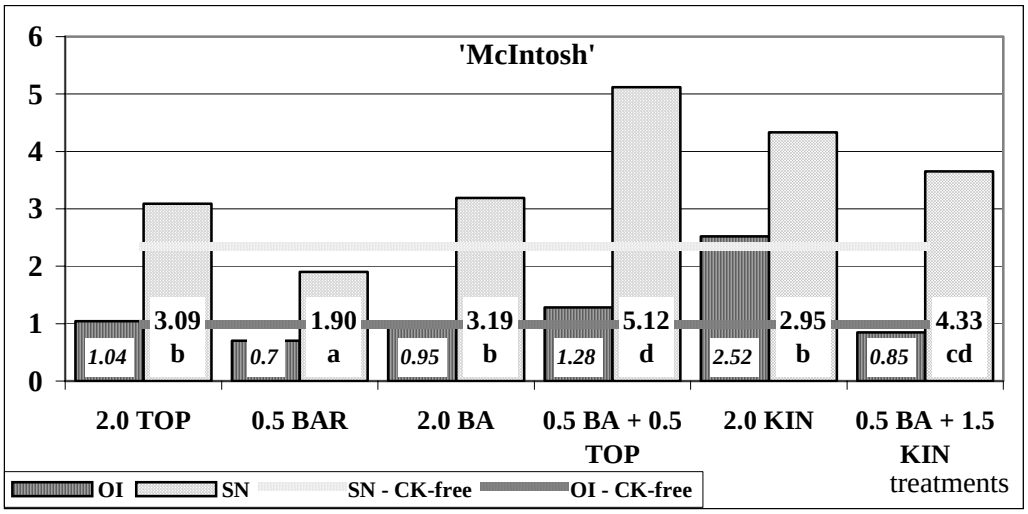
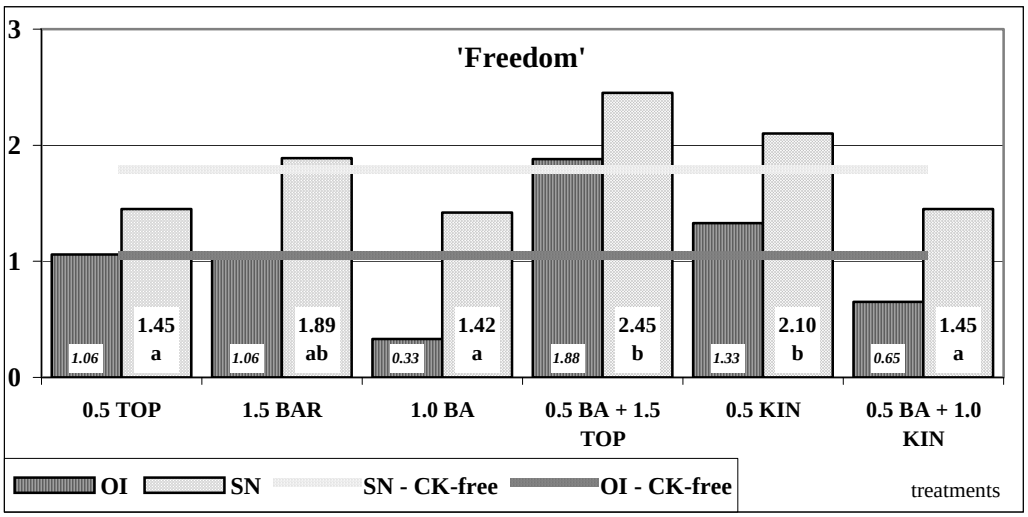
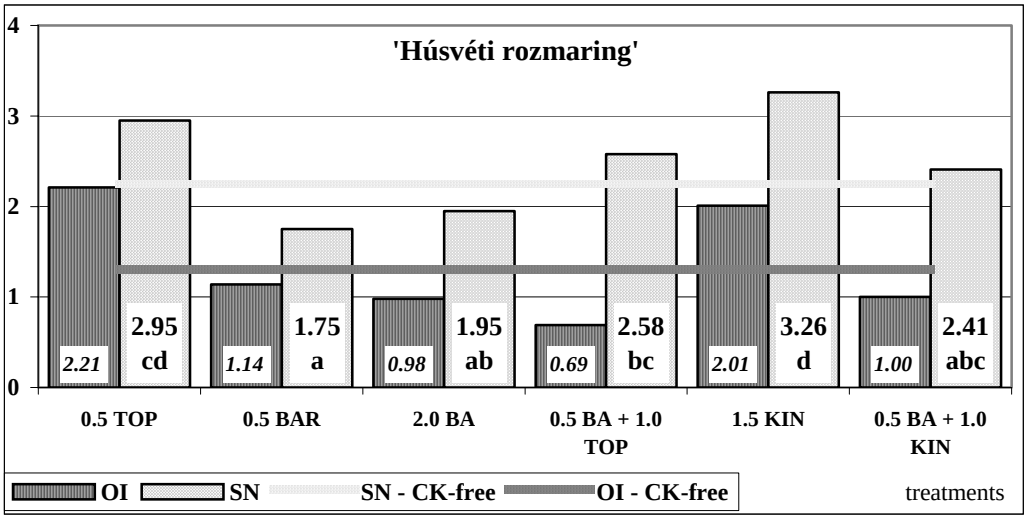
Figure 1 Post-effects of different cytokinins applied in their most effective concentration on the shoot regeneration of 'McIntosh', 'Freedom' and 'Húsvéti rozmaring' after 7-week-long regeneration







GROWTH PROMOTING EFFECTS OF TRIACONTANOL IN THE MICRO-PROPAGATION OF HORTICULTURAL PLANTS

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Introduction

Triacontanol is a component of epicuticular waxes of different plant species. Its stimulating effect on plant growth was demonstrated by increasing vegetative growth and clorophyll content of treated plants. Triacontanol was used in combinations with various chemicals to test their effects on callus formation and regeneration of rice, but no significant activity of triacontanol was found. Studies with triacontanol, however, on 'Fuji' apple in tissue culture proved a significant stimulating effect onto the growth of this plant. Since no other works were found in the literature dealing with the *in vitro* application of triacontanol, we have started to investigate its effects in the micropropagation process of different plants. In previous experiments with lemon balm, apple and cherry rootstocks triacontanol proved to be stimulatory. The main purpose of the present work was to explore the applicability of triacontanol in micropropagation of other horticultural plants as well.

Materials and Methods

Triacontanol [$CH_3(CH_2)_{28}CH_2OH$] the long chain primary alcohol was applied in a concentration range of 2-20 µg.l^{-1} alone or in a combination with other growth regulators in both multiplication and rooting phases of micropropagation.

Sterile shoots of selected clones of gerbera (*Gerbera jamesonii*), asparagus (*Asparagus officinalis*) and raspberry (*Rubus sp.*) cv. Malling Exploit served as initial material. Murashige-Skoog medium (1962) was used supplemented with 0.5 mg.l^{-1} BA plus 0.1 mg.l^{-1} IAA in the case of raspberry, 6 mg.l^{-1} Kinetin plus 0.1 mg.l^{-1} IBA in the case of gerbera and 2 mg.l^{-1} Kinetin plus 0.1 mg.l^{-1} NAA for asparagus in the multiplication phase. Hormone free basal medium was applied in the root induction phase. Both the multiplication and rooting media were supplemented with 0, 2, 5, 10 and 20 µg triacontanol per liter and sterilized by autoclave at 121 °C, 1.2 bar for 20 minutes. Illumination of the cultures was supplied by cool white fluorescent tubes at the light intensity of 50 µM·m^{-2}·s^{-1} for 16 hours/day with a constant temperature of 22 °C.

For evaluation the number and length of shoots and roots was recorded, fresh mass, dry weight and chlorophyll content of the plants was measured after four weeks of culture.

Results

Effects of triacontanol in the multiplication phase

Even the lowest concentration (2 µg.l^{-1}) of triacontanol enhanced significantly the total shoot number of asparagus. Higher concentrations up to 20 µg.l^{-1} did not give any further increase. Although differences were not significant, growth of fresh mass and dry weight of the crowns was obvious. Shoots originated from 2-5 µg.l^{-1} treatments were stronger, thicker than that of the other treatments.

In the multiplication of gerbera also the 2-10 $\mu g.l^{-1}$ concentration range proved to be effective. A slight increase of the shoot number was followed by the increment of fresh weights and dry matter contents. The value of these parameters reached the peak at 5 $\mu g.l^{-1}$ triacontanol concentration and although at 10 and 20 $\mu g.l^{-1}$ showed a tendency of decrease, they still were significantly higher than of the control. In the case of raspberry positive effects of triacontanol were manifested at the highest concentrations. The number of shoots was markedly increased at 20 $\mu g.l^{-1}$. Rise in the fresh weights and dry matter content was recorded at the concentration range of 5-20 $\mu g.l^{-1}$.

Triacontanol effect in the root induction phase

The number of roots of asparagus was significantly enhanced by 2 $\mu g.l^{-1}$ triacontanol and the higher concentrations did not result any further increase.

A stable elevation of both the proportion of rooted plants and the number of roots per plantlet was recorded in all treatments starting from the lowest concentration in the experiments conducted with gerbera.

Rooting of raspberry shoots was very poor in all treatments. No correlation was found between the triacontanol concentration and the rooting parameters.

Changes in the pigment content of triacontanol treated plants

The increasing triacontanol concentrations induced a continuous, significant growth in the photosynthetic pigment content of the asparagus plants in the multiplication phase. In case of raspberry even the lowest concentration enhanced the chlorophyll content and the elevation was remarkable at 10 and 20 $\mu g.l^{-1}$.

There were only slight changes in the chlorophyll content of the leaves of gerbera in the course of experiments.

Conclusion

The present work indicates that triacontanol proved to be an effective growth regulator in the micropropagation of different plants. During the multiplication phase it increased both the number of shoots and the fresh weight of plants.

In most cases triacontanol promoted rooting by increasing the number of roots and the rate of rooting as well. Roots of the treated plants were usually shorter and stronger than that of the control. As a consequence of this the survival rate during the acclimatization process proved to be higher too.

The chlorophyll content of plantlets was also enhanced by triacontanol. Our results clearly indicate that triacontanol is a useful growth regulator in both the multiplication and root inducing phases of micropropagation.

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IN VITRO CULTURE AND CALLUS PRODUCTION OF SEVERAL *Salvia* SPECIES

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Introduction

The genus *Salvia* (*Labiatae*) includes more than 900 species spread out all over the world. In ethnobotany, some of them are known for their medicinal potential in certain cases yet commercially utilised but most of them are totally unstudied. The ornamental potential is also important: some species and variety are used for gardening and pot plant production. Only few reports of *in vitro* culture on the genus *Salvia* were found in literature. In 1990 Olszowska and Furmanowa reported the micropropagation of *S. officinalis*; while a micropropagation protocol was reported in 1993 by Hosoki and Tahara for *S. leucantha,* in 1997 by Molina *et al.* for *S. canariensis* and in 2004 by Skaia and Wysokinska for *S. nemorosa.* The possibility to manipulate the *in vitro* production of rosmarinic acid from cell cultures of *S. officinalis* and *S. fruticosa* was demonstrated by Kintzios *et al.* (1999) and the same acid and the lithospermic acid B were already produced from transformed cells of *S. miltiorrhiza* (Chen *et al.*, 1999). By the other side in 2003 Savona *et al* reported the identification of a new secoisopimarane diterpenoid in *S. cinnabarina* and in 2004 Bisio *et al*. described other new terpenoids in *S. wagneriana* and it seems that these compounds have an interesting biological activity (Romussi G., data not published). Taking into consideration also these two last species, in the frame of an international project on the valorisation of the genus both for ornamental and medicinal purposes, several new species were introduced *in vitro* for propagation studies and with the aim to produce biomass for secondary metabolites extraction.

Materials and Methods

Axillary and apical shoots of *Salvia cinnabarina, S. wagneriana, S. dolomitica, S. horminum, S. sclarea, S. chamaedroydes, S. elegans, S. coccinea* and *S. mellifera* grown in the Hanbury Botanic Garden (Ventimiglia, Italy) were sterilised with ethanol (70%) for 30 sec then with a solution of NaOCl (1% of free chlorine) for 15 min and then rinsed twice in distilled sterile water; the explants were cultured in MS (Murashige and Skoog, 1962) without hormones. Aseptic explants of all species were used for a screening to compare the growth in the presence or in absence of BA (0.3 $mg.l^{-1}$). With two sample clones of *S. wagneriana* and *S. dolomitica* a factorial experiment was done in order to compare the presence of BA (0.3 $mg.l^{-1}$) or Kin (1 $mg.l^{-1}$) respect to a hormone-free control. Four replications with 10 shoots each were prepared for each thesis. After 40 days the multiplication rate, the height of the shoots and the presence of roots were recorded. All the experiments were carried out in growth chamber at 24°C±1 with a photoperiod of 16 hours of light at 30 $\mu M.m^{-2}. s^{-1}$. The plants were transferred in a peat-perlite substrate and placed for 20 days in the greenhouse with relative humidity of 80% supplied by a mist system. They were then transferred at a lower relative humidity (60%) for other 20 days.

Experiments for callus induction were performed with the species *S. cinnabarina, S. elegans, S. wagneriana, S. cacaliifolia, S. somaliensis, S. corrugata*. Leaf fragments were lied onto a medium containing MS (1962) base medium plus 2,4D 0.5 $mg.l^{-1}$, Ki-

netine 0.5 mg.l^{-1}, 30 g sucrose or 50 g sucrose and cultured in light (24°C±1 with a photoperiod of 16 hours of light at 30 µM.m^{-2}. s^{-1}) or in dark. After repeated subcultures of 30 days each the development of the friable callus was detected.
Cell cultures in Erlenmayer flasks were established from the callus of *S. cinnabarina* using the same media in comparison with a medium with 30 g sucrose but without hormones (MSO). The cultures were renewed every 7 days and pH was determined at each renewal, the cells were cultured at 80 rpm in the light (16 h photoperiod, 25 µM.m^{-2}.s^{-1}).

Results and Discussion

The propagation experiment made with the species *S. dolomitica* and *S. wagneriana* permitted to define a positive effect on multiplication either by the utilisation of BA or K; the best results was obtained in the medium containing BA (Tab.1). In the media containing the cytokinins the explants were short and any effect on the rooting potential was observed. Comparing the two species it was possible to say that the total multiplication rates were similar while the height of the explants was significantly higher in *S. wagneriana* than in *S. dolomitica* (Tab.2). A higher rhizogenic activity was detected in *S. wagneriana* respect to *S. dolomitica* but the percentages were for both species at appreciable levels.
The results of the multiplication rate screening on all the species tested permitted to observe a large variation in the multiplication rates (Fig. 1); the shoots grew well in total absence of growth regulators but reached multiplication rates 2 or 3 times higher when cultivated with BA (0.3 mg.l^{-1}) without missing the rooting potential. Complete plants can be immediately transferred to acclimatization without further *in vitro* treatments. After 50 days a mean value of acclimatization percentage of 80% was recorded for all species and they were transferred to pot successfully.
Callus development was obtained from all the species but *S. wagneriana*, *S. cinnabarina* and *S. elegans* produced a higher amount of undifferentiated material than the others. *S. cinnabarina* callus showed a dark colour, was very friable and some roots appeared from the clusters; *S. elegans* grew better in the presence of light, callus was more compact, green and abundant; *S. wagneriana* callus showed a brownish-white colour and a compact behaviour. As preliminary results it was possible to observe that the higher amount of sucrose permitted the development of a major quantity of callus in volume, either in solid and in liquid medium. The pH of the cell cultures maintained stable during subcultures for the media containing-hormones while in absence of hormones (MSO) a severe decrease appeared after 21 days of culture (Fig. 2). The evaluation of the fresh and dry matter production is in progress. The material was extracted and the evaluation of the secondary metabolites is also in progress.

Aknowledgements

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Table 1 Micropropagation: factorial experiment, hormonal effect on *in vitro* performances of *S. dolomitica* and S. *wagneriana*

	Multiplication rate (No shoots / explant)	**Height (cm)**	**Rooting (%)**
MS0	1.39 a	7.42 b	94.04 a
K (1 $mg.l^{-1}$)	1.84 b	5.29 a	93.06 a
BA (0.3 $mg.l^{-1}$)	3.32 c	5.34 a	87.37 a

Table 2 Micropropagation: factorial experiment; features of the two species tested, average of all the media

	Multiplication rate (No shoots / explant)	**Height (cm)**	**Rooting (%)**
S. dolomitica	2.28 a	5.55 a	86.68 a
S. wagneriana	2.08 a	6.48 b	96.3 b

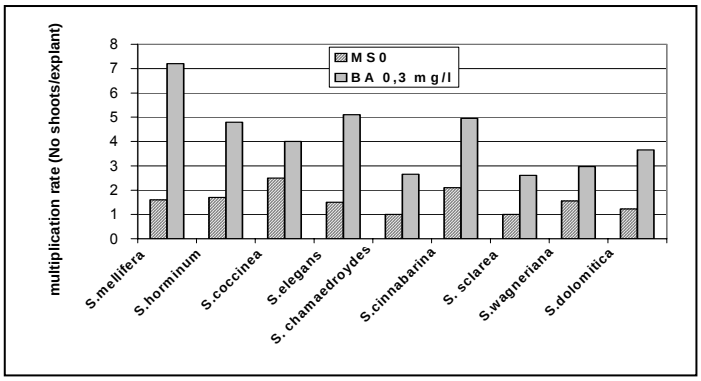

Figure 1
Screening of the multiplication rates (No of shoot per explant) in the presence or absence of BA

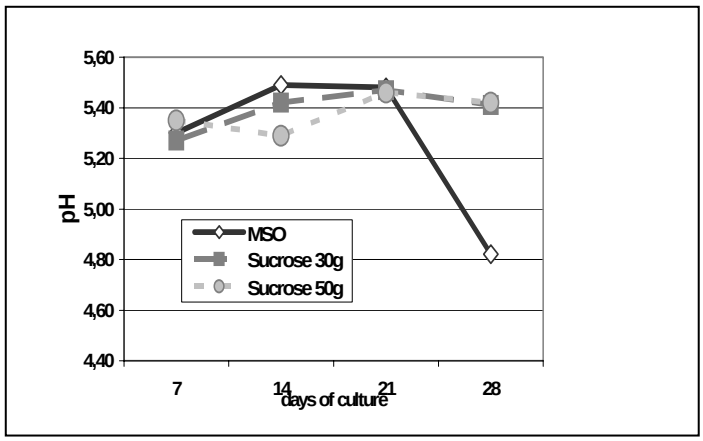

Figure 2
Ph levels of the cell cultures of *S. cinnabarina* related to the sucrose concentration in the liquid medium

USE OF SYNTHETIC ANTIOXIDANTS TO IMPROVE REGENERATION CAPACITY OF PLANT TISSUE CULTURES

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Many biologically active substances were used to increase both callus initiation level from different explants and followed regeneration frequency: phytohormones, several extracts from plants, amino acids, $AgNO_3$ and others. Among them especially interest revives effectiveness of antioxidants, which increase plant resistance for to biotic and abiotic stresses. It is known that antioxidants prevent cell death that is connected with high peroxidase activity and development of free radicals during of the plant tissue growing in culture conditions. We elaborated approach that includes combination of specific seeds sterilisation method and using of silver nitrate and synthetic antioxidants ambiol and fenoxan during different stages of calli induction, growing and regeneration.

Especially attention was paid for a possibility to establish calli cultures from seeds, material that are ready to use at any time. Barley, flax and red clover seeds were used for calli culture induction. Two-stage sterilisation was implemented: 1) pre-treatment with K_2MnO_4 solution in water, concentrations and continuance depends from seeds size; 2) final sterilisation in 50% of bleach solution for 20 minutes. Barley embryos were dissected directly from the sterilised seeds and put on calli inducing media. In they turn, sterilised flax and clover seeds were preliminary germinated on the MS basal medium without phytohormones, for flax the medium was supplemented by 10 $mg.l^{-1}$ $AgNO_3$. Different parts of seedlings were used as explants: for flax leaves and stem segments, for red clover – petiole. As a calli inducing media for all mentioned species the MS basal medium with 2 $mg.l^{-1}$ of 2.4 D were used. Barley calli inducing media was supplemented by 10 mg/l $AgNO_3$. For regeneration as optimal was found the MS basal medium with addition of specific combination of phytohormones and antioxidants: for barley -1 $mg.l^{-1}$ kinetin and 0.5 $mg.l^{-1}$ NAA with 10 $mg.l^{-1}$ fenoxan or 0.2 $mg.l^{-1}$ ambiol, for flax - 1 $mg.l^{-1}$ BAP with 0.5 $mg.l^{-1}$ ambiol or 1 $mg.l^{-1}$ fenoxan. For red clover best was the MS basal medium without addition of any phytohormones and antioxidants.

STUDIES ON THE INTERACTION OF SOME FACTORS FOR RASPBERRY (*Rubus idaeus* L.) MICROPROPAGATION

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Introduction

In vitro propagation is a method currently applied to a number of woody species in Romania. Raspberry micropropagation was studied to replace the common propagation method and to produce elite virus – free plants as well.

Introduction in *Rubus* certification scheme of *in vitro* propagation led to necessity that various aspects of raspberry tissue culture to be systematically studied to develop an efficient micropropagation regime. Several research developed micropropagation systems for raspberry propagation (Anderson, 1980; Donnelly *et.al.*, 1980; Sobczykiewicz, 1984; Snir, 1981; Welander, 1985; Desjardins and Gosselin, 1987; Hoepfner and Nestby, 1991), but multiplication rates are not always high enough for economical purposes. Anderson 1980 concluded that although most of the raspberry cultivars behave similarly on tissue culture media an optimal medium composition must be matched to each cultivar in order to obtain the best results in propagation.

The goal of this study was to find some factors which will influence the multiplication ability of some raspberry cultivars in order to improve the techniques of clonal propagation.

The investigations aimed at a statistical comparison between ratio and concentration of growth regulators as well as to assess the genotype influence and the effects of ascorbic acid on the multiplication process.

Material and Methods

The biological material included plants developed in the differentiation phase of 8 raspberry cultivars. Murashige-Skoog (1962) the most commonly use basic medium, in work on raspberry micropropagation was used. Because in the literature, for multiplication a cytokinin level of 0.05–4.0 mg.l^{-1} is mentioned, 1.0, 2.0 and 3.0 mg.l^{-1} N^6-benzyladenine (BA) with a constant level of 0.1 mg.l^{-1} 3-indolylbutiric acid (IBA) was chosen. Each treatment was tested either in the presence or absence of L - ascorbic acid (25.0-50.0 mg.l^{-1}) as an antioxidant to minimize the yellowing of cultures (Sobczykiewicz, 1984). The cultures were grown in a growth chamber under 16 hours of light from fluorescent tubes and at 22±2 °C room temperature. The multiplication rate was estimated as number of plantlets per explants at the end of 3 subculturing periods. The results were statistically measured by variance analysis and Duncan's test while for finding the present quantitative relationship between the experimental variables, the correlation and regression methods were used.

Results and discussion

The observations have shown that on an ascorbic acid free medium, the multiplication rate (MR) was dependent to BA. Therefore, in case of 1.0 mg.l^{-1} BA, the mean of multiplication rate has varied from 0.33 (Malling Exploit cv.) to 1.67 (Romy cv.) and for a concentration of 3.0 mg.l^{-1} BA, the average values of the multiplication rate were 0.92 (Willamette cv.) and 5.25 (The Latham cv.), Table 1.

Yet, the appraisal of cultivars investigated for the treatments 1.0, 2.0 and 3.0 mg.l^{-1} BA and 0.1 mg.l^{-1} IBA on acid ascorbic free medium are not significant (Duncan's test).

The analysis of the results variance has emphasized a very significant influence of interaction between cytokinin BA and ascorbic expressed in their synergic action. It is therefore explainable the average values of the MR much higher on the media having the auxin/cytokinin ratio of 0.1 mg.l^{-1} IBA/3.0 mg.l^{-1} BA and 50.0 mg.l^{-1} ascorbic acid. It is relevant that 5 cultivars of 8 tested have had a MR higher than 6.0 reaching maximum 41.92 with Romy cv. (Table 1).

Although it is ascertained that high concentration of cytokinin may result in extreme, undesirable bushiness, 3.0 mg.l^{-1} BA and 50.0 mg.l^{-1} ascorbic acid had positive effects. The positive effects and tendency to a very good multiplication rate were significantly expressed by Duncan's test in the average values higher than 20.0 with Latham, Schopska Alena and Romy cvs.

Because the variance analysis showed the significance of all correlated factors, the regression analysis established the quantitative relationship for each cultivar.

The regression equations between the multiplication rate and factors involved in its variance for Romy cv. have distinguished a straight relationship between the values of the multiplication rate and variability of BA and ascorbic acid concentration values. The studies went to prove that the positive effects of Murashige-Skoog medium (1962) reached the maximum when the auxin/cytokinin ratio was 0.1 mg.l^{-1} IBA/3.0 mg.l^{-1} BA and 50.0 mg.l^{-1} ascorbic acid. The multiplication rate was directly correlated to BA and ascorbic acid concentrations, the interactions between them having a very significant influence.

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Table 1 Influence of N^6–benzyladenine and ascorbic acid concentrations on *in vitro* multiplication rate of the raspberry shoot

BA mg.l^{-1}	**Ascorbic acid** mg.l^{-1}	**Multiplication rate**							
		Gradina	**Hertiage**	**The Latham**	**Malling Promise**	**Willamette**	**Schopska Alena**	**Romy**	**Malling Exploit**
1.0	0	1.17 e-r	0.587-r	1.0 e-r	0.83 e-r	0.33 f-s	1.42 e-r	1.67 e-r	0.33 f-s
	25	1.0 e-r	1.50-r	0.9 e-r	1.0 e-r	1.67 e-r	1.58 e-r	1.42 e-r	0.33 f-s
	50	2.08 e-r	3.83-r	2.08e-r	0.92 e-r	1.83 e-r	2.58 e-r	3.92 d-r	0.33 f-s
2.0	0	1.42 e-r	1.25 e-r	1.25e-r	0.92e-r	1.42e-r	0.83e-r	1.67e-r	0.33f-s
	25	3.58 e-r	1.67 e-r	3.83d-r	0.33f-s	0.33f-s	3.67e-r	8.08d-k	1.75e-r
	50	4.50 d-r	5.25 d-r	6.08d-r	1.33e-r	1.67e-r	6.83d-q	14.75b-f	1.08e-r
3.0	0	1.58e-r	1.75e-r	5.25d-r	1.17e-r	0.92e-r	1.83e-r	2.66e-r	0.33 f-s
	25	7.0d-n	3.83d-r	6.92 d-o	1.25e-r	1.83e-r	7.58d-l	18.08 b-d	1.0 e-r
	50	**13.17b-g**	**15. 91b-e**	**24. 08 c-j**	6.92d-o	3.08e-r	**21.58be**	**41.92a**	3.67e-r

MORPHOGENETIC FEATURES OF GRAPEVINE HYBRIDS OBTAINED BY *IN VITRO* IMMATURE EMBRYO GERMINATION (*Victoria* x *Sultanina*)

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Introduction

In grapevine, the main applications of *in vitro* zygotic embryo culture are for obtaining new early ripening cultivars (Valdez *et al.,* 1997), new seedless varieties, intergeneric hybrids (Ramming *et al*., 2000), or genotypes with different ploidy level (Yamashita *et al.,* 1998). The high degree of variability induced by tissue culture *per se* and by the origin of the explants (embryos obtained from hybridization), requires the checking of ploidy within the regenerated plants (Faure and Nougarède, 1993). Several authors suggested that in the case of species with small chromosomes and difficult to stain, as is the case with grapevine, the ploidy level should be evaluated indirectly by measurements of stomata deriving from epidermal layer (L1) or measurement of pollen grains deriving from cortical layer (L2), instead of chromosomes count in root cells deriving from medullar layer (L3). However, when chimerism is suspected in grapevine plants, all of these approaches are to be considered.

In this paper we present some aspects concerning the grapevine plants regenerated from immature embryos cultured *in vitro* and establishment of their ploidy level by indirect methods and flow cytometric analysis.

Materials and Methods

In 1997 were obtained 35 hybrid plants by *in vitro* germination of immature embryos derived from abortive ovules of *Victoria* (seeded) x *Sultanina* (seedless), and two years later were planted in the field. Although all the plants were obtained from the same crossing and on the same culture medium, three of them were different in their morphological features. These plants were subjected to determination of the density and length of stomata and the pollen diameter. Furthermore, young leaves, not fully expended were collected and used for intact nuclei extraction and DNA staining in CyStain PI absolute P. Nuclear DNA content was analyzed with Becton-Dickinson Analyser and estimated by the fluorescence of stained propidium iodite nuclei.

Results and Discussion

The three distinguished hybrid plants (named VxS, OX and XX) were characterized by using the international code of descriptive characteristics for *Vitis sp.* (O.I.V., 1984). Each feature was evaluated and the results are presented in Table 1. OX hybrid is characterized by wider leaves, thicker and darker than those of VxS hybrid. XX hybrid is significantly different from all grapevine plants, having thicker branches and stems, shortened nodes, thick and coarsely serrated leaves, dark green in colour, and a leaf surface higher with about 50% in comparison to VxS and 25% in comparison to OX, respectively.

In order to determine the ploidy of specific L1 epidermal layer, the stomata size was measured. The colodium print from the lower epidermis of the leaves, analysed to optical microscope, revealed a lower length of stomata, with a lower degree of length varia-

tion, and also a lower density of the stomata for OX and XX hybrids in comparison to VxS hybrid.
Regarding the pollen size, the measurements showed the significant differences between the mean value of the diameter for a diploid grapevine (20.4 µ, as reported by Zhang *et al*., 1998) and both the lower mean value obtained with VxS hybrid (17.9 µ) and higher mean values with OX (22.1 µ) and XX (24.8 µ) hybrids. Moreover, with VxS the maximum value measured for the pollen diameter corresponded to the mean value for a diploid plant, while with OX and XX hybrids the highest value of the pollen diameter were closer (26.4 µ and 29.7 µ respectively) to those specific for the unreduced gametes (31 µ).
The amplitude of variation was higher and more relevant when the pollen volume was calculated. If it is considered that a volume ratio of 1.9 is specific for diploid grapevine (Dermen, 1954), than all the three analyzed hybrids showed values different from diploid level (higher value of 2.1 for VxS hybrid and lower values of 1.7 for OX and XX hybrids, respectively). These results could be explained by a more frequent presence of bigger pollen grains, double in diameter and volume (2.0-2.6 times more) for OX and XX hybrids, in comparison with normal grapevine plants, which is specific for plants with a different degree of ploidy (Table 1).
When samples of nuclei from leaf tissue obtained from the three grapevine hybrids were analyzed by flow cytometry, were produced distinct and consistent peaks (159.5, 160.6 and 169.4 respectively) for the DNA quantity of nuclei in G0/G1 phase of cell cycle. These values were lower than those obtained with a diploid sample (203.7). The same determinations were performed with mixtures in equal proportion between control nuclei extract and hybrids nuclei extract. In all these cases (diploid - VxS; diploid - XO; diploid - XX), two peaks were obtained, one corresponding to the control and another to the hybrid. The DNA ratio was directly compared and variation of the DNA amount of each sample was detected. Our results clearly indicated that all the three morphologically distinct hybrids regenerated *in vitro* from zygotic immature embryos (VxS, OX and XX) are neither diploids, nor polyploids, but they are likely hypoploids.
The anatomical distortions exhibited by the three investigated grapevine hybrids are common to plants with either excessively high or low ploidy. The sub-unit value of the DNA index indicates a lower quantity of genetic material in comparison to the diploid plants that could be explained by either a reduction of chromosome number during meiosis, or a genetic material loss during *in vitro* culture, but without affecting the viability of the regenerated plants.

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Table 1 Characteristics of three morphologically distinct hybrid plants *Victoria* x *Sultanina*, and their DNA content

Characters	**Hybrid VxS**	**Hybrid OX**	**Hybrid XX**
Leaf surface (cm^2)	101-150 cm^2 = 2	150-200 cm^2 = 3	250-300 cm^2 = 5
Length of leaves	11-13 cm = 3	13-16 cm = 4	18-21 cm = 6
Shape of leaves	Round	Pentagonal	Heart shaped
Number of lobes	5 lobes	3 lobes	3 lobes
Colour of leaves	Light green = 3	Green = 5	Dark green = 7
Level of coarsely	Smooth = 1	Smooth = 1	Coarse = 2
Shape of leaf-tooth	Straight = 2	Straight = 2	Concave-convex = 4
Shape of petiole sinuous	Large opened = 1	Lyre shape = 3	"U" shape = 2
Inflorescence	Present, big	Present, small with few buds	1-2 / plant, small, on the tendril
No stomata/optical field	11-52	6-24	4-34
Density of stomata/optical field	26-30	16-20	5-10
Mean of stomata density	28.9	15,6	6,8
Variation of pollen diameter/ mean value µ	13.2-23.1/ 17,9	16.5-26.4/ 22.1	16.5-29.7/ 24.8
Diameter ratio (maxim value/mean value)	1.3	1.2	1.2
Variation of pollen volume/mean value μ^3	1.203-6.450/ 3.001	2.350-9.629/ 5.700	2.350-13.710/ 7.982
Volume ratio (maxim value/mean value)	2.1	1.7	1.7
FC analysis G Mean	159.48	160.66	169.39
Index between reference material (hybrid) and a diploid sample (G Mean = 203.68)	0.78	0.79	0.83

STUDIES ON THE ABILITY FOR LONG-TERM SHOOT REGENERATION BY DIRECT AND INDIRECT ORGANOGENESIS IN SOME IMPORTANT *Fragaria* AND *Rubus* CULTIVARS

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Introduction

Although significant advances have been made towards understanding of the key factors involved in promoting direct and indirect organogenesis, long-term shoot regeneration is still difficult to maintain in many plant species. *Fragaria x ananassa* and *Rubus idaeus* are among the species to which regeneration protocols can be efficiently applied for either shoot formation directly from leaf, petiole and stipules, or indirectly from tissue-derived callus. Since in most strawberry and raspberry cultivars investigated for their *in vitro* regeneration ability the occurrence of first shoots is found after four to five weeks, in both direct and indirect organogenesis, it is almost unanimously considered that they are avoiding any risk of genetic instability. Therefore, transformation can rely on such protocols giving early shoot regeneration. When shoot formation is maintained over three months, which generally happens only with calli, the genetic variation should be considered, and occurrence of somaclonal variants from at random mutated cells become possible.

During the last decade, we investigated systematically the ability for long-term shoot regeneration from leaf and petiole-derived calli and further behaviour of regenerants in some important *Fragaria* and *Rubus* cultivars, such as Aiko, Addie, Coral, Earlibelle, Elsanta, Fresno, Premial, Redgauntlet, Senga Sengana, and respectively Autumn Bliss, Bulgarski Rubin, Cayuga, Heritage, Ruvi, Schopska Alena, Veten, Willamette.

Material and Methods

Leaf and petiole explants, and respectively leaf and petiole-derived calli, were subcultured monthly on media supplemented with the combinations of cytokinins (BA and TDZ) and auxins (2,4-D and IAA) which proved to be the most adequate for promoting shoot formation.

The assessment of long-term ability for shoot regeneration was done by calculating both the frequency of explants or calli forming shoots and number of shoots per explant or callus after each subculture on media with the initial composition, or media with an increased concentration of cytokinin.

Results

With almost all strawberry and raspberry cultivars, and for both direct and indirect organogenesis, the average number of shoots regenerated per leaf explants was highest between the 6^{th} and 8^{th} month in culture. The mean number of shoots regenerated per explant within the first two months was significantly higher with calli compared with leaf discs or petiole segments. As expected, by analogy with their behaviour during micropropagation, both leaf and petiole-derived calli of strawberry showed a generally higher potential of organogenesis than that of leaf and petiole-derived calli of raspberry. Irrespective of the genotype, most of the petiole-derived calli showed their highest frequency of shoot regeneration with 1 to 4 weeks later than leaf-derived calli.

With each subsequent subculture, a generally decrease of organogenesis ability was noticed in all strawberry and raspberry cultivars tested. However, excepting strawberry cvs. Addie and Premial, and respectively raspberry cvs. Autumn Bliss, Heritage and Ruvi, all the other cultivars investigated, expressed their ability for shoot formation from their leaf and petiole-derived calli for at least three subcultures. In almost all cvs. of raspberry and several cvs. of strawberry, petiole-derived calli showed a significantly higher ability for long-term organogenesis, as compared to leaf-derived calli, which was found to be correlated with both their behaviour in the first 6-8 weeks in culture, and potential of proliferation.

Since the commutation from shoot formation to rhizogenesis, after 2-3 subcultures, was considered as an indication of unbalanced ratio between growth regulators in favour of the auxin, the transfer of calli on media containing higher concentration of BA was found to be beneficial in some cvs. of both strawberry and raspberry. However, a constant decrease in the average number of shoots regenerated per callus was observed, especially in raspberry and in leaf-derived calli.

A surprising observation was that the transfer for 1-2 weeks, in darkness at 10-15 °C, of calli showing no longer potential for organogenesis, resulted in the recovery of shoot formation ability in several strawberry cvs. (Aiko, Coral, Fresno, Redgauntlet), and even in two cvs. of raspberry (Schopska Alena and Veten). Thus, when cultured on 22.20 μM BA and 9.05 μM 2,4-D, shoots were regenerated in small number from such calli even 4 months after their organogenesis ability seemed to be arrested.

Depending on genotype, shoots regenerated from calli after long-term in culture were apparently normal in all their characteristics, or exhibited abnormal morphology, such as dwarf. In some cases, these shoots were unable to form adventitious buds and to form roots.

Discussion

Subculturing of leaf and petiole-derived calli was found to be more suitable for maintaining organogenesis ability over subsequent subcultures, as compared to the subculturing of leaf and petiole explants, in both strawberry and raspberry cultivars investigated. However, strawberry cultivars showed a generally higher ability for long-term organogenesis as compared to raspberry cultivars.

Since significant differences were found over subsequent subcultures in the frequency of calli showing organogenesis ability and of number of shoots regenerated per callus, among various cultivars of strawberry and raspberry, the role of genotype should be considered as very important. As large differences in the shoot regeneration ability were found even among calli derived from leaf and respectively petiole explants, the origin of calli seems also to influence the long-term organogenesis.

The knowledge about the regeneration potential and dynamics of shoot formation over consecutive subcultures in various is o great importance for practical applications, as either genetic uniformity can be preserved in regenerated plants, or genetic variation could be exploited. The results of the evaluation made for long-term regeneration ability of many strawberry and raspberry cultivars of major interest for genetic improvement by in vitro techniques, allows a better design of the experiments aiming at the induction and selection of somaclonal variants, or at genetic transformation.

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With some of the strawberry and raspberry cultivars, when TDZ (6.81 μM or 9.08 μM) was used instead of BA to induce organogenesis, in combination with IAA (2.86 μM or 5.71), direct shoot formation was promoted very early. Thus, the first true shoots were visible after 3-4 weeks in culture, with 1-3 weeks earlier comparing with regeneration subsequent to callus formation.

IN VITRO REGENERATION OF *Hagenia abyssinica* (BRUCE) J.F. GMEL. (*ROSACEAE*) FROM LEAF EXPLANTS

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Introduction

H. abyssinica (Bruce) J.F. Gmel is a dioecious monospecific tree belonging to the family *Rosaceae*. The female and male trees are distinguished only after flowering. The species is distributed between the altitudinal range of 2450 and 3250 m.a.s.l. (Hedberg, 1989) in Ethiopa, Kenya, Tanzania, Uganda, Sudan, Congo, Malawi, Burundi and Rwanda (Friis, 1992). The demand for timber from this tree is high and the female inflorescence has widely been used for its potent anti-tapeworm activity. Since tree improvement by conventional breeding is a slow process, specific characters can be improved by genetic engineering. Developing regeneration and transformation protocol is a prerequisite for introducing new genes. Recently, we have developed micropropagation protocol for this species (Feyissa *et al*., 2005). However, there are no reports on *in vitro* regeneration from leaf explants. The objective of this study was to develop an *in vitro* regeneration protocol for *H. abyssinica* to be used later for transformation.

Material and Methods

Four to five weeks old *in vitro* shoots from seedlings and sprouts of mature trees were used. The shoots were multiplied on MS medium containing 2.2 μM BAP and 0.5 μM IBA. Young compound leaves were excised and wounded or each leaf was cut into three segments and cultured in 90 mm Petri dishes containing 25 ml medium, and incubated in the dark. The medium consisted of MS salts containing 3% sucrose and different concentrations of NAA, 2,4-D and TDZ. The pH was adjusted to 5.5, solidified with 0.25% (w/v) gelrite. After three or four weeks, the cultures were transferred to shoot regeneration medium. The regeneration medium was growth regulators free or contained lower concentration of TDZ. When shoots appeared cultures were transferred to light. To study the effect of explant type, entire petioles, petiole segments and leaflets were used. The cultures were kept under a 16-h photoperiod from cool white fluorescent tubes with 33 μmol $m^{-2}s^{-1}$ at 24/18 °C day/night temperature. To examine the effect of genotypes, complete leaves derived from four other genotypes were used. After two or three months regeneration percentage and the number of shoots per explant were calculated. A total of five Petri dishes (40 leaves or 60 segments) per treatment were used and each experiment was conducted twice. Histological study was carried out and sections were examined under light microscope. Shoots were multiplied, rooted, and acclimatized following the method described by Feyissa *et al.* (2005).

Results

Callus was formed at the cut surfaces and several shoot primordial were observed on explants with lower concentration of auxin/cytokinin (0.01, 0.1, 0.5 μM NAA and 0.05, 0.1 μM TDZ) or cytokinin alone (0.05, 0.1 μM) after three weeks (Fig. 1a). Callus induction was directly proportional to the concentration of growth regulators. NAA and 2,4-D promoted callus induction but high concentrations were toxic to the explants.

TDZ promoted callus induction and shoot regeneration but inhibited shoot elongation (Fig. 1b). Significant variation in the rate of shoot regeneration was observed between the treatments (Table 1).

The rate of regeneration was significantly different among explants of seedling origin and mature trees. Regeneration of 96% to 100% was obtained between 1.0 and 10 μM TDZ from explants of mature origin. The highest number of shoots per explant was 8.4 ± 4.8 at 1.0 μM TDZ. Complete leaf performed best in both shoot regeneration and number of shoots per explant among different explants tested (Table 2). The five genotypes showed strong significant variation in rate of regeneration in the range of 1.3% to 98.8%. Shoots were multiplied on multiplication medium (Fig 1c). From histological studies, active cell divisions and xylem differentiation were observed in two weeks culture. Differentiated cells formed meristematic regions that could be observed from fifth weeks old cultures onwards. Ninety seven percent of shoots of seedling origin and 93% of shoots of mature tree origin rooted with no significant difference. After two months, 93% of plantlets of seedling origin and 90% of plantlets of mature tree origin survived (Fig. 1d).

Discussion

In our study, the main factors that affected morphogenesis were growth regulators concentrations, type of explants, age of donor plant and genotype. In many woody plants, including recalcitrant species, callus induction and plant regeneration have been achieved using TDZ (Huetteman and Preece, 1993). The inhibitory effect of TDZ on shoot elongation has been reported by many researchers recommending that TDZ concentration must be reduced and/or other cytokinins or combinations of cytokinins and auxins must be used for further shoot elongation (Bhagwat *et al*., 1996; Preece and Imel, 1991). NAA promoted callus induction and shoot elongation but the calli turned brown and died. The lower regeneration rate of explants of seedling origin as compared to mature tree origin indicates that explants of different age require different concentrations of growth regulators. This protocol is significant for future tissue culture and genetic transformation research on *H. abyssinica* and to understand the mechanisms that control the regeneration process in this species. The toxic effect of exogenous auxins needs to be studied further.

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Table 1 Percentage of regeneration and number of shoots produced by mature plant derived explants at higher concentrations of TDZ

NAA (μM)	TDZ (μM)	Entire leaf		Segments	
		% of regeneration	No. of shoots per explant	% of regeneration	No. of shoots per explant
0.0	1.0	98.8^{a}	8.4 ± 4.8^{a}	79.2^{b}	5.2 ± 2.9^{ab}
0.0	5.0	96.3^{a}	7.0 ± 3.5^{b}	91.7^{a}	5.5 ± 3.0^{a}
0.0	10.0	100.0^{a}	5.4 ± 3.2^{c}	75.0^{b}	4.4 ± 2.1^{bc}
1.0	0.5	52.5^{bcd}	2.6 ± 1.6^{d}	13.3^{fg}	1.7 ± 0.8
1.0	1.0	47.5^{cdef}	3.4 ± 1.8^{d}	28.3^{de}	2.5 ± 1.5^{d}
1.0	2.5	42.5^{def}	2.2 ± 1.1^{d}	30.0^{de}	2.2 ± 0.9^{d}
1.0	5.0	60.0^{bc}	2.1 ± 1.1^{d}	33.3^{d}	2.1 ± 1.3^{d}
1.0	10.0	37.5^{ef}	3.2 ± 2.0^{d}	28.3^{de}	2.5 ± 0.9^{d}
1.0	20.0	62.5^{b}	2.7 ± 1.4^{d}	50.0^{c}	1.8 ± 0.9^{d}
5.0	0.5	10.0^{g}	2.5 ± 0.6	11.7^{fg}	2.2 ± 1.1
5.0	1.0	15.0^{g}	2.8 ± 1.0	10.0^{g}	3.8 ± 1.0
5.0	2.5	35.0^{f}	2.8 ± 1.7^{d}	28.3^{de}	2.9 ± 1.7^{cd}
5.0	5.0	37.5^{ef}	2.0 ± 1.1^{d}	20.0^{efg}	4.4 ± 2.9
5.0	10.0	50.0^{bcde}	3.9 ± 1.2^{cd}	21.7^{ef}	4.0 ± 2.9
5.0	20.0	35.0^{f}	3.7 ± 3.2^{cd}	15.0^{fg}	3.0 ± 1.3

Means within each column followed by the same superscript, a-g, are not significantly different at 5% probability level

Table 2 Effect of different explants on rate of regeneration

Explant	NAA (0.01 μM) TDZ (0.1 μM)		TDZ (1.0 μM)	
	% of regeneration	No. of shoots per explant	% of regeneration	No. of shoots per explant
Complete leaf	93.8^{a}	5.0 ± 2.3^{a}	98.8^{a}	8.4 ± 4.8^{a}
Complete petiole	38.8^{d}	3.7 ± 2.4^{b}	28.8^{c}	5.5 ± 3.7^{b}
Leaf segment	59.2^{b}	3.5 ± 1.6^{b}	79.2^{b}	5.2 ± 2.9^{b}
Petiole segment	46.7^{c}	3.2 ± 1.9^{b}	15.0^{c}	5.2 ± 2.6^{b}
Leaflet	10.6^{e}	2.6 ± 1.4^{b}	22.8^{c}	2.8 ± 1.2^{b}

Means within each column followed by the same superscript, a-e, are not significantly different at 5% probability level

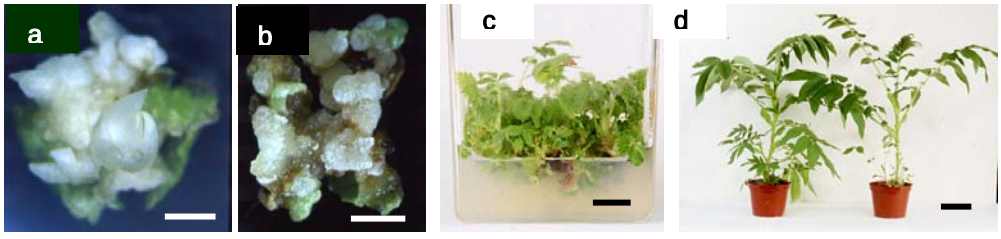

Figure 1 ***(a)*** Shoot primordia by direct regeneration after three weeks on culture (*Bar* =2.5 mm)
(b) Callus formation after five weeks on culture (*Bar* = 5 mm).
(c) Shoots of mature tree origin on multiplication medium after five weeks (*Bar*=1 cm)
(d) Four-month-old plants in the greenhouse, mature tree origin (left) and seedling origin (right) (*Bar* = 11 cm)

CLONAL PROPAGATION OF SMALL FRUITS UNDER *IN VITRO* CONDITIONS

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Introduction

Micropropagation can be used as a system for rapid and effective planting material production in biologically valuable species of small fruit, such as highbush blueberry (*Vaccinium corymbosum* L.), lingonberry (*Vaccinium vitis-idaea* L.) and raspberry (*Rubus idaeus* L.). Production of high quality plants in different cultivars of Vaccinium sp. and Rubus sp. in a large amount is needed to satisfy the incresing interest for small fruit cultivation and plantation establishment in Slovakia.

Material and Methods

In our experiments we tested the regeneration ability of different cultivars in *Vaccinium corymbosum* L.- cultivars ´Berkeley´, ´Bluecrop´, ´Blueray´, ´Darrow´, ´Duke´, ´Brigitta´; *Vaccinium vitis-idaea* L. cv. ´Red Pearl´, ´Koralle´, ´Linnea´, ´Sanna´, ´Ida´ and Rubus idaeus L. cv. ´Miral´, ´Sajana´, ´Bojana´, ´Afrodita´ and ´Bulharský rubín´. For direct regeneration of shoots isolated meristems, dormant apical and axillary buds were used.

In *Vaccinium sp*. primary explants were cultivated on Anderson culture medium (AN) with different concentration of zeatin and 2iP. Influence of different pH of culture media was also searched.

Adventitious organogenesis was induced on the leaf and stem explants of in vitro plants on AN medium with zeatin 2.19 $mg.l^{-1}$ and 4.38 $mg.l^{-1}$or thidiazuron 2.2 $mg.l^{-1}$. For shoot multiplication explants were tranferred on AN medium with 0.5 $mg.l^{-1}$ zeatin, 30 $mg.l^{-1}$ sucrose and 8 $mg.l^{-1}$ Phyto agar, pH 4.5.

For *Rubus sp*. shoot induction explants were cultivated on modified MS medium with 1$mg.l^{-1}$ BAP and 0.1$mg.l^{-1}$ IBA. For shoot multiplication the same medium was used.

For adventitious shoot induction leaf and stem cuttings, MS and AN culture medium and TDZ, BAP and Zea in different concentrations were tested.

For the rooting of shoots modified MS with 1$mg.l^{-1}$ IBA was applied.

To search a clonal fidelity of vegetatively propagated plant material RAPD analyses and flow-cytometry analyses were performed using *in vitro* obtained clones of *Vaccinium sp*. after several months of *in vitro* cultivation.

Results

Axillary and adventitious organogenesis are the most generally applied techniques for rapid and effective plant production in *Vaccinium sp*. Intensity of the shoot proliferation depends not only on the genotype but important role plays also type of primary explant and type and concentration of cytokinin in culture medium. Using zeatin 2 $mg.l^{-1}$ significantly higher multiplication of shoots was achieved in comparison with 2-iP, with the highest multiplication rate on medium with pH 5 in cultivar Duke.

Experiments on adventitious organogenesis in *Vaccinium sp*. confirmed that zeatin 2.19 $mg.l^{-1}$ and 4.38 $mg.l^{-1}$ was the most effective for shoot formation from leaf explants.

In *Rubus* the best shoot induction and multiplication was achieved by cultivation of isolated meristems and dormant buds on modified MS medium containing 1mg.l^{-1} BAP and 0.1mg.l^{-1} IBA. The highest number of shoots produced on explant after 2 months of cultivation was in Bulharsky rubin (13.9).
Adventitious bud induction was obtained in cultivars Miral, Afrodita and Bulharsky rubin with the best organogenesis on MS medium containing 0.5 mg.l^{-1} TDZ and 0.2 mg.l^{-1} 2,4-D. Regenerated microshoots were rooted in vitro or ex vitro and succesfully acclimatized under *in vivo* conditions.
Flow-cytometry analyses did not reveal changes in ploidy level in tested clones. Similarly, RAPD analyses showed no differences between DNA profiles of maternal and in vitro plants originated either via axillary or adventitious organogenesis. However it was found that RAPD is suitable for identification of blueberry cultivars, since it was able to discriminate between tested cultivars resulting in characteristic polymorphic banding patterns among cultivars.

Discussion

Zeatin, 2-iP and thidiazuron are the most frequently used cytokinins for *in vitro* shoot induction in *Vaccinium* species. Our results confirm the findings of other authors that zeatin is more effective than 2-iP and thidiazuron for induction of shoots in *Vaccinium sp*. Debnath and McRae (2002) found that zeatin induced multiple shoot formation at concentrations 4.38 and 6.57 mg.l^{-1} in lingonberry. Reed and Abdelnour-Esquivel (1991) show in their paper more effective influence of zeatin in comparison with 2-iP in blueberry.
For *Rubus idaeus* micropropagation MS medium with 1mg.l^{-1} BAP and 0.1mg.l^{-1} IBA was proper, the same like it is mentioned in the paper of Donnelly *et al*. (1980) and Palonen and Buszard (1998).
Random amplified polymorphic DNA analysis (RAPD) has been reliably used in cultivar and clone characterization for different *Vaccinium sp*. (Arce-Johnson *et al*. 2002). But for detection of somaclonal variability in microclonally propagated plants the others more specific markers (SSR, EST-PCR) are necessary to use.

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IN VITRO PROPAGATION OF *Centarea jacea* L.

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Introduction

In a previous study, we have been studying the regeneration of *C. jacea* L. (*Asteraceae*) using TDZ as growth regulator for the promotion of *de novo* formed shoots on leaf explants (Lê, 2004a). The results showed that the ability to undergo organogenesis exhibited by leaf explants should be useful for the propagation of true-to-type of sensitive and resistant clones of *C. jacea* L. (Lê and Nowbuth, 2004b). However, the average shoot number per explant, in terms of usable shoots, was not sufficiently high to be practical.

The aim of this work was to determine the optimal conditions for producing usable shoots in order to improve the propagation potential of *C. jacea* L.

Material and Methods

Leafy shoots taken from *in vitro* regenerated microplants of sensitive clone of C*entaurea jacea* L were placed on basal medium containing Murashige and Skoog (1962) basal salt mineral supplemented with 1 $mg.l^{-1}$ thiamine, 0,5 $mg.l^{-1}$ pyridoxine, 0,5 $mg.l^{-1}$ nicotinic acid, 100 $mg.l^{-1}$ myo-inositol. In order to proliferate new shoots, different combinations of growth regulators were tested with respect to the basis of organogenic responses of the explants (Table 1). All media contained 3% sucrose with pH adjusted to 5.7-5.8 and solidified with 0.7% agar before autoclaving at 121 °C (1.1 $kg.cm^{-2}$) for 15 minutes. Cultures were maintained at 20 °C day/ 18 night ± 1 °C under cool white fluorescent lamps at 55 $\mu mol.m^{-2}.s^{-1}$ with a 16 h light and 8 h dark. Experiments were set up with 16 explants/treatment and were conducted three times. Observations on the number of usable shoots and their growth were recorded. Results were subjected to analysis of variance and significant differences in values were calculated where effects were statistically significant.

Results and Discussion

As shown in Table 1, explants failed to produce shoots when the growth regulators were absent in the medium. The induction of shoot proliferation was stimulated when cytokinin was incorporated alone or in combination with an auxin in the culture medium. In this regard, medium supplemented with BAP as sole source of growth regulator seemed to be the most effective (4.2 ± 0.53) in terms of usable shoots, while adding the auxins in combination with BAP to the medium has led to moderate shoot proliferation. A combination of BAP and IAA produced an average of 4.1 ± 0.50 shoots/explant while a medium containing BAP and IBA yielded 3.1 ± 0.50 shoots/explants. NAA containing-medium appeared to be less effective causing the low proliferation rate of shoots (2.1 ± 0.3 shoots/explant) during the course of this study. In addition, auxins added to the medium had led to high percentage of callus formation at the basal end of the explants. Vitrification phenomenon was also observed particularly on both IBA and NAA containing-media in subsequent subcultures.

The beneficial effect of cytokinin described here is similar to those reported by Cuenca *et al.* (1999) for *C. paui* (3.3± 0.3 shoots/explant) and Cuenca and Amo-Marco (2000) for *C. spacii* (3.8± 0.5 shoots/explant) with the same concentration of BAP. Hosoki and

Kimura (1997) have also obtained shoot formation on *C. macrocephala*, but at a low rate of proliferation (2.1 ± 0.1 shoots/explant).

Table 1 Effect of Plant Growth Regulators (PGRs) on the proliferation rate of new shoots and the percentage of explants that generated callus formation

Treatments	Number of shoots / Explant*	Explants with Callus (%)
Control	1.2 ± 0.13c	0
BAP (4.44 µM)	4.2 ± 0.53a	39
BAP (4.44 µM)+ IAA (0.57 µM)	4.1 ± 0.50a	56
BAP (4.44 µM)+ NAA(0.54 µM)	2.6 ± 0.40b	98
BAP (4.44 µM)+ IBA(0.49 µM)	3.4 ± 0.65a	92

*/ Means with different letters are significantly different according to Duncan's multiple range test at 5% level

Conclusion

The results obtained in this study indicate that the presence of PGRs in the culture medium promotes the organogenic ability of sensitive clone of *C. jacea* L. However, further experiments would be needed in order to verify the influence of the studied PGRs on others genotypes of *C. jacea* L., particularly on resistant clones.

Effect of PGRs on shoot proliferation in *C. jacea* L.

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SUCCESSFUL MICROPROPAGATION OF *Astilbe* (SIMPLICIFOLIA HYBRIDS)

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Perennial plants become more and more important in tissue culture. Many perennial plants are woody which can make tissue culture problematic. Therefore a study has been done to develop a tissue culture system for *Astilbe*. An *in vitro* culture was initiated from either meristem tips or flowers. It was found to be very important to have the material used for the initiation in the right stadium. Over 80 percent of the initiated explants were killed by bacteria or fungi. After dissinfection with a 0.8 % active chlorite solution a population could be established by subculturing every 5 weeks. Different types of cytokinine for multiplication and auxine for rooting were tested. The best results for multipliation were found on MS medium with addition of 2 mg.l^{-1} Kinetine. Rooting of the plantlets was the most successful on MS medium with addition of 2 mg. l^{-1} IAA. The best circumstance was 25 ºC at 16 hrs/3500 lux. Rooted plantlets were successfuly transferred into soil.

A BIOLOGICAL TEST TO EVALUATE, IN 10 DAYS, CHEMICALS TO PREVENT MICROCUTTING HYPERHYDRICITY

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Introduction

Scientists and industrials involved with *in vitro* tissue culture are faced with the hyperhydricity phenomenon also called vitrification (1), whose consequence is the hypertrophy of the meristems, leaves and stems resulting from a drastic increase of the water content in the tissues. Hyperhydricity limits the success of plant regeneration from *in vitro* meristem culture, adventitious buds and somatic embryos. Indeed, the vitrified cuttings become necrotic and dy more or less quickly. Moreover, hyperhydricity makes the propagation of *in vitro* shoot tips in bioreactors impossible. Being an aleatory phenomenon, hyperhydricity renders a planification of the industrial i*n vitro* production of woody plants usually impossible. Despite intensive research on this topic, hyperhydricity of *in vitro* cuttings remains still a major problem, as reflected by 141 publications referring to hyperhydricity over the 3 last years. This paper describes a biological test to evaluate in ten days the capacity of chemicals to prevent hyperhydricity. The test consist in the comparison of the behaviour of standardized microcuttings of M26 apple rootstock cultured on two control media "a liquid medium without BAP (positive control - non vitrifying medium) and on a liquid medium with BAP (negative control - vitrifying medium) with the same latter medium containing the chemical to be evaluated. The cuttings produced in the test conditions have been characterized focusing the attention on the morphological development of the 3 uppermost leaves as well as the ploidy level, the mitotic index and the efficiency of the photosynthetic apparatus. EM2 (2) and Dextran sulfate 5000 (3) have been evaluated as antivitrifying chemicals.

Material and Methods

A stock culture of M26 apple rootstock cuttings are intensively propagated on a solid LP (4) culture medium (BAP 4.4 μM and agar Bacto Difco 7g.l^{-1}) during 30 to 40 days. The standarddized cuttings isolated from the stock culture consisted of a 2.5 to 3 cm long stem with 3 to 4 leaves. Those are transferred in test tubes containing 15 ml of the LP liquid medium **a/** with BAP (vitrifying medium); **b/**without BAP (non vitrified) and **c/** on the liquid medium with BAP with the chemical to be tested.

The morphological development of the newly developped leaves is recorded every day considering the stage of vitrification, as described by Pâques *et* Boxus (5). The ploidy level and the mitotic index are established using a Partec PA II cytometer, as described by Ochatt *et al.*(6). The photophysical and photochemical characteristics of the hyperhydric and non hyperhydric leaves are estimated by the fast chlorophyll a fluorescence OJIP transients recorded using a Plant Efficient Analyser (PEA, Hansatech Instruments Ltd, King's Lynn, Norfolk, PE 30 4NE, UK) according to the procedure described by Strasser et al. (7). It is even possible to make the measurements with a 1s illumination time through the glass tube or the Petri dish without touching the plant material and keeping therefore the sterile conditions. EM2 is prepared and used as described by Pâques and Boxus (2) and the Dextran sulfate 5000 is used according to the recommendation of Nairns *et* Furneaux (3).

Results

The behaviour of the cuttings grown on the positive and negative control media looks quite different:

- on the liquid culture medium + BAP (negative control) more than 80% of the cuttings develop typical hyperhydric curled leaves after 10 days of culture. All cuttings looks vitrified after

30 days on that medium. The new apical leaves are longitudinally curled and water soaked. All the tissues of the stem and leaves look hypertrophied with very wide lacunae network.

- on the liquid medium - BAP (positive control) 70% of cuttings look non hyperhydric ("normal").The newly apical leaves look normal. The tissues of the stem and leaves are compact without any aberration as described in hyperhydric tissues. The hyperhydric leaves versus non hyperhydric ones have been characterized probing the nuclear DNA content and the chlorophyll a fluorescence emission.
- the ploidy level of the leaves is not affected by hyperhydricity. However, the cellular cycle is disturbed as revealed by the mean mitotic index, which is always higher in hyperhydric leaves.
- the analysis of the chlorophyll a fluorescence points out significant differences between hyperhydric and non hyperhydric leaves. Hyperhydricity mainly induces a decrease in chlorophyll content per leaf area and a decrease in the redox reactions of the photosynthetic electron transport chain (dark reactions). Only in an advanced stage the quantum yield for primary photochemistry starts to decrease as well. The most sensitive experimental indicator is the photosynthetic performance index PI. It includes the three parameters: the density of the photosynthetic reaction centres and the efficiency of the light and the dark reaction. Therefore the PI decreases as a first very sensitive sign as soon as one or more of the three parameters decrease.

The evaluation of EM2 and Dextran sulfate sodium (500.000KDa) in the liquid medium with BAP confirms that the two chemicals are protecting 80% of the cuttings against the hyperhydricity maintaining or increasing the proliferation rate in comparison to the positive control medium. However, the buds developed in liquid with Dextran are hyperhydric while they are non hyperhydric when the liquid medium contains EM2.

Discussion

It is confirmed that BAP is a determinant factor inducing hyperhydricity of microcuttings grown on a liquid medium. A maximum of 10 culture days is enough to reveal vitrification in liquid medium. Our test allows to probe very quickly both the potential of antivitrifying chemicals and the gelling agent quality used in *in vitro* culture.

The morphological aberrations of the hyperhydric tissues grown on a liquid medium with BAP are similar to those described in the litterature. Contrasting data for grasspea by Ochatt *et al*. (6), Hyperhydricity was not associated in apple with the appearance of a peak of endoreduplication. This could explain why hyperhydricity is partially reversible on M26 rootsock if it isnot too advanced. However, the mitotic index is higher in hyperhydric tissues of M26 indicating the perturbbation of the cell cycles, probably at the cytodieresis of daughter cells, as a result of perturbations of the cell wall properties in hyperhydric tissues (5, 9).

The phenotyping by using the fast fluorescence OJIP transient and its analysis by the JIP-test allows a fast *in vivo* screening of many plants and cultivars before visual symptoms appear.

The role of BAP must be elucidaded considering the relations between hyperhydicity, the modification of the mitotic index and the cell wall properties. Particular attention will be focused on the antagonist effect of EM2 and sodium dextran sulfate (500. 000KDa) able to reduce Hyperhydricity, maintaining or increasing the proliferation rate. A possible interaction between EM2 and sodium dextran sulfate (500.000KDa) and BAP is presently under investigation.

Acknowledgements

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SESSION II:
ADVANCED PROPAGATION TECHNIQUES

TEMPORARY IMMERSION BIOREACTOR: AN EFFICIENT TECHNOLOGY FOR SCALING-UP PLANT PRODUCTION

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Abstract

The use of liquid medium in plant tissue culture has many advantage among them is an ideal technique for mass production because reduces manual labor and facilitates the automation process. The major disadvantage is the hyperhydricity, which could produce a severe physiological disorder.

Temporary immersion has been shown to reduce problems usually encountered in liquid culture. Based on this concept, a collective of researcher belong to Bioplant Center adapted a semi-automated system for large-scale propagation of plants. This bioreactor has been named as Twin Flasks system (BIT®) and grouped into the systems with complete immersion by pneumatic driven transfer of liquid medium without medium replenishment (Etienne & Berthouly, 2002).

BIT® is relatively simple and easy to use. They enable contact between all parts of the explants and the liquid medium. The culture environment is renewal by forced ventilation during each immersion period. The way to carried out pneumatically-driven temporary immersion is to connect two glass or plastic flasks, from 250 ml to 20 liters, by silicone tubing, and apply overpressure to push the medium into the other recipient. For special type of plants, a forced aeration in the culture recipient can be used. The injection of CO_2 permits to improve the photomixotrophic culture. There are different designs according to proposal.

BIT® has been used for *in vitro* propagation of a wide range of tropical crops: eg: *Ananas, Saccharum sp, Musa sp, Colocasia sp, Araceaes, Eucaliptys sp, Rosaceae, Bromelias, Paeony.*

Temporary immersion has positive effects on all stages of shoot proliferation and somatic embryogenesis. Multiplication rate and plant growth are generally better than conventional micropropagation (semi-solid and liquid). Plant material performs better during the acclimatization phase. Survival rates and plant vigor in greenhouse were generally increased with material regenerated from BIT®.

In order to establish a micropropagation procedure and increase the efficacy of BIT® - technology, different parameters should be optimized. Among them, the immersion time, immersion frequency, the volume of nutrient medium, the volume of culture container, the duration of proliferation phase, the use of plant growth retardant, the number of cycle in BIT®.

Till now, plants regenerated by BIT® have not showed somaclonal variation detected by molecular probes and evaluations in the field. These conditions of *in vitro* culture improved the quality of regenerants in all species studied.

A research on the effect of temporary immersion on physiology is essential to optimize culture conditions in this simplified bioreactor. Plantain and pineapple have been used as reference plants to study physiological behaviour in this new technique of culture.
In the headspace container, the CO_2 concentration increase in comparison with conventional micropropagation after non-immersed stage. Especially in BIT® there was a higher CO_2 and a lower O_2 peak during the elongation phase compared to the multiplication phase. These results indicate also a stronger respiratory activity in BIT® during the elongation. The concentration of C_2H_4 in BIT® was also possible to detect at the end of elongation. In BIT®, the concentrations were lower than on semi-solid medium.
BIT® did not seem to improve photosynthetic capacity of shoots before transplanting. Shoot growth did not totally depend on the photosynthesis process. They appeared to use more nutrients in the culture medium than those from photosynthesis. TIB-derived shoots showed a remarkable nutrient uptake indicating higher photomixotropic metabolism.
BIT® increased respiratory activity of shoots, but this fact did not seem to affect the intrinsic quality of plantlets. For this reason, a good understanding of temporary immersion culture and its physiology at the moment of transfer to *ex vitro* conditions can provide guidance in how to optimize the acclimatization procedure in order to reduce plant losses. Studies along this idea continue in progress.
Apart from being used for plant micropropagation, temporary immersion represents a useful tool to study different metabolic processes. For example, carbon and nitrogen metabolism has been characterized in this system during the growing of plantain plants in elongation and acclimatization phases.
Many temporary immersion systems are also adapted to produce secondary metabolite. The beneficial effect of BIT® to produce bromelin, an important protease from pineapple, has been also assessed.
The simplicity and low cost of BIT® is compatible with large-scale propagation. It permits important lower labor, better biological yield and consecutively reduces production cost.

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PATTERN FORMATION DURING EMBRYO DEVELOPMENT IN NORWAY SPRUCE

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Somatic embryos of conifers has become a useful tool for clonal propagation. Somatic embryos are also valuable for studying regulation of embryo development. The somatic embryo system includes a stereotyped sequence of developmental stages, resembling zygotic embryogeny, which can be synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages. The genetic regulation of embryonic pattern formation has been studied in the model plant *Arabidopsis*. In contrast hardly anything is known about the regulation of embryo development in gymnosperms. Gymnosperms have several disadvantages as experimental organisms. They have large genomes, about 200 to 400 times bigger than *Arabidopsis*. Furthermore, they have large size and a long generation time. Molecular data suggest that extent seed plants (gymnosperms and angiosperms) share a last common ancestor about 285 million years ago. From an evolutionary point of view it is important to learn more about the regulation of embryogenesis in gymnosperms.

The sequence of embryo development in gymnosperms can be divided into three phases (Singh, 1978). (1) Proembryogeny - all stages before elongation of the suspensor. (2) Early embryogeny - all stages after elongation of the suspensor and before the establishment of the root meristem. (3) Late embryogeny - establishment of the root and shoot meristems and further development of the embryo. The pathway of somatic embryo development resembles zygotic embryogeny, even though the origin of the embryo is different. Embryogenic cultures of Norway spruce are established from zygotic embryos. The cell lines proliferate as proembryogenic masses (PEMs) in the presence of auxin and cytokinin. Somatic embryos differentiate from PEMs after withdrawal of auxin and cytokinin. This stage corresponds to early embryogeny. The embryos develop further, to a stage corresponding to late embryogeny, in the presence of abscisic acid.

The variation in transcript abundance during somatic embryiogenesis in Norway spruce suggest that high level of DNA metylation is important for the differentiation of somatic embryos from PEMs. Furthermore, down-regulation of genes involved in auxin metabolism is important for apical-basal polarization during early embryogeny and down-regulation of genes involved in cell wall formation is important for differentiation of primary meristems (Stasolla *et al*., 2004).

Formation of the apical-basal embryonic pattern during early embryogeny proceeds through the establishment of three major cell types: the meristematic cells of the embryonal mass, the embryonal tube cells and terminally differentiated suspensor cells. The suspensor cells are degraded by programmed cell death (Bozhkov *et al*., 2004; Suarez *et al*., 2004). Metacaspase is essential for maintaining the proper balance between cell proliferation and programmed cell death. Differentiation of the outer cell layer in the embryonal mass is regulated by *PaHB1*, which encoded protein is highly similar to those from the HD-GL2 angiosperm counterparts (Ingouff *et al.*, 2001). Suggesting similarities in the definition of the outer cell layer in seed plants. Like in angio-

sperms, a proper function of the outer cell layer in Norway spruce requires a specific expression pattern of a lipid transfer protein gene (Sabala *et al*., 2000).
During late embryogeny *PaHB2* participates in the maintenane of the radial pattern by specifying cell identity in the cortical layers (Ingouff *et al.*, 2003). The pattern of expression of *Pavp1* during maturation of Norway spruce somatic embryos is similar to that of angiosperm VP1 homologues during somatic and zygotic embryogenesis (Footitt *et al*., 2003).
Taken together, the regulation of embryo formation has many similarities in gymnosperms and angiosperms, but there are also differences which have to be studied more in detail.

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CYTOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF SOMATIC AND ANDROGENIC EMBRYO FORMATION IN FLAX

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Introduction

Flax (*Linum usitatissimum* L.) is an ancient cultivated species with an important impact in world economy. Traditionally cultivated for its main product –fibre and seed oil – this species has gained a new interest in the emergent market of functional food due to its high content in fatty acids, mainly α-linolenic acid, and lignan oligomers.
More research on that plant, particularly with a prospect for formation of bipolar structures in culture from somatic tissue and preparation of dihaploid plants via androgenesis represent a valuable contribution to flax breeding and cultivation. Intensified research can lead also to better understanding of plant cell totipotency and the embryo induction and development *in vitro* conditions.

Material and Methods

Experiments were carried out with 2 linseed oil cultivars Szegedi 30 and Flanders and fibre cultivars Carolin, Viking, Super, Belinka, Red Wing and two breeding lines PR FGL and San Elias 192/22. The donor plants for experiments with androgenesis were grown in field conditions at the experimental field of the Institute of Plant Genetics and Biotechnology in Nitra. For androgenesis and somatic embryogenesis in flax the procedures described previously were used (Pretova and Williams, 1986; Dedičová *et al*., 2000; Preťová and Obert, 2000; Obert *et al.,* 2004).

Results

Somatic embryogenesis

We induced direct and indirect formation of somatic embryos from zygotic immature and mature flax embryos and from hypocotyls segments of 6 days old flax seedlings. The direct induction of somatic embryos from late heart and early torpedo flax zygotic embryos involved three main stages: inhibition of the main zygotic embryo shoot-root axis, internal stimulus for embryogenic development and continuing stimulus for mitotic division. A broad spectrum of morphogenic structures was produced and appeared to be the manifestation of the same phenomenon –cell totipotency.
Somatic embryogenesis via direct formation was also achieved from the hypocotyl segments after 2,4D treatment. After first subculture on a hormone free medium embryo-like structures appeared on the cut ends of the segments. More structures were formed on the "shoot" end of the segment and less on the "root"end. The formed structures were liberated from the primary tissues after 3 weeks when they reached heart stage. Approximately a third of these somatic embryos reached the cotyledonary stage and was capable to germinate.
When 28-days-old zygotic flax embryos were used as the primary explants or higher concentration of 2,4D (5 $mg.l^{-1}$) was applied, first a callus was produced and later on hormone free medium embryo-like structures appeared. The resulting embryos mostly have had weakly formed shoot apices.
The formation of somatic embryos can be strongly influenced also by the level of hydroxyl radicals in the medium. Also the chitinase activity was correlated with somatic embryogenesis in flax.

Androgenesis

The induction of bipolar structures derived from microspores was the highest on the N6 medium, preferentially after cold pre-treatment (7 days at 8 ºC). The most responsive flax cultivar was the Red Wing and PR FGL 77. Our experiments resulted in 62% anther response and 155 microspore derived flax plants were transferred to soil. The most responsive was the late uninucleate microspore stage. The bipolar structures developed in similar way as it was in experiments with somatic embryogenesis.

Disscussion

Very often the forming structures in our experiments were arrested in the globular stage. The transition from the globular stage to the heart stage is an embryogenesis check-point. Numerous embryogenic mutants are blocked at this stage (Mayer *et al*., 1998). Even not all zygotic flax embryos excised in their globular stage were able to pass this check-point in culture (Preťová, 1986) and it was named a "critical point" of *in vitro* cultivated flax embryo development. As a consequence of this phenomenon a weak formation or lack of well developed shoot pole was observed. A striking resemblance to the *stm* mutants described for *Arabidopdis thaliana* L. (Mordhorst *et al*., 2002) was noticed. In both experiments a wide range of bipolar structures that could be classified as "embryo-like" structures has been formed. This fact shows that *in vitro* conditions the morphogenic response possesses much more plasticity than it is available to the cell within the complete plant body under the strict control and cell to cell coordination with regard to high extent of cell, tissue and organ differentiation (Preťová and Obert, 2005).

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PHENOLIC COMPOUNDS AS A BIOLOGICAL MARKER IN SNOWDROP (*Galanthus* sp.) ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

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Introduction

Many flavonoids and other phenolic compounds are flower pigments while some of them are involved in the control of IAA activity and, consequently in plant growth and development (Murphy *et al.*, 2000). Snowdrops (*Galanthus* sp.) are valuable, endangered spring flowering ornamental bulbous plants with traditional uses in Alzheimer's disease. Moreover snowdrop lectin can be used as novel insect control.
In the aim to estimate the role as a biological marker of differentiation the composition and concentration of phenolic compounds were studied in snowdrop's tissues during organogenesis and somatic embryogenesis processes.

Materials and Methods

Snowdrops were propagated in liquid (fed batch system) or solid MS media cultures. For initiation of cultures bulb scale explants were used. The concentration of growth regulators (BAP, NAA, Picloram) varied from 0.5-25 μM. A high performance liquid chromatography (HPLC Schimadzu LC-10AS) with UV detection was used for phenols analyses (Swiderski *et al*., 2004).

Result and Discussion

The results of the experiment indicated that the influence of cytokinin and auxin in liquid media is different from what happens in agar solidified media.
The liquid media with cytokinin resulted in the increase of fresh weight (FW) while the liquid media with auxin led to decrease of the FW.
On the media with auxin a numerous somatic embryos were formed in liquid (fed batch culture) system; on the media containing more cytokinin than auxin adventitious bulblets were developed on solid media (Fig. 2 and 3).
The differences in the level of secondary compounds among different calluses were both qualitative and quantitative (Fig. 1). The highest level of phenolic compounds was noticed in callus tissues grown on the media containing 25 μM Picloram. Moreover phenolic compounds were dominant in globular embryogenic callus in contrast to non-embryogenic callus (Fig. 1). Similar differences in phenolic compounds concentration were also observed in juvenile plant of *Betula* (Keski-Saari and Julkunen-Tiitto, 2003).
The level of phenolic compounds was higher in tissues propagated in liquid media than on the solid media (Fig. 1). The difference in phenolic compounds concentration was independent on the origin of tissues (i.e. bulbs, scales, shoots, ovary tissues) and the type of growth regulators used for induction of morphogenesis. As the snowdrop tissue growth value (GV) in fed batch liquid culture was higher then the GV on solid media (Bach *et al.,* 2003) phenolic compounds level could serve as a marker of biological quality of snowdrop regeneration. In our previous experiments carried on tulips were obtained similarly results (Bach *et al.,* 2004).

Acknowledgement

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Figure 1 The level of phenolic compounds in different stages of snowdrop morphogenesis:

A - buds and adventitious bulblets (origin from bulb scale, 100%MS, 3% sucrose)

B - buds and adventitious bulblets (origin from bulb scale, 100%MS, 6% sucrose)

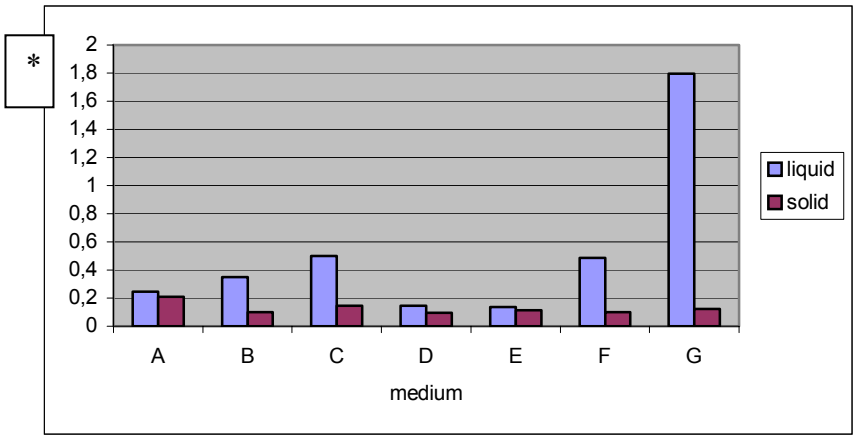
C - buds and adventitious bulblets (origin from bulb scale, 50%MS, 3% sucrose)

D - undifferentiated vascular bundle's callus and bulbs (origin from bulb scales, 10 μM BA, 1 μM NAA)

E - undifferentiated vascular bundle's callus and bulbs (origin from shoots, 10 μM BA, 1 μM NAA)

F - differentiated vascular bundle's callus (origin from ovary, 25 μM Picloram, 1 μM BA)

G - differentiated vascular bundle's callus (origin from bulb scales, 25 μM Picloram, 1 μM BA)



*Concentration of flavonoids and other phenolic compounds were shown in estimated units as the combined area of chromatography peaks per 1 g fresh weight of analysed tissue

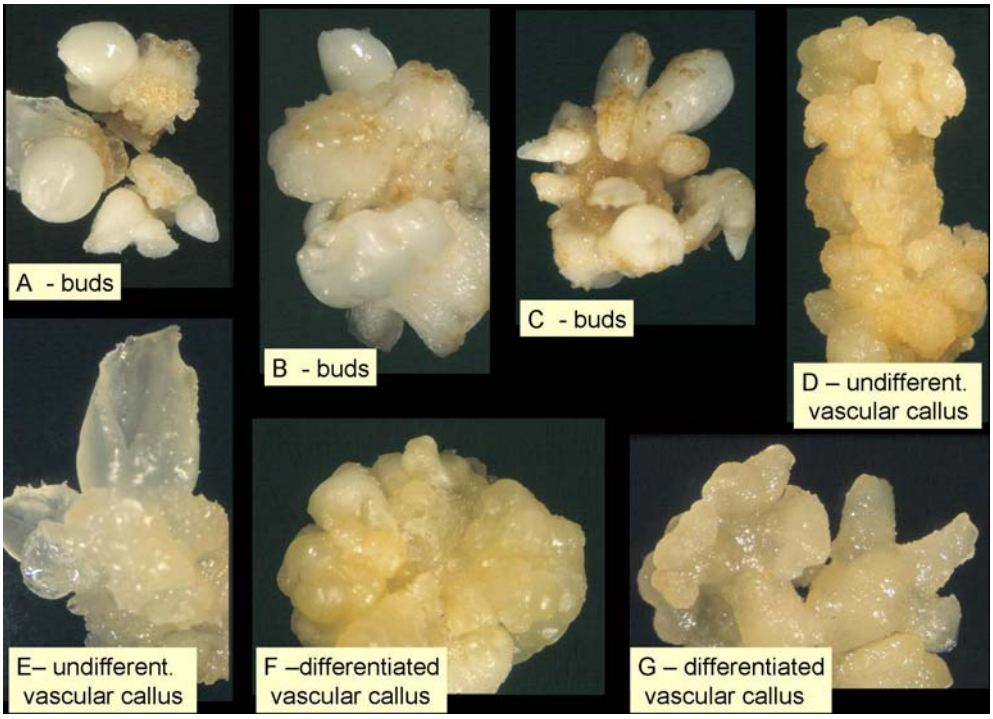


Figure 2 Cultures of *Galanthus* sp. in liquid media

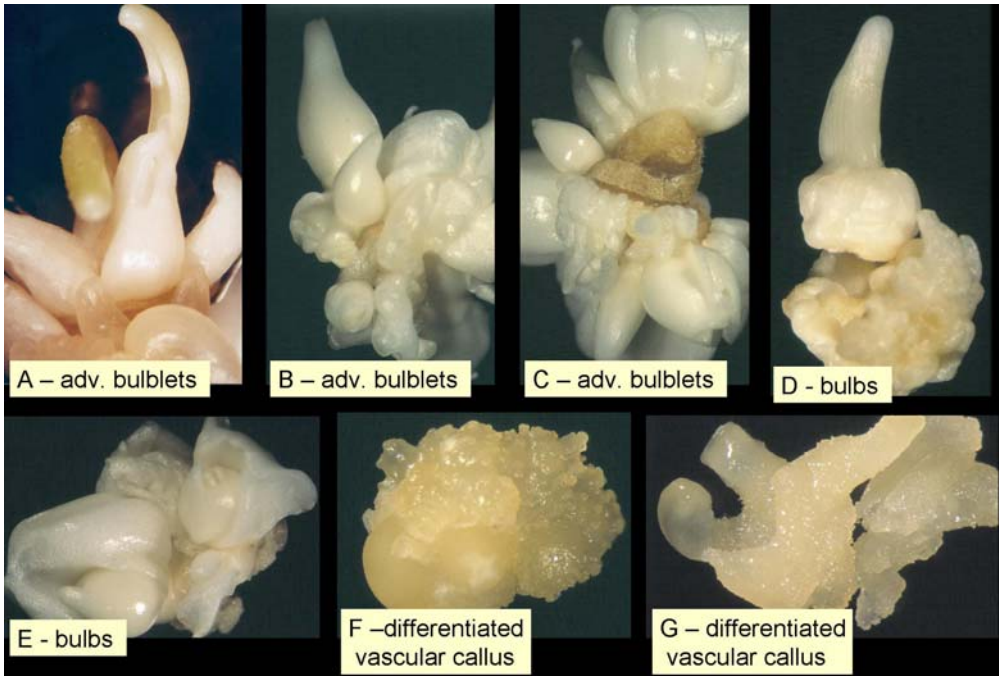


Figure 3 Cultures of *Galanthus* sp. in solid media

EMBRYOGENESIS OF MARITIME PINE: AN OVERVIEW

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Introduction

Maritime pine (*Pinus pinaster*) covers more than 4 million hectares in south-western Europe. In Portugal, this species occupies 33% of the forest area (1 million hectares) and it provides the raw material of choice for most wood products-based industry. Clonal propagation allows the exploitation of maximum genetic gain achieved in breeding programs and it can be used commercially for large scale propagation of selected genotypes. However, clonal propagation of maritime pine is difficult by conventional means and therefore, alternative propagation methods have been widely investigated. One of such methods, somatic embryogenesis, is a tissue culture technique for embryo multiplication. However, with a few exceptions, somatic embryogenesis in conifers has remained inefficient and strongly genotype-dependent. In the present work, the establishent of a somatic embryogenesis system from immature zygotic embryos of maritime pine is described. Molecular biology tools for analysing somaclonal variation as well as to identifying genes important for embryo development have been used to address some of the problems that remain difficult to solve in the available somatic embryogenesis system for maritime pine and other pine species. An overview of the different research areas going on in our lab related to embryogenesis of pine will be presented.

Materials and Methods

Embryogenic cultures of *P. pinaster* were established from pre-cotyledonary zygotic embryos of selected open pollinated trees as previously described (1). Established cultures were maintained on proliferation medium consisting of DCR basal medium (2) supplemented with BAP, 2,4-D, casein hydrolysate, L-glutamine and 2% (w/v) sucrose, solidified with 0.2% (w/v) Phytagel, in the dark at 22±2ºC. Subculture of the embryogenic cultures was performed every 2 weeks. Mature somatic embryos were obtained by culturing aliquots of freshly prepared embryogenic suspensions on filter paper discs placed on the surface of maturation medium containing ABA and a higher concentration of gelling agent. Cotyledonary somatic embryos were obtained after 12-18 weeks in the dark. The isolated embryos were subsequently transferred to post-maturation and germination conditions and plantlets were then transferred to a substrate of peat:perlite:pine bark (1:1:1) and grown in a mist chamber prior to transfer to the greenhouse.

Previously available Simple Sequence Repeat (SSR) markers (3) as well as newly developed SSRs (4) are being used to analyse DNA isolated from embryogenic cultures with different ages subjected or not to cryopreservation (5) and plants derived from somatic embryogenesis following described conditions (3, 4). Sequence-Specific Amplification Polymorphism (SSAP), a technique that requires information on retrotransposon specific sequences, is also being applied in maritime pine to evaluate genetic variation during somatic embryogenesis.

Somatic and/or zygotic embryos at several stages of development were used for differential gene expression analysis using differential display. Complementary DNA clones corresponding to transcripts found in specific developmental stages prior to cotyledon formation have been selected for further characterization. Full length sequences have been obtained using RACE and *in situ* hybridization has been performed for three of the cDNA clones. A putative Rab-GTPase gene is being further characterized by localization of GFP-fusions in pine protoplasts and genetic complementation using yeast mutants.

Results and Discussion

Somatic embryogenesis initiation has been obtained from all tested open-pollinated seed families (a total of 20) of maritime pine. Although embryogenic cultures can be established from a wide range of seed families, initiation rates varied greatly with the family. A highly strong dependence on the genotype has been observed during the somatic embryo maturation step. Less than 30% of the established lines were able to produce cotyledonary embryos when subjected to maturation conditions and some of the obtained embryos showed morphological abnormalities. Apparently normal somatic embryos have gone through post-maturation and germination treatments and recovered plants were transferred *ex-vitro*. Some of the plants continued to grow similarly to seedling-derived plants but others showed plagiotropic growth or reduced apical dominance and this behaviour was observed across different families and embryogenic lines. The growth of these plants will be followed over the next years and compared to the growth observed in plants derived from seedlings derived from the same trees.

The DNA analyses using different molecular markers are still underway but, up to now, no variation has been confirmed in the embryogenic cultures or in the somatic embryo plants.

Several genes that are differentially expressed along embryo development have been identified. We have focused on some identified transcripts that are expressed in specific early stages of embryogenesis (before cotyledon primordia formation). After searching for homologies with known sequences, three cDNA clones presenting high homologies with a Rab-GTPase, an F-box protein containing a Kelch repeat and a lipid transferase were selected for further studies. The expression profile for these clones has been confirmed using real-time PCR and full-length cDNA sequences have been successfully obtained. Studies for localization of expression within the embryo and for functional analysis have been initiated and preliminary results will be presented.

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MICROSPORE EMBRYOGENESIS AND ALBINISM IN BARLEY

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Introduction

Microspore embryogenesis consists in the regeneration of haploid plantlets from microspores, which are initially destined to develop into pollen grains. The microspore enters the embryogenic process developing into haploid embryo, which further regenerates a haploid plantlet. Haploid microspore derived plants are thus spontaneously or artificially diploidized and then produce totally homozygous diploid plant.

The process of microspore embryogenesis can be decomposed into 4 steps: 1/ the pre-treatment, or induction phase, destined to switch the pollen fate from the initial gametophytic program to the alternative sporophytic embryogenic program; 2/ the culture phase, which consists in the embryogenic development of the reoriented microspore; 3/ the regeneration phase allowing the development of microspore derived embryos into haploid plantlets; 4/ the diploidization phase.

In barley, the protocol of microspore embryogenesis has been widely improved for years in most cultivated lines. The best results were obtained in the winter lines, especially in the winter cv. Igri, which represents the model genotype for the improvement of microspore embryogenesis through anther culture. The main problem affecting the efficiency of microspore embryogenesis in barley is the production of albino plantlets in various proportions according to the cultivars. Both winter and spring lines are concerned though the latters are more sensitive. For example, the winter cv. Igri produces up to 90 % of green haploid plantlets, whereas the spring cv. Cork usually generates 99 % of albino plantlets, reducing the potential improvement of barley by anther culture in these cvs.

Optimizing the protocol aiming at decreasing the proportion of albino plants

The protocol was mostly improved focusing on the pre-treatment step. In Igri, using 4 weeks of anther cold pre-treatment (4°C) in the dark and in water, we obtained only 50% of green plants. But reducing the duration to 4 days and increasing the osmotic pressure of both the pre-treatment and the culture media, we could enhance the green plant proportion up to 88% in Igri. Several osmolytes were tested among which mannitol, sorbitol, PEG and sucrose. Mannitol at 180 mosm/kg provided the best results in terms of yield and albinism. In this respect, the percentage of chlorophyllous regenerated plants was not modified in Cork and remained lower than 1%, whatever the used compound.

The influence of copper has been tested because copper is involved in many biochemical processes dealing with chlorophyll synthesis and is indispensable to pollen development. When copper sulphate was used during the pre-treatment and the culture media at 10 µM, the yield of anther culture in Igri was improved by 400% considering the

number of regenerated plantlets and the rate of green plants reached 89%. The most striking result was obtained in Cork, since we could get up to 20.3% of green plants. Copper sulphate increases the survival of the microspores in the anther and further synchronizes the first sporophytic division of the reoriented microspores.
Finally, the effect of the anther/spike position was investigated. In both Igri and Cork, the anther response decreased from 76.6 to 31.5% in Igri and from 58.8 to 32.0% in Cork when the donor spike was far away from the main shoot. Amazingly, anthers collected from spike of the second tiller enabled to drastically improve the proportion of green regenerated green plantlets, reaching 90% in Igri and 39% in Cork. The current experimental design aims at combining the "tiller effect" and the "copper sulphate effect" in order to further decrease the amount of albino plant regeneration following microspore embryogenesis, especially in recalcitrant varieties.

Biological origin of albinism

In order to study the difference of behaviour between winter and spring barley cultivars during microspore embryogenesis, especially on albinism, plastid features and plastid DNA were followed during the microspore embryogenic process in both Igri and Cork.
In Igri, before pretreatment, microspore plastids contained DNA in the stroma and included starch and a single thylakoid. After pretreatment and during the culture phase, plastids slowly differentiated forming thylakoids, degrading starch reserves and conserving DNA in the stroma. During regeneration pro-chloroplasts further differentiate in fully functional chloroplasts containing amounts of chlorophyll comparable to the donor plant. In Cork, at the time of sampling most of microspore plastids were deprived of thylakoids and less than 2% of plastid sections contained DNA. During the culture phase, plastid DNA was not restored and plastids mainly accumulated starch reserves and plastoglobules, whereas thylakoids did not develop. In the microspore derived embryos of Cork, most of plastids remained undifferentiated and embryos regenerated a high proportion of albino plantlets.
These results indicate that numerous events occurring before the regeneration phase could explain the formation of albino plantlets in the spring cultivars, especially the plastid DNA fragmentation during pollen development occurs more rapidly in Cork than in Igri. Whatever, the microspore plastids are affected as early as the sampling stage but the process seems to be reversible using appropriate compounds such as copper.

Prospects

In order to identify the reason why chlorophyll is lacking in albino microspore derived plantlets, it would be necessary to further investigate the successive step(s) of chlorophyll biosynthesis in these plants, looking for missing enzymes. Afterwards, it should then be possible to check whether plastids or microspores are deprived of the corresponding gene(s).

IN VITRO BEHAVIOUR OF SE- AND PEM-TYPE AVOCADO EMBRYOGENIC CULTURES

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Introduction

Embryogenic cultures of avocado have been classified into two groups (SE and PEM) based on their morphology in presence of auxin (Witjaksono and Litz, 1999). PEM-type cultures consisted of proembryogenic masses (PEM) with occasional development of proembryos and somatic embryos at early stages while SE-type cultures consisted of somatic embryos, from globular to cotyledonary stages, and low frequency of proembryos and PEMs.

The aim of this investigation was to characterize SE- and PEM-type embryogenic cultures by monitoring growth habit and ability to produce mature somatic embryos under different culture conditions.

Material and Methods

Embryogenic cultures of avocado (*Persea americana* Mill.) were initiated from immature zygotic embryos, cv. Anaheim, in MS medium (Murashige and Skoog, 1962) supplemented with 0.41 μM picloram and 6 $g.l^{-1}$ agar (Pliego-Alfaro and Murashige, 1988). Each cell line, originated from one embryo, was maintained in the same culture conditions with change to fresh medium at monthly intervals.

For stimulating maturation of somatic embryos, embryogenic supensions were initiated in liquid maintenance medium according to Witjaksono and Litz (1999). Suspension cultures were maintained on a rotary shaker at 120 rpm and subsequently sieved through two screens. The 1-2 mm fraction was cultured on B5m medium (consisting of MS medium with the macroelements of Gamborg *et al.* (1968)) solidified with 6.8 $g.l^{-1}$ Gelrite during three recultures of five weeks each (Perán-Quesada *et al*., 2004).

The ability of embryogenic cell lines to regenerate mature somatic embryos was evaluated by quantifying the production of white-opaque (w-o) somatic embryos.

Three typical cell lines of each culture type (SE or PEM) were used in this study. Ten to twenty cultures were used per cell line and treatment and the experiments were repeated twice.

Results

Avocado embryogenic cell lines cultured on maintenance medium differed in their morphology and could be divided into SE- and PEM-type according to Witjaksono and Litz (1999) (Fig. 1). However, growth at the end of each subculture, in terms of fresh weight increase, was similar for both types of cultures.

Maintenance medium composition differently affected the appearance of SE- and PEM-type cultures. PEM-type cell lines retained their basic morphological characteristics independently of variations in basal medium (MS or B5m) or gelling agent (agar or Gelrite); however, SE-type cultures showed its typical morphology in agar-gelled media while in Gelrite-gelled media their appearance became more similar to that of PEM-type cell lines.

Significant differences in the ability to differentiate mature somatic embryos were found between both culture types. Eighty percent of cultures belonging to SE-type were capable of regenerating w-o somatic embryos while in PEM-type cell lines only 3.33% of cultures developed these structures.

Time in suspension showed an important effect on the subsequent response to maturation treatment. The best results were obtained at 9 and 14 days, for SE- and PEM-type cultures respectively, coinciding in both cases with the linear growth phase of the suspensions.

Large variations in the ability of embryogenic cultures to regenerate mature somatic embryos were found to be related with initial suspension density. While for SE-type cell lines optimum results were obtained at 0.4 g, PEM-type cultures gave the best results at 4 g. At these conditions, cultures capable of differentiating w-o somatic embryos increased up to 33.13%.

Discussion

Avocado embryogenic cultures developed different morphology in presence of auxin. As reported von Arnold *et al*., (1995) in *Picea abies*, it might be related to the developmental stage at which embryogenic cell lines keep proliferating in culture. SE-type cell lines proliferate at a more advanced stage than PEM-type cell lines.

Morphological differences observed under maintenance conditions were associated to differences in the subsequent culture ability to produce mature somatic embryos. In *Picea abies*, von Arnold *et al*. (1995) have proposed that embryos of undeveloped cultures were blocked in their development and had not reached the stage where they could respond to maturation treatment. Results obtained in the present study revealed that differences in regeneration capacity between embryogenic cultures could also be explained on the basis of different culture requirements (Egertsdotter and von Arnold, 1993). Several treatments, such as increasing time in suspension or initial suspension density, have improved the ability to produce mature somatic embryos from PEM-type cell lines. Further investigation should be made for optimizing maturation of somatic embryos from PEM-type cell lines.

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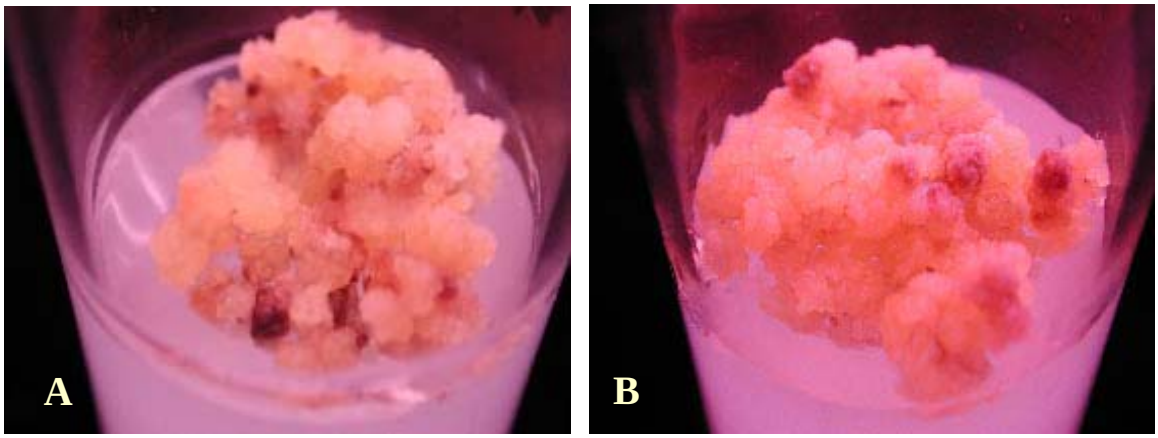

Figure 1 Avocado, cv. Anaheim, embryogenic cultures in maintenance conditions.
A. SE-type culture
B. PEM-type culture

POLYAMINE METABOLISM DURING ZYGOTIC EMBRYOGENESIS IN SCOTS PINE

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Introduction

The polyamines (PAs) spermidine, spermine and putrescine are widespread in higher plants. Their role in plant embryogenesis has been shown but until now these studies have mainly been focused on somatic embryogenesis (SE). It has been shown that PA levels in embryogenic cultures of pines (*Pinus* sp.) fluctuate as somatic embryogenesis proceeds. Proliferation of the embryogenic cell masses has been accompanied by a high level of putrescine compared to both spermidine and spermine (Sarjala *et al.*, 1997; Minocha *et al.*, 1999), while transfer onto maturation medium has resulted in an increase in the spermidine/putrescine ratio (Minocha *et al.*, 1999). However, the reactions of embryogenic tissues to exogenous PAs have proved complicated. The changes in growth and PA pools have varied between species, and shown high dependence on the developing stage of somatic embryos (Santanen and Simola, 1992; Minocha *et al.*, 1993; Sarjala *et al.*, 1997; Kong *et al.*, 1998). The general aim of the study was to improve understanding of the PA metabolism during the zygotic embryo development of Scots pine (*Pinus sylvestris* L.) and to achieve this goals, we studied PA metabolism at transcriptome, proteome and metabolome level.

Material and Methods

Developing zygotic embryos of the Scots pine (*Pinus sylvestris* L.) were excised from one-year-old immature seed cones which were derived from elite pine clones. Scots pine specific primers were developed for PA biosynthesis genes *Adc* (arginine decarboxylase), and *Samdc* (S-adenosylmethionine decarboxylase). At the different developmental stages PAs were analyzed by HPLC and the expression of PA biosynthesis genes were tested by real time RT-PCR and by *in situ* hybridization. For polyamine data covering two years the statistical regression models were fitted.

Results

According to the statistical regression models the fluctuation of PAs showed an increasing trend in the early stages but a decreasing trend in the late stages of embryogenesis. Only the free putrescine fraction remained stable during embryo development. PA biosynthesis strongly preferred the ADC pathway: both *ADC* gene expression at the mRNA transcript level and ADC enzyme activity were substantially higher than putative *ODC* gene expression or ODC enzyme activity, respectively. The *ADC* gene expression increased during zygotic embryo development at both mRNA and enzyme activity levels. The *ADC* mRNA transcripts were localized in the dividing cells of apical and axillary meristems, and they were specifically associated with the spindle apparatus of the metaphase and anaphase cells of late embryos.

Discussion

The statistical regression modelling of PA data in the present study emphasized the concave curvature in all free and soluble-conjugated fractions of the examined PAs except free Put. The fluctuation of PAs seemed to show an increasing trend at the early stages but a decreasing trend at the late stages of embryo development. Only the free Put fraction remained stable throughout the period of embryo development. The statistical regression modelling of PA data in the pre-

sent study emphasized the concave curvature in all free and soluble-conjugated fractions of the examined PAs

We also found a high PA content in developing Scots pine zygotic embryos. This is in accordance with the high PA contents reported in tissues undergoing rapid cell division, active metabolism and somatic embryogenesis (Egea-Cortines and Mizrahi, 1991; Kakkar and Sawhney, 2002). Spermidine, which was the most abundant PA in our samples, was also the main PA in the zygotic and somatic embryos of *Pinus radiata* (Minocha and Minocha, 1995; Minocha *et al*., 1999b).

Both the *ADC* gene expression at the mRNA transcript level and the activity of the ADC enzyme were detected in developing zygotic embryos. The higher rate of ADC activity compared to ODC was prominent, which is in agreement with the reports of somatic embryo development in red spruce (Minocha *et al*., 1996) as well as the zygotic embryogenesis of Norway spruce (Santanen and Simola, 1999). We found the highest ADC and ODC activities in the supernatant fraction containing soluble proteins, but some were also detected in the pellet. We also found that *ADC* expression increased during embryo development at both the mRNA and the enzyme activity levels. This suggests an involvement of transcriptional regulation in the regulation of *ADC* gene expression, but does not rule out post transcriptional regulation of the gene.

In the present study, we were able to localize *ADC* gene expression specifically in the mitotic cells of the shoot apical and axillary meristems of developing zygotic embryos. To our knowledge, this has not been reported earlier in the zygotic embryos of higher plants. There is evidence that PAs are involved in the regulation of cell division in animals (Bello-Fernandez *et al.*, 1993), micro-organisms (Theiss *et al.*, 2002) and plants (Kaur-Sawhney *et al.*, 1980). In mammalian cells, the progression of the normal cell cycle is dependent on PAs (Oredsson, 2003), and PA contents have been reported to vary throughout the cell cycle in *Helianthus tuberosus* (Serafini-Fracassini, 1990). The specific localization may indicate that ADC is involved either in chromatin condensation or in mitotic spindle formation during cell division in Scots pine embryos. However, the molecular basis of interaction between PAs and the microtubular system is still unknown and has not been shown in plants before. It has been suggested that, because PAs are multivalent cations, they can act as electrostatic bridges between the highly charged linear chains including DNA and microtubules (Tang *et al.*, 1997), but this does not explain why ADC was expressed specifically in the mitotic cells of shoot and axillary meristems in late embryos, but not in early embryos or in root meristems.

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THE SOMATIC EMBRYOGENESIS RECEPTOR KINASE GENE (*SERK*) IN HIGH EMBRYOGENIC CALLUS CULTURES OF *Cyclamen persicum* MILL. CV. HALIOS

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Introduction

Cyclamen persicum Mill cv. Halios is a commercial cultivar that is mass produced as pot-plant using F1 hybrid seeds. In order to reduce the propagation cost and to increase the homogeneity of the crop, it could be suitable to establish a simple and rapid protocol to produce somatic embryos and then artificial seeds.

Moreover, in these recent years, there are many reports of genes involved in the regulation of somatic embryogenesis; among them, particularly interesting seems to be *SERK (SOMATIC EMBRYOGENESIS RECEPTOR KINASE)*, a Leu-rich repeat, receptor-like kinase (LRR-RLK). This gene is involved in the precocious stages of embryogenesis and has been shown to be a marker of embryogenic cells in *Daucus carota* (Shah *et al.*, 2001), *Dactylis glomerata*, and *Arabidopsis* (Shah *et al.*, 2002), in which, *SERK* genes exist as a gene family. The identification of *SERK* genes in maize (Baudino *et al.*, 2001) and also in *D. glomerata* indicates that *SERK* is present in monocot as well as dicot plants and this gene was found in other several species: *Citrus unshiu, Heliantus annus, Oryza sativa, Medicago truncatula* (Nolan *et al.*, 2003).

The aim of the present research is to study this gene in *Cyclamen persicum* in order to induce or increase embryogenic potential in superior genotypes.

Material and Methods

Immature ovules at different stage of development, taken from the mother plants (*Cyclamen persicum* Mill. cv. Halios , Morel® - France), were used as starting materials for the callus induction. They were surface sterilized first with ethanol (70%, 30 sec), then in NaOCl solution (1.2%, 20 min). They were rinsed twice in sterile distilled water (10 min each).

The aseptic explants were cut longitudinally and plated in Petri dishes on semisolid embryogenic induction medium according with the protocol of Schwenkel *et al.* (1998), containing ½ MS micro and macro elements, MS vitamins, 30 $g.l^{-1}$ sucrose, 5 $g\ l^{-1}$ glucose, 100 $mg.l^{-1}$ caseinhydrolisate, 2 $mg.l^{-1}$ 2,4-D, 0.8 $mg.l^{-1}$ 2-iP, 4 $g.l^{-1}$ phytagel, pH=5.6. The same medium, without any growth regulator, was used for the regeneration phase. The cultures were maintained at 24°C, in darkness and were subcultured monthly.

RNAs were isolated from the callus and the somatic embryos cultured *in vitro* on regeneration medium according to the protocol of Kiefer *et al.* (2000). The first-strand cDNA synthesis was performed on 2.5 μg of total RNAs using oligodT and M-MuLV (Invitrogen® - USA). PCR based strategies were performed using 2 μL of the RT reaction as a template and using degenerated primers designed on nucleic acid or protein

alignment (CODEHOP strategy-Rose *et al*., 1998) of the *SERK1* gene in all the species were this gene was already sequenced. PCR products of interest were purified using the High Pure PCR Product Purification Kit from the Roche ® Diagnos- tics Corporation and were performed according to the instructions of the manufacturer. The fragments were ligated onto p-GEM T-Easy vector (Promega, Madison, Wis.); all clones were sequenced.

As a control of the size of the expected product we considered *Arabidopsis* genomic DNA and pGEM-T cloned *SERK1* plasmid cDNA. The 3' and 5' regions of the gene were kept using the Racing strategy.

Results and Discussion

The protocol of sterilization allowed to reach the 98% of sterility. The ability to induce callus and to regenerate somatic embryos in *Cyclamen persicum* Mill. cv Halios in the suggested medium is confirmed; all the characteristic phases of this process could be clearly described.

There is an evident clonal behaviour in all the callus lines obtained: the calli show different colours and compactness related to the embryogenic potential. From a high embryogenic line it was possible to extract high quality RNA and cDNA to be used in the PCR strategy. We successfully cloned a fragment (750 bp) of a *Cyclamen SERK* cDNA (Fig. 1) which showed a high homology with the sequences of the other species: 93% with *Citrus unshiu*, 92% with *Medicago truncatula* and 88% with *Oryza sativa*.

It seems that in *Cyclamen persicum*, this gene exist in two allelic forms. This is also confirmed from the sequencing of the fragments obtained from the racing of the cDNA 3' region. The sequencing and the analysis of the cDNA 5' region is still in progress.

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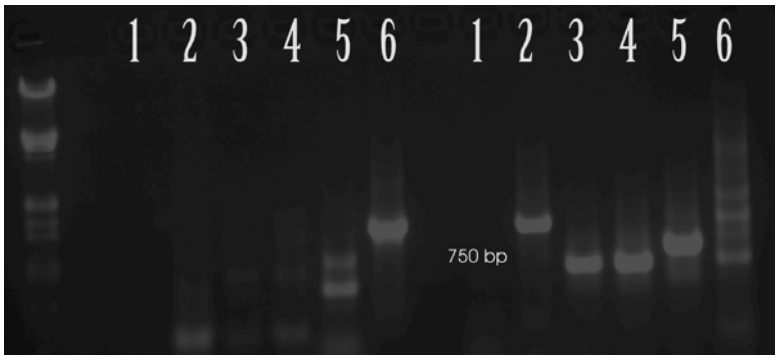

Figure 1 Left: Primary (Primers D1-H2)-Right: Nested PCR (P2-H2)
Molecular Marker (λDNA-ECOR1 HindIII digest)
Lanes 1: control
2: DNA *Cyclamen persicum* Mill.
3 and 4: cDNA *Cyclamen persicum* Mill.
5: *Arabidopsis thaliana* DNA
6: *Arabidopsis thaliana* pGEM-T cloned *SERK1* plasmid cDNA

A NEW REGENERATION SYSTEM FOR GENETIC MODIFICATION OF LEGUMES

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Introduction

The regeneration system used for genetic modification of most of the legumes, is based on multiple shoot formation of the axillary buds of the cotyledonary nodes of seed. As a result most of the plants are either escapes or only partly transformed and as a consequence will not pass the transgenes to the next seeds generation.

Olhoft *et al.* (2003) stated that the efficiency of soybean transformation has to be improved 5-10 times before one person can produce 300 transgenic lines per year. Meristems are highly complex, multicellular structures and meristematic cells represent only a very low proportion of the total cells in the explant. The efficiency of the organogenic system for genetic modification could be improved if the number of shoots per explant is increased or if the number of meristematic cells in explants is increased.

This abstract describes the new regeneration system developed for pea and soybean and how it has been for the production of genetically modified pea plants.

Material and Methods

One node cuttings derived from seedlings of pea and soybean were multiplied on TDZ containing medium. This resulted in a tissue covered with buds and or small shoots (Tzitzikas *et al*., 2004; Zinhui *et al*., 2005). From here on described as bud containing tissue tissue (BCT). BCT was multiplied in a repetitive way on TDZ supplemented medium. Shoots were obtained by transferring the tissue to GA (pea) or BA (soybean) containing medium.

In pea BCT was used for *Agrobacterium* mediated transformation using AGL1(pLUC-legj). The aim is to produce pea plants with an altered protein composition.

Results

In pea culture of stem tissue with one node (1-cm size) on a medium containing 4 $mg.l^{-1}$ TDZ result in multiple shoots. Subcultured of the multiple shoots on the same medium resulting in the formation of a green hyperhydric tissue which is fully covered with small buds (BCT) BCT is multiplied by subculturing fragments on the same medium. Plants are regenerated by transferring BCT to a medium supplemented GA_3. Plants were rooted on a medium supplemented with 0.5 $mg.l^{-1}$ NAA or IAA or IBA. Rooting was inhibited if the BCT was maintained for a prolonged period of time. Both rooted and non-rooted plants were acclimatized successfully in the greenhouse (Tzitzikas *et al*., 2004).

In soybean BCT initiation occurred only if the axillary buds were not removed from the cotyledonary node. The best BCT induction was achieved by pretreating the seeds for one week on medium supplemented with 0.1 $mg.l^{-1}$ TDZ, followed by culture of the cotyledonary node on medium supplemented with 0.5 $mg.l^{-1}$ BA for 4 weeks. A medium supplemented with 0.1 mg/L TDZ resulted in the proliferation of BCT. BCT tissue

was maintained in this way for 12 months. Three hundred thirty six shoots were obtained when 1g of BCT tissue was subcultured on medium supplemented with 0.5 $mg.l^{-1}$ BA. Medium without growth regulators was optimal for rooting. The regenerated plants grew normal in the greenhouse (Zinhui *et al*., 2005).

In pea BCT was infected with the *Agrobacterium* strain AGL1(pLUC-legJ). Selection of transgenic tissue was based on luciferase activity only. In total independent transgenic plants were obtained. Most of them were able to produce seeds in the greenhouse. In all tested plants the luciferase transgene was transmitted through seeds.

Discussion

The previous described transformation systems for legumes usually use mature or immature seeds as starting material and meristems of the cotyledonary nodes as target tissue. As the meristematic cells represented only a small portion in the explant, the chance of transforming such cells is low. Furthermore because a meristem is a multicellular structure there is a chance of obtaining chimeric transformed plants. BCT contains much more meristematic cells than a single cotyledonary node and can be used as target tissue for genetic transformation. The cyclic nature of repetitive subculture of MBT allows purification of a completely genetically modified plant from a partly transformed meristem. This approach was successful in pea. More reseach is needed whether it is also useful for other legumes.

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MICROSPORES: A MULTIPURPOSE HAPLOID CELLS

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Isolated microspore cultures have the remarkable quality to resemble the alternation of generations in the life cycle of angiosperms, i.e. the change between the diploid sporophytic and the haploid gametophytic generations. Although the natural destination of microspore development is to differentiate into mature pollen and accomplish fertilisation, isolated and *in vitro* cultured microspores or young pollen grains can either differentiate into mature, fertile pollen (the male gametophytes) by culture in a rich medium without stress, or divide repeatedly and develop into embryos (sporophytes) after a stress treatment.

As experimental systems, microspore cultures are used to investigate pollen development and pollination, embryogenesis, totipotency, cytodifferentiation, cell cycle, and the role of stress in development. As a tool in genetic engineering, they can be used to produce doubled haploids (recombinant inbreds) for plant breeding and gene mapping, to induce and select for mutants and to create transgenic plants.

The development of isolated, defined wheat microspores undergoing *in vitro* embryogenesis has been followed by cell tracking. Three types of microspores were identified on the basis of their cytological features at the start of culture. Type 1 microspores had a big central vacuole and a nucleus close to the microspore wall, usually opposite to the germ pore. This type was identical to the late microspore stage in anthers developing *in vivo*. Microspores with a fragmented vacuole and a peripheral cytoplasmic pocket containing the nucleus were defined as type 2. In type 3 microspores the nucleus was positioned in a cytoplasmic pocket in the centre of the microspore. Tracking revealed that, irrespective of origin, type 1 microspores first developed into type 2 and then into type 3 microspores. After a few more days, type 3 microspores absorbed their vacuoles and differentiated into cytoplasm-rich and starch-accumulating cells, which then divided to form multicellular structures. Apparently the three types of microspores represent stages in a continuous process and not, as previously assumed distinct classes of responding and non-responding microspores.

A modified suppression subtractive hybridization method was used to isolate genes involved in the formation of embryogenic microspores. Subtracted cDNA pools were created using already constructed cDNA libraries prepared from poly(A^+) RNA isolated from non-stressed tobacco microspores and microspores stressed in a starvation medium for 6 days at 33 °C. In total 435 randomly picked plasmid and phage clones were screened by differential reverse Northern for their expression profiles. The potential of the method was demonstrated by the isolation of 92 differentially expressed cDNAs from stressed microspores. Further analysis of selected clones by multiple tissue Northern and *in situ* hybridization confirmed their stronger expression in stressed microspores and microspore derived embryos, but not in mature pollen. Sequence analyses of these clones revealed several genes involved in metabolism, chromosome remodelling, transcription and translation, while about 35% of sequences did not significantly match to any known gene or published ESTs. Based on the identity and expression profiles of identified clones, the formation of totipotent (embryogenic) microspores by stress is discussed.

ALTERNATIVE METHODS FOR LONG-TERM CONSERVATION OF NORWAY SPRUCE AND OAK EMBRYOGENIC LINES

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Introduction

Long-term preservation of embryogenic lines is important both for conservation of germoplasm and biodiversity and for technical reasons of plant propagation. The most common way for conservation is the cryopreservation, but it usually claims controlled prefreezing, and the cryostat is not affordable for all propagation laboratories. This paper is a revue of most commonly used conservation methods applied to embryogenic lines of oak and Norway spruce. Comparisons between cryopreservation (with and without controlled freezing) and encapsulation were made, and the genetical implications of each procedure were analyzed. The long-term culture of embryogenic lines (living collection) is considered as reference for the physiological status of cultures and for the conformity of regenerated plants.

Material and Method

The biological material was represented by embryogenic lines of oak (*Quercus robur*) and narrow crowned Norway spruce (*Picea abies f. pendula*) from the collection of the biotechnology lab of Forest Research Institute, Research Station of Simeria (Palada-Nicolau, 1999; Palada-Nicolau and Hausman, 2000). The cryopreservation of Norway spruce ESM without controlled freezing was performed according the method of Hargreaves and Smith (1994) with modifications. Various combinations of freezing solutions and cryoprotective agents were tested. The results were compared with them obtained in the classical cryopreservation technique using controlled freezing (Kartha *et al.*, 1988). The cryopreservation of oak embryos was performed without controlled freezing, according to the method of Tutkova and Wilhelm (1999) with modifications. The embedding in calcium alginate was performed according to the protocol proposed by Kinoshita and Saito (1990) for the conservation of buds. The recovery of embryos after cryopreservation was assessed by microscopical observation with the fluorescein-diacetate coloration.

Results and Discussion

Cryopreservation of narrow-crowned ESM with and without controlled prefreezing. The Norway spruce embryogenic tissue (72 monoembryonal origin embryogenic lines, belonging to 10 maternal progenies) was maintained in long-term culture (living collection) by subcultures performed every 7 days on GDX medium. As living collection is a time-consuming technique, it was necessary to conserve the most of lines and multiply them only before starting the synchronic maturation procedure. According to the method of Kartha *et al*. (1988) it is rather easy to cryopreserve Norway spruce ESM using a cryostat programmed for a controlled freezing from 0 ^{o}C to -35 ^{o}C, after a treatment of cryoprotection based on sucrose 5% and DMSO. This technique, applied to common Norway spruce ESM, resulted in 98-100 % recovery of embryogenic tissue. The FDA test showed the viability of a small part of cryopreserved tissue, mostly small, spherical cells of embryo heads, but also suspensoral and isolated embryogenic cells. The restriction of viability to a small number of embryogenic cells can explain in some extent the genetic drift of cryopreserved embryogenic lines, if we accept some variability inside the cell line. The most important expression of such genetic drift was the recovery of cell lines that varied in maturation efficiency (either better or worse than before cryotreatment), maturation quality and structural composition, but the regenerated plants presented a rather good conformity with the source. Because of the lack of a cryostat, it was necessary to develop a cryopreservation method without controlled freezing. Hargreaves *et al*. (1995) proposed such a method, based on cryoprotection with sorbitol and DMSO and using a prefreezing of 2 h at -70 ^{o}C. We have tried to develop a cryopreservation method using only a cryoprotective treatment and a lab freezer up to -20 – 22 ^{o}C. Two variants of cryoprotection treatments were tested, based on sucrose 5% and on sorbitol 0.4M. DMSO was used in the same way in both variants. As for the prefreezing, we tried to simulate the program of the cryostat by immersion of cryoprotected tissue for one hour in alcohols cooled at -20 ^{o}C. Three variants were tested: absolute ethanol, isopropanol and a mixture 1:1 of them. For the immersion of cryotubes containing ESM pieces in the alcohol solutions, a home-made device, inspired by “Mr. Frosty” (Nalgene) of M. Wagner was used. 10 ESM pieces of cca 200 mg of each variant of cryoprotection and freezing (in total 60) were processed from each of 3 embryogenic lines tested, totally 180 pieces. The test of recovery was made after a week in liquid nitrogen, by cultivating the ESM pieces on GDX medium with the progressive decreasing of su-

crose concentration for promoting the re-growth. FDA observations were made occasionally, in order to see how many and which type cells survived. The differences among cell lines showed that the efficiency of recovery was dependent of genotype or of cell line quality (senescence degree, growth rate, etc.). Sucrose generally gave better results as cryoprotector than sorbitol. As for the freezing media, isopropanol was slightly better than ethanol and the mixture of the two alcohols did not ameliorate the results. In fact, the role of the immersion solution is to stabilize the freezing regime and to control the freezing speed, depending of the technical characteristics of the freezer. The continuous survey of the temperature was made with a temperature logger placed inside the freezer.

A combined method of encapsulation and cryopreservation of oak somatic embryos. Three oak embryogenic lines were used in the experiment (Q.r.1., Q.r.3., and NL 100), all of them belonging to an embryogenic system based on direct embryo induction and proliferation by serial adventitious embryogenesis, on hormone-free medium. Before starting the procedure, the cultures with a good proliferation rate and good dispersion of embryos were partially synchronized and the embryos were separated in 4 development stages (Palada-Nicolau and Hausman, 2001). Only the stages 1 and 4 were used for encapsulation and cryopreservation. The alginate beads containing each one embryo (or a 1 mm embryo cluster in the case of stage 1) were conserved for a short to medium time period (one to three months) at 4 ^{o}C, in refrigerator. The recovery and re-growth in this case was in the range of 85% to100%, depending of time and conservation conditions. It is very important to maintain a constant humidity inside the plastic Petri dishes containing the beads, otherwise, the dried beads will not re-growth. After a one month cultivation of beads on P 24 hormone-free medium, the small embryos (stage 1) proliferated by serial embryogenesis, forming embryo clusters or colonies, and the big (stage 4) ones continued maturation and started to germinate. In order to conserve the oak embryogenic lines for a long time period, and perform the cryopreservation without controlled prefreezing, the alginate beads were immerged in liquid nitrogen. Cryoprotection treatments with sucrose were applied to embryos, before encapsulation. After one week in liquid nitrogen, the beads were cultivated on hormone-free medium at 25 ^{o}C for one month, in order to test the viability and re-growth.

The following results were obtained: (1) Only the small stage 1 embryos recovered after cryotreatment. The surviving rate was around 10-15% (maximum 27% and minimum 6.7%) and the re-growth rate was around 5%, with a maximum of 6.7% and a minimum of 2.8%. The embryo clusters recovered better than the isolated embryos. **(2)** There were differences among genotypes: Q.r.1 and NL 100 gave better results than Q.r.3. **(3)** The cryoprotection treatments were not benefic Moreover, the immersion of embryos in sucrose solution before encapsulation was harmful. **(4)** As a consequence of the recovery and re-growth of cryopreserved oak embryos, 16 new embryogenic lines were obtained. **(5)** Their germination and conversion ability was not affected by cryotreatment. **(6)** Regenerated oak plants were obtained out of cryopreserved embryos derived from the somaclones Q.r.1 and NL 100.

Conclusions

A simple cryopreservation method for Norway spruce ESM somaclone was developed, using only a cryoprotective treatment and a lab freezer up to -20 – 22 ^{o} C. The differences among cell lines showed that the efficiency of recovery was dependent of genotype or of cell line quality (senescence degree, growth rate, etc.). Sucrose generally gave better results as cryoprotector than sorbitol. As for the freezing media, isopropanol was slightly better than ethanol and the mixture of the two alcohols did not ameliorate the results. A combined method of encapsulation and cryopreservation of oak somatic embryos, in order to conserve them for a long period. Alginate beads containing each one embryo or a small embryo cluster were conserved for a short to a medium time period (one to three months) at 4 ^{o}C, into refrigerator. The recovery and re-growth in this case was in the range of 85% to100%. Only the small stage 1 embryos recovered after cryotreatment. The surviving rate was around 10-15% (maximum 27% and minimum 6.7%) and the re-growth rate was around 5%, with a maximum of 6.7% and a minimum of 2.8 %. The embryo clusters recovered better than the isolated embryos. There were differences among genotypes. The cryoprotection treatments were not benefic. Moreover, the immersion of embryos in sucrose solution before encapsulation was harmful.

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IN VITRO RESCUE OF INTERGENERIC HYBRIDS BETWEEN *Melandrium album* AND *Lychnis coronaria* (*CARYOPHYLLACEAE)*

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Introduction

Intergeneric hybridization is useful for transferring valuable characters across species to create new combinations of forms and colors in the progeny. The aim of this study was to evaluate hybrids between *M. album* and *L. coronaria* obtained by means of *semi in vitro* cross pollination.

Material and Methods

Placental pollination was used to overcome incompatibility and to obtain intergeneric hybrids. Pollen grains isolated from aseptically collected anthers of *L. coronaria* were directly placed on ovules remaining on the placenta of *M. album*. After pollination placentae were transferred to test tubes containing ½ MS medium supplemented only with 3% sucrose. Hybrid cotyledonary embryos which were developed one month after pollination were dissected from the ovules and transferred to MS medium supplemented with 1 $mg \cdot l^{-1}$ IAA. After two months of culture, plantlets with green leaves and root systems were transferred to soil. Some of the hybrids grew normally, over wintered and produced flowers during the next summer. Hybridity of the *M. album* x *L. coronaria* plants was confirmed by comparison of their morphological characters and by RAPD analysis. Petal color, bract and leaf color were described using the Royal Horticultural Society color chart.

Results

As a result of pollen tube growth through the ovule micropyle, several vigorous embryos and subsequently hybrids were obtained. Stylar barriers could be overcome by intraovary pollination. Hybrids are intermediate between its parents with respect to majority of 25 characters scored. RAPD pattern in agarose gel of *M. album* x *L. coronaria* hybrids visualized DNA bands originating from both parents. In the second growth season plants started to flower, however, due to male and female sterility hybrids did not produce seeds. This experiment will be continued by polyploidization of the F1 hybrids with the aim of obtaining viable seeds.

Discussion

Morphometric flower characteristics of hybrids were intermediate between parent plants, probably because the parents are closely related and genetically similar species 2n = 24.

Intergeneric hybrids often show abnormalities i.e. malformation of cotyledons and leaves, inhibition of root growth and susceptibility to diseases. Many of our hybrids were also weak, grew slowly and died at juvenile stage.

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IN VITRO AND MICROSCOPIC TOOLS FOR INTERSPECIFIC HYBRIDISATION IN WOODY ORNAMENTALS

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Introduction

Interspecific hybridisation is recognized as one of the most important source for evolution and domestication of flowering plants. Also for woody ornamentals, these techniques are used to introduce new genetic variation in the cultivated plants. But the difficulty of creating interspecific hybrids increases along with the phylogenetic distance between the parents. Different pre- and postzygotic barriers have been met from the first attempts on.

Within the *Hibiscus*, *Buddleia*, *Ligustrum* and *Hydrangea* genera, a lot of interspecific crosses were done (and will be done). The prezygotic barriers (no germination of the pollen of the parent plants, inhibited growth of the pollentube through the style of the seed parent) were analysed using light and fluorescence microscope. For overcoming the postzygotic barriers (malformation of endosperm, inhibition of germination of the seeds), the 'embryo rescue' technique was performed on the 4 genera, in order to replace endosperm and thus allow maturation of the hybrid embryo. The hybrid character of the obtained seedlings is then analysed by flowcytometry, AFLP and GISH.

Not only the phylogenetic distance, but also the difference in ploidy levels between the two parents is one of the reasons of incongruity in interspecific crosses. For example, in the cross *Buddleia globosa* x *Buddleia davidii*, the ploidy levels are different. In this study, *in vitro* research efforts with regard to chromosome doubling of *Buddleia globosa* are also performed.

Material and Methods

The aim of the interspecific crosses within the 4 genera *Hibiscus*, *Buddleia*, *Ligustrum* and *Hydrangea* is summarized in Table 1. Before performing the interspecific pollination, pollen of each parent plant was collected and transferred to a germination medium containing 15% sucrose, 10mg/100ml H_3BO_3, 10mg/100ml KH_2PO_4 and 10mg/100ml $CaCl_2$ in water. After overnight incubation, the pollen germination is analysed with a light microscope (LEICA DM IRB). To study the growth of the pollen tube through the style of the seed parent, pollen tubes (4 samples per cross) were stained with aniline blue 72 hrs after pollination. The stained material was squashed on a glass slide and observed with a fluorescent microscope (LEICA DM IRB). Characterization of the prezygotic barriers was performed as described by Cuevas *et al* (1994).

In order to overcome the postzygotic barriers, immature embryos (*Hibiscus* and *Ligustrum*) and ovules (*Hydrangea*, *Buddleia*) were transferred to the germination medium 11 weeks after pollination. Preceding *in vitro* initiation, *in vivo* material was rinsed in 70% ethanol, sterilised for 20 min in a 10% NaOCl solution with 0.005% teepol and finally rinsed 3 times in autoclaved water. The media that were used are presented in Table 2. Cultures were maintained at 23 ± 2°C under a 16 hrs photoperiod. Tissue culture plants were subcultured every 6 weeks. After rooting *in vitro*, plantlets were acclimatised for 12 weeks (on basal medium) and transferred to greenhouse.

When flowering, the seedlings obtained after 'embryo rescue' were analysed by flowcytometry and AFLP. Flow cytometry was performed using a Partec PAS III as described by De Schepper *et al.* (2001). AFLP (Amplified Fragment Length Polymorfism) was performed as described in Van Huylenbroeck *et al,* (2000). The primercombinations used for the different genera are presented in Table 3.

Another (microscopic) technique to study the introgression of pollen parent DNA in the hybrid is GISH (Genomic *In Situ* Hybridisation) This GISH-technology is at this time optimized for *Hydrangea macrophylla*. The chromosome preparations were made following the protocol described in Khrustaleva and Kik (2001). The chromosomes were stained with 1 $\mu g.ml^{-1}$ DAPI.

Chromosomes were measured using Micromeasure software for Windows version 3.3. A karyotype for *Hydrangea macrophylla* was constructed according the nomenclature described in Levan *et al.* (1964). FISH was performed on *Hydrangea macrophylla* 'Fasan' as described in Lim *et al.* (2001), using the pTa71 probe (Gerlach and Bedbrook, 1969).

For the chromosome doubling of *Buddleia globosa*, mitosis inhibitors oryzalin (ORY) and trifluralin (TRI) (filter sterilised and added after autoclaving) were used in different concentrations. In a first method, seeds of *B. globosa* were sown on medium 70 (see Table 2). When the 2 cotyledons had emerged, a drop of an ORY (0.3 mM or 3 mM) or TRI (0.3 mM or 3 mM) solution was applied between them. In a second method, seeds were sown on medium 70 which was enriched with ORY and TRI in different concentrations (10 µM, 100 µM and 1mM). And in a third method, *B. globosa* shoots were multiplicated on WPM medium (see Table 2), to which concentrations of ORY and TRI (10 µM and 100 µM) were added. Flow cytometry was performed to analyse the ploidy of the obtained plants.

Results and discussion

For *Ligustrum*, *Hibiscus* and *Buddleia*, no prezygotic barriers were observed. All the interspecific pollinations that were performed, resulted in germination of the pollen and growth of the pollen tube to the ovules through the style of the seed parent. After 'embryo rescue', a lot of (possible hybrid) seedlings were obtained. These F1 plants are now evaluated in the field to analyse their hybrid character or acclimatized in the greenhouse.

For *Hydrangea*, on the other hand, it was not possible to visualize the growth of the pollen tube through the style of the seed parent with the used protocol. A better protocol will be optimized. Also the 'embryo rescue' protocol for *Hydrangea* needs more optimalization. Only a few F1 seedlings were obtained. They are now evaluated in the field to study their hybrid character.

Within *Hibiscus* and *Buddleia*, hybrid F1 plants of 3 interspecific crosses already flowered. Those plants (*Hibiscus syriacus* x *Hibiscus paramutabilis* (6 plants), *Buddleia davidii* x *Buddleia weyeriana* (50 plants) and *Buddleia weyeriana* x *Buddleia davidii* (7 plants) are completely molecular characterized (AFLP and flowcytometry) and used to generate an F2 population (self pollination). The obtained F2 plants are now acclimatized in the greenhouse and will be evaluated in the field starting this summer. Normally, a segregation of interesting traits (leaf morphology, flower colour) will be observed. Also this summer, new self pollinations of the F1 plants will be performed in order to have a much larger F2 population in which the segregation is more explicit.

Next to AFLP, GISH (Genomic *in situ* hybridisation) is a powerful technique to analyse the hybrid character of the seedlings. This GISH technology first is optimized for *Hydrangea*. A karyotype was constructed for *Hydrangea macrophylla*. This karyotype consists of 6 metacentric chromosomes, 8 submetacentric chromosomes and 4 subtelocentric chromosomes. One of the subtelocentric chromosomes bears the Nucleolar organisation region (rDNA). In further research, a karyotype for the other *Hydrangea* parent plants will be developed and the obtained hybrids will be analysed with GISH.

The *in vitro* chromosome doubling of *Buddleia globosa* resulted in tetraploid plants (5%) acclimatized in the greenhouse. When flowering, they will be used in a cross with *B.davidii*.

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Table 1 The objectives of the interspecific crosses within the genera *Hibiscus*, *Buddleia*, *Ligustrum* and *Hydrangea*. The interspecific pollinations were performed in insect free plastic greenhouses

Genus	Objectives of the interspecific crosses
Hibiscus	Introgression of the growth vigour of *H.paramutabilis* and *H. sinosyriacus* into *H.syriacus*. Introgression of the intense flower colour of *H.rosa-sinensis* and *H.hamabo* into *H.syriacus*.
Hydrangea	Introgression of the leaf shapes of *H.quercifolia* and the intense flower colour of *H.macrophylla* into *H.paniculata*, enlargement of the *paniculata* sortiment, which is most winter hardy and resistant.
Buddleia	Introgression of the yellow flower colour of *B.weyeriana* and *B. globosa* into *B.davidii*.
Ligustrum	Hybridisation of the evergreen *L.japonicum* and *L.lucidum* with the winter hardy species, creation of winter hardy species with a lot of berries.

Table 2 Composition of the media, used for 'embryo rescue' of *Hibiscus* (medium 70), *Ligustrum* (medium 60), *Hydrangea* (medium 41) and *Buddleia* (medium 70) and composition of WPM medium, used for multiplication of *Buddleia globosa* shoots. Media were autoclaved (121°C, 500 hPa, 30 min), Meli-jars (De Proft *et al*, 1985) contained 100 ml medium per jar

		Medium 41	Medium 60	Medium 70	WPM medium
		Per liter	Per liter	Per liter	Per liter
water	ml	1000	1000	1000	1000
MS* + vit. (1/2)	mg		2202.6	2202.6	
B5** + vit.	mg	3164			
WPM****	mg				2462.6
sucrose	g	20	30	30	30
2-Ip*****					25 µM
PPM***	ml	1		1	
agar	g	7	7	7	7
charcoal	g				2.5
pH		5.8	5.8	5.8	5.8

* **MS** = Murashige and Skoog medium, micro- and macroelements including vitamins (Duchefa Biochemie). This MS medium is used at half strenght (full strenght: 4405.2 mg per liter medium) (Murashige T., Skoog F., 1962)

** **B5** = Gamborg B5 medium, micro- and macro-elements including vitamins (Duchefa Biochemie). (Gamborg O., Miller R., Ojima, K., 1968)

*** **PPM** = Preservative for Plant tissue culture Media. (Plant Cell Technology)

**** **WPM**= Woody Plant Medium. (Duchefa Biochemie)

***** **2-IP**= 6-τ-τ- (Dimethylallylamino)-purine (cytokinine)

Table 3 Primer combination used to perform AFLP on the obtained seedlings after interspecific hybridisation within *Hibiscus*, *Buddleia*, *Ligustrum* and *Hydrangea*

Genus	Primer combinations
Hibiscus	E- AAC + M- CAT / E- ACC + M- CAT / E- AAG + M- CTA
	E- AGC + M- CTA / E- ACA + M- CTG / E- AGC + M- CTG
Buddleia	E- ACA + M- CTG / E- ACG + M- CAA
Ligustrum	E- ACT + M- CAT / E- AGG + M- CTA / E- ACG + M- CAA
Hydrangea	E-ACT + M-CAT / E-AGG + M- CTT / E- ACG + M- CAA

TISSUE CULTURE AND MUTAGENESIS IN CROP IMPROVEMENT

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The essential component of any conventional breeding program is the exploitation of genetic variation and use it to recombine the desired genes from crop varieties and related species by plant breeders for the development of new varieties having desirable agronomic traits such as high yield, resistance to abiotic and biotic stresses. As the human population grows and environment degrades further, plant breeders need to adopt new technologies for sustainable agriculture production. Plant tissue culture (somatic embryogenesis, micropropagation, doubled haploids) has a great potential in plant improvement, provided plants can be readily regenerated in large numbers (Jain 2002). It provides the options to reduce costs in generating the useful traits and pre-breeding materials for plant breeders, as well as shortening the screening program, e.g. for salt and drought tolerance. Micropropagation technology is more expensive than the conventional methods of plant propagation, and requires several types of skills. It is a capital-intensive industry, and in some cases the unit cost per plant becomes unaffordable. The low cost alternatives are needed to reduce production cost of tissue-cultured plants. Induced mutations with physical and chemical mutagens have been quite effective for a significant increase in plant production among both seed and vegetatively propagated crops (Jain 2005) under the auspices of IAEA. This is clearly shown by IAEA mutant variety database (available at: http://www-mvd.iaea.org/). More than 2300 mutant varieties have officially been released in many countries including China, India, Japan, Russia, The Netherlands, and USA who have contributed greatly in the application nuclear technology.

At IAEA under the Food and Agriculture program, Coordinated Research Projects (CRPs) and Technical Cooperation Projects (TCPs) are being carried out in Member States, mainly involved the use of tissue culture, mutation, breeding and recently molecular markers, in improvement of both seed and vegetatively propagated crops. The overall achievements of CRPs and TCPs are:

Banana CRP: Research tools were developed for germplasm characterisation and improvement through induced mutations, somaclonal variation, somatic embryogenesis, cryopreservation, and genetic engineering (Jain and Swennen, 2004). For example, screening techniques were developed for disease (Fusarium wilt, black sigatoka) and nematode resistance. DNA flow cytometry protocol was developed for polyploidy detection, monitoring of cytochimera dissociation, and chromosomal stability.

***Tropical and subtropical fruit crops CRP*:** Among several fruit crops, seedless citrus mutant lines were identified in Iran. In Malaysia, papaya M2 field evaluation, some plants showed reduction in plant height, change in flesh colour, and trees showing resistance to malformed top disease (resistant ratings between 0 and 1 as compared to control 4). The Pakistani group observed plant height reduction in mutated guava lines.

***Under-utilized and neglected crops CRP*:** Only a limited number of crops produce the bulk of food consumed in the world, e.g. wheat, rice, corn, barley and others. Many under-utilized and neglected species are extremely important for food production and locally well adapted to marginal lands and constitute an important part of the local diet, providing nutritional elements. The major results of CRP are: 17 putative root rot di-

sease tolerant mutants of cocoyam were obtained; a new strategy for shortening generation cycle was developed in bamabara groundnut; 3 selected taro mutants had desired traits-high tolerance to leaf blight disease, early maturing, heavy corms and good taste; and 22 okra mutants showed resistance to yellow vein mosaic virus.

Technical Cooperation Projects (TCPs): In Thailand, 4 stable flower colour mutants of Canna (*Canna hybrida*) [Pink Peeranuch, Yellow Arunee, Cream Prapanpongse, Orange Siranut], 3 mutants of Portulaca (KU1, KU2, KU3), and 16 mutants of chrysanthemum ornamental plants induced by gamma treatment have been released.

The potential of nuclear applications together with tissue culture in crop improvement will be discussed.

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IDENTIFICATION AND CHARACTERISATION OF A K^+_{ATP} CHANNEL IN MITOCHONDRIA ISOLATED FROM THE EMBRYOGENIC CULTURES OF DIFFERENT CONIFEROUS SPECIES

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Introduction

Somatic embryogenesis (SE) is a multi-step regeneration process starting with the formation of embryogenic cell masses, followed by somatic embryo development, maturation and plant regeneration. Therefore, SE represents a unique experimental model for studying different biochemical and developmental processes. This is possible because manipulations of culture conditions allow to better correlate morphogenetic changes to physiological alternations [12]. Recently published reviews [14, 13] are helping with better understanding of a serious bulk of information related to physiological and molecular events occurring during *in vitro* embryogenesis (embryo development).

Mitochondria, together with chloroplasts, are the main organelles involved in the energetic state of the cells. However, their role in SE has been almost ignored. To our knowledge, there is only one publication [9] dealing with the isolation of mitochondria from embryogenic suspension cultures of *Larix* x *leptoeuropea*, aiming the purification of the mtDNA for genetical studies.

Energy coupling is fundamental for ATP production by mitochondria. Indeed, its synthesis is under control of proton permeability systems, located on the inner membrane, such as the plant uncoupling mitochondrial protein (PUMP) [8], the ADP/ATP carrier (AAC) [2] and the recently described ATP-sensitive K^+ channel, together with the K^+/H^+ antiporter [3].

The aim of the present work was to detect the presence of a K^+ channel in mitochondria isolated from embryogenic cell masses of three different coniferous species (*Picea abies*, *Abies cephalonica* and *Pinus nigra)* and to characterise it.

Material and Methods

Embryogenic cultures of *P. abies* (genotype AFO 541, obtained from Dr. Bercetche, AFOCEL France) were grown on medium according to Gupta and Durzan [7]. Proliferation medium was supplemented with 5 μM 2,4-dichlorphenoxyacetic acid, 2 μM kinetin, 2 μM 6-benzylaminopurine and 58 mM sucrose and 0.75% agar.

Two embryogenic cell lines (6 and 8) of *A. cephalonica* were cultivated on MS [10] proliferation medium with ½ macro- and micronutrients, supplemented with 0.1% (w/v) casein hydrolysate, 3.4 mM L-glutamine, 58 mM sucrose, 4.4 μM BA and solidified with 0.3% Phytagel [1].

Embryogenic cultures of *P. nigra* (cell line E146) were maintained on medium DCR [6] containing 9 μM 2,4-D, 2.2 μM BA, as well as 0.5% (w/v) casein hydrolysate and 342.2 μM L-glutamine.

All the embryogenic cultures were maintained at 24°C in the dark. Regular subculturing of the proliferating cultures was performed in the case of *P. abies* every week and 3-5 day-old cultures were used for isolation of mitochondria. Cultures of *A. cephalonica* and *P. nigra* were subcultured in two-week intervals and 7-8 day-old cultures were used for isolation of mitochondria. For each isolation of mitochondria 40-60 g of fresh embryogenic tissue were used.

Mitochondria were isolated from different plant materials as described in (4) and resuspended in 0.3 M sucrose, 20 mM HEPES-Tris (pH 7.2) and 0.1% (w/v) BSA.

Electrical potential ($\Delta\Psi$) measurements were evaluated as variation of fluorescence ($\Delta F/F$), using safranin O as probe, in the above described medium (0.1mg mitochondrial protein/ml). Swelling experiments were performed as absorbance changes at 540 nm of the mitochondrial suspension (0.2 mg mitochondrial protein/ml) in 0.2 M KCl, 20 mM HEPES-Tris (pH 7.2) and 0.1% (w/v) BSA.

Results

Crude mitochondria, isolated from the three different coniferous species, represented four different embryogenic cell lines. They showed typical features of intact plant mitochondria, such as integrity (ca. 90%), basal oxygen uptake (ca. 100 nmol.min^{-1} per mg of protein), respiratory control ratio (ca. 2.5) and partial KCN-resistant respiration. However, some peculiar characteristics were observed: i) high stimulation by exogenous inorganic phosphate on $\Delta\Psi$; ii) halved oxygen uptake, during the state 4 respiration, with respect to the basal one; iii) extremely low activity of external NAD(P)H dehydrogenase found in *P. abies*.

In order to investigate the presence of a K^+_{ATP} channel, potassium-inward activity was followed both as swelling of mitochondria resuspended in a KCl-based medium and KCl-induced $\Delta\Psi$ depolarisation after malate plus glutammate energization. Both activities were clearly shown in all tested lines, although with different magnitudes. In *A. cephalonica* and *P. nigra* isolated mitochondria, the KCl-induced $\Delta\Psi$collapse was accompanied by a small stimulation of O_2 consumption. On the contrary, in *P. abies* mitochondria, such a $\Delta\Psi$ depolarization was associated to an inhibition of O_2 uptake.

A spontaneous potassium-outward activity, evidenced as $\Delta\Psi$ formation, was found in all tested cell lines, while the previously described stimulation by CsA [11] was very slight. This phenomenon was prevented by the presence of 40 mM KCl in the assaying medium and completely dissipated by uncouplers (FCCP), suggesting that the K^+ gradient was converted to ΔpH by the K^+/H^+ antiporter [5].

Mitochondria isolated from embryogenic cultures of *P. abies* showed the highest K^+ fluxes and, therefore, the deeper characterization of K^+_{ATP} channel was performed with above mentioned cell lines.

Similarly to observations obtained with mitochondria from mammals and angiosperms, the K^+ channel here described was able to transport other monovalent cations (K^+>Na^+>Li^+>Rb^+) and was inhibited by ATP and regulated by reducing agents (DTE).

It has been suggested that the plant K^+ channel is voltage-dependent, being closed by the formation of $\Delta\Psi$. However, if energy dissipating systems are operating, the possibility to obtain this effect on isolated mitochondria is limited. *P.abies* mitochondria possess a high rate of KCN-resistant respiration (ca. 50%) and internal rotenone-insensitive NADH dehydrogenase. Both activities are responsible for non-coupled respiration and can explain the high rate of K^+-inward flux and the lack of inhibition by $\Delta\Psi$.

In the above described scenario, the mitochondrial non-coupled respiration pathways seems to be expressed in synergy with the inward-activated potassium fluxes, this behaviour could be related to metabolic conditions referring to environmental stresses and/or developmental changes.

Future perspective will be focused on understanding if these mitochondrial features are related to the artificial growth condition of *in vitro* cultures, or if they depend on the developmental stages of cells, such as described manifestation of programmed cell death in embryogenic cultures of *P. abies* [4].

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INFLUENCE OF ZINC SULPHATE ON BARLEY DOUBLED HAPLOID PRODUCTION

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Introduction

The use of doubled haploid (DH) plants had been proved to be very useful in plant breeding programs, since homozygous plants are obtained in a few months leading to a significant reduction in the time to release new cultivars. Efficient protocols for DH production have been developed in barley via anther and/or isolated microspore cultures. However, the low rate of embryogenesis, and plant regeneration, as well as the high percentage of albinism of cultivars of high agronomic value hinder the application of this methodology. It should be noted the importance of developing protocols of gametic embryogenesis applicable to a wide range of cultivars.

Modifications of stress pre-treatment and composition of culture medium (carbohydrate and nitrogen source, growth regulators, etc.) led to a significant improvement of the number of barley DH plants (for review see Malupszynski *et al*., 2003). Less attention has been given to the micronutrient composition of the media. Recently, positive effects of the incorporation of a high concentration of copper sulphate into the pre-treatment, and induction medium of anther cultures (Wojnarowiez *et al*., 2002) have been reported.

Zinc is another micronutrient which is an essential component of many proteins involved in regulation of gene expression in different mechanism of plant growth and development, such as oxidative stress prevention, carbohydrate fixation, chlorophyll synthesis, phosphate transport, development of tapetum and pollen cells, etc. (Kobayashi *et al*., 1998; Cakmak, 2000; Yanagisawa, 2004).

We have studied the influence of the concentration of Zn^{2+} in the pre-treatment and culture media on barley doubled haploid production. A three to six-fold increase in the concentration of Zn^{2+} in the culture medium favoured the production of green plants of genotypes with different gametic embryogenesis response.

Material and Methods

The winter two-rowed Igri and Reinette, and the winter six-rowed Hop cultivars were used for this study. Igri is a model variety for gametic embryogenesis, whereas Reinette and Hop are medium and low responding ones, respectively A well-established standard protocol described by Cistué *et al.*, (2003) was followed for evaluation of the anther culture response. In order to evaluate ***the effect of different concentration of Zn^{2+} on the pre-treatment medium,*** dissected anthers from the same spike, were randomly distributed in 0.7 M mannitol solidified with 8 $g.l^{-1}$ Agarose Sea Plaque (pre-treatment medium), containing 0 (Control), 30, 90, 180, 300 and 600 µM of $ZnSO_4.7H_2O$. Anthers were pre-treated for four days at 25 °C in the dark. Eighteen anthers pretreated with the same concentration of Zn^{2+} were transferred to 1.5 ml liquid FHG medium (30 µM $ZnSO_4.7H_2O$), supplemented with 200 $g.l^{-1}$ of Ficoll Type-400 (Sigma). To study ***the effect of concentration of Zn^{2+}along pre-treatment and culture media***, the eighteen anthers pretreated with 90, 180, 300 and 600 µM of $ZnSO_4.7H_2O$ were cultured in induction medium containing the same amount of Zn^{2+} than in pre-treatment medium.

Furthermore, control cultures were pretreated and cultured with 0 and 30 µM of $ZnSO_4.7H_2O$, respectively. Experiments consisted of 12 to 20 replicates of 18 anthers per each concentration tested and genotype. Variables as number embryos (EMB), green plants (GP), as well as percentage of regeneration (pREG), and percentage of green plants (pGP) were recorded.

Results and Discusion

Although the incorporation of 30 to 600 µM Zn^{+2} in the pre-treatment medium did not significantly increased gametic embryogenesis capacity as compared with the control (0 µM Zn^{+2}), it slightly improved the number of embryos and green plants of cultivars Igri and Hop. A significant genotype effect was observed when the concentration of Zn^{+2} was raised along pre-treatment and induction culture media from 30 µM (control) up to 600 µM. Around a 1.3 to 2-fold increase in the number of embryos and green plants for Igri and Reinette cultivars, respectively, were obtained with a concentration of 180 µM Zn^{+2}. No statistically significant differences for number of green plant were obtained for the cultivar Hop (probably due to its low gametic embryogenesis response), although a 3-fold increase in this variable was produced with 90 µM Zn^{+2}. All these results show that the optimal concentration of Zn^{+2} in the microspores pre-treatment and induction media were higher than used previously, and probably there is an interaction between genotype and zinc concentration.

In this study we have increased the gametic embryogenesis capacity of different cultivars of barley by raising the concentration of zinc sulphate in the culture media. Similar results were reported in microspores of barley cutivar Igri with increased concentration of copper sulphate (Wojnarowiez *et al.*, 2002). The role of zinc in gametic embryogenesis is unknown, however it has been described the importance of this micronutrient in animal embryogenesis (Falchuk and Montorzi, 2001).

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GAMETIC AND SOMATIC EMBRYOGENESIS THROUGH ANTHER CULTURE IN *Citrus*

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Anther culture is the most commonly used method to produce haploids, doubled-haploids and homozygous pants in *Citrus* (Germanà, 1997; Germanà, 2003b). Haploid plants, with a gametophytic set of chromosomes in the sporophyte, have potential use in mutation research, selection, genetic analysis and genetic transformation. The possibility of obtaining triploid somatic hybrids (important for the seedlessness of their fruits) by fusion between haploid and diploid protoplasts is another important application of haploidy in *Citrus* breeding. Doubled haploids are also important in genome mapping and in exploring "gametoclonal variation".
Haploids can be induced in woody plants mainly through two strategies: 1) gynogenesis, where they arise from the female gamete, and 2) androgenesis (anther culture, isolated microspore culture) where they are regenerated from the male gametes.
Androgenesis has been successfully induced in genus *Citrus*, but only in few genotypes. In *Citrus* and their relatives, haploid calli and plantlets have been recovered by anther culture from *Poncirus trifoliata* (L.) Raf. (Hidaka *et al.*, 1979) and *Citrus madurensis* Lour. (Chen *et al.*, 1980); haploid, diploid and triploid plantlets and highly embryogenic homozygous calli have been obtained from three cultivars (Nules, SRA 63 and Monreal) of *Citrus clementina* Hort. ex Tan. (Germanà *et al.*, 1994; 2000; Germanà and Chiancone, 2003); haploid, but albino embryoids of Mapo tangelo (*Citrus deliciosa* x *Citrus paradisi*) (Germanà and Reforgiato, 1997), haploid and diploid calli, embryoids and leafy structures but no green plants of *Citrus limon* (L.) Burm. f. (Germanà *et al.*, 1991), haploid embryoids of *Clausena excavata* (Froelicher and Ollitrault, 2000) and triploid and homozygous embryoids of *Citrus reticulata* (unpublished) have been also achieved.
Anther culture has been also employed to obtain somatic embryos and the regeneration of many woody plants. Particularly, anther culture is usually employed to establish somatic embryogenic cultures of *Vitis* (Cersosimo *et al*., 1990). Actually, in *Citrus*, by anther culture, not only homozygous haploid, diploid and triploid calli and plantlets have been recovered, but also heterozygous regenerants have been obtained (Germanà, 1997). In fact, the production of embryogenic callus can be also obtained through *in vitro* culture of anthers of *C. aurantium* (Hidaka *et al*., 1981; Germanà, 2003a), *C. sinensis* (Hidaka, 1984b), *C. aurantifolia* (Chaturvedi and Sharma, 1985), *C. madurensis* (Ling *et al.*, 1988), *C. reticulata* (Germanà *et al*., 1994; Germanà, 2003a), *Poncirus trifoliata*, the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (Deng *et al*., 1992) and *C. paradisi* (unpublished).
In this last case, anther culture could be regarded as a method of obtaining somatic embryogenesis (Germanà, 2003a). Crop genetic improvement through biotechnology requires efficient procedures of regeneration *in vitro*, together with the modern techniques of cellular and molecular biology. Particularly, in *Citrus*, highly embryogenic somatic calli are valuable for propagation or genetic improvement. In fact, they can be used for somatic hybridization by protoplast fusion, for genetic transformation, for

synthetic seed production, for clonal propagation, for *in vitro* germplasm preservation, but also for basic research in plant physiology and biochemistry.
This report summarizes the status of research on anther culture technique to obtain gametic and somatic embryogenesis in *Citrus.*

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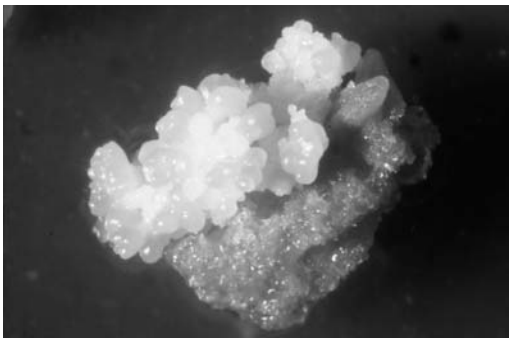

Figure 1
Gametic embryogenesis from anther culture of *Citrus clementina*

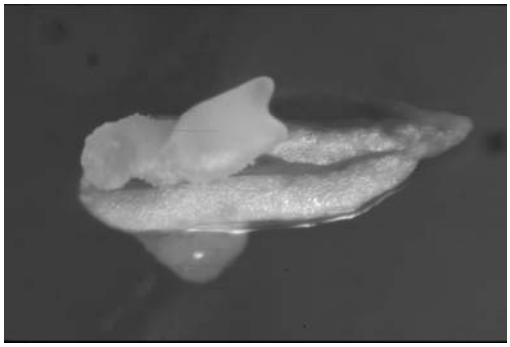

Figure 2
Somatic embryogenesis from anther culture of *Citrus limon*

HIGH-FREQUENCY EMBRYOGENESIS FROM ISOLATED MICROSPORES *IN Brassica rapa oleifera*

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Abstract
Thirty different varieties of turnip oil rapeseed were tested for response to microspore culture. The varieties were from Bangladesh, Canada, Finland, India and Sweden. From a total of thirty genotypes tested, twenty-five produced embryos. Only 'CR25'and 'CV-2' produced more than 1000 embryos per 100 isolated buds. Eight varieties produced from 100 to 1000 embryos and 14 varieties produced less than 100 embryos per 100 buds.
In case of low-responsive varieties yield was considerably improved by changing by changing the culture media after 3 days. In contrast, this practice did not significantly improve the embryo yield of high-responsive varieties.
From the very heterogeneous variety 'CR25'we obtained homogenous lines from microspore derived embryos and we tested 16 of them for response to microspore culture. There was a very large variation in response between the different lines. In four experiments the line 'HL14' produced an average of 8900 embryos per 100 isolated buds, which is probably the highest embryo yield reported from microspore culture in turnip oil seed rape.
The germination of lines from microspore-derived embryos was also studied in four varieties. Compared with microspore-derived embryos in other *Brassica*, a very large part of the developed plants were diploid. This indicates that there is a very high rate of spontaneous chromosome doubling very early in the microspore culture.

CELL SUSPENSION CULTURE OF WHEAT

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Introduction

Cell suspension culture is more suitable for physiological, biochemical and molecular biological investigations of growth and differentiation processes than callus culture grown on solid medium (Ozawa *et al*., 1996). It can be used as a source of protoplasts suitable for genetic manipulations. It was shown that protoplasts isolated from cell suspensions of *Gramineae* can be induced to undergo divisions and form colonies and callus tissues. However, the suspension cultures are very often non-morphogenic in nature (Vasil *et al.*, 1991). The goal of the present study was to investigate selected exo- and endogenic factors influencing the growth and differentiation of hexaploid winter wheat cell suspension culture and to improve known from the literature methods of its establishment for a long time (Fellers *et al*., 1995; Pauk *et al*., 1994; Qiao *et al*., 1992; Redvay *et al*., 1990). Studies were carried out on wheat cultivars temporary cultivated in Poland (Marcińska *et al*., 2001a).

Material and Methods

Liquid cultures were initiated from the callus derived from immature embryos and inflorescences of winter wheat (*Triticum aestivum* L.). The flasks with cultures were held in climatic cameras on shakers with large working area. Various culture conditions were optimised: size and shape of culture vessels, suspension density, supplements of various nutrients (NaCl, $AgNO_3$ or $CuSO_4$), increased concentration of sucrose, biologically active substances (zeatin, indolile acetic acid) and the effect of the temporary exposure of the culture to low temperatures. Studies of viability and morphology of cells were done with light and fluorescent microscopes. Images were analysed with LUCIA computer analysis programme (area and circularity of cells). An increase in fresh and dry weight of the suspension and the number of cell aggregates during short and long term cultures were determined. Moreover, changes in pH, conductivity and osmolality of the medium were studied. During a long term culture, chosen biochemical parameters such as protein, lipid, phenol and carbohydrate contents were determined with the column, gas, laminar and high pressure liquid chromatography. The linear correlation between the level of these parameters and the increase in cell mass, number of aggregates and green centres on its surface was established.

Results and Discussion

The growth kinetic of the cell suspension culture was dependent on the initial mass of callus, while it was not correlated with the vessel size. It is probably connected with aggregation of cells, but not free space in the flask. Zeatin stimulated differentiation in suspension with the higher density of cells while indolile acetic acid (IAA) had an opposite effect. This could be related partially to nitrogen transport and metabolism. NaCl stimulated while $AgNO_3$ or $CuSO_4$ slowed down an increase in cell density. The two latter salts effected the higher number of cell aggregates while had no influence on the morphology of cells. It is suggested that different acting pathways of these salts on the cell metabolism exist. A temporary treatment with low temperature stimulated morphogenesis. Cooling probably caused the re-establishment of the morphogenetic potential of cells, weakened during the long time culture. When the culture time was prolonged, the

total amount of proteins in cells increased while it decreased in the culture medium. This indicates that proteins were mostly held inside the cells and only a small amount of them was secreted to the medium. This effect can be connected with biochemical aspects of morphogenesis. A higher phenols concentration in the medium than in cells had a positive effect on cell mass production and negative on the number of aggregates in the suspension, while their concentration in cells had no clear effect on suspension differentiation. Higher accumulation of carbohydrates in cells stimulated the differentiation of aggregates. It is suggested that carbohydrates participate in the activation of the secondary metabolic pathways in cell suspension culture. The increase in phospholipids (PL) content in cells accompanied the increase in total mass of the suspension, while the share of galactolipids (GL) decreased at the same time. Besides of the cytokinins from the isopropenoid group, the presence of aromatic cytokinins from the benzylaminopurine group was detected (Marcińska *et al*., 2001b). So far, they were found in a limited number of species, and never in wheat. Microscopic studies of the suspensions confirmed the occurrence of morphogenic centres with a high number of small cells. Protocols known from literature were modified according to the obtained data. During the initiation of the basic liquid culture, suspensions should be supplemented with fresh medium every two weeks until they reach the optimal mass and volume. Further stabilisation of culture is achieved when part of the medium is replaced with the fresh one in one week long intervals. Culture was fully stabilised when the suspension divided by half and supplemented with the fresh medium to the initial volume intensely grew. In most cases, a significant linear correlation was obtained between suspension growth parameters and physiological parameters of the medium: pH, electric conductivity and osmolality. On the basis of the presented results we can regard wheat suspension as an important plant material which can be studied for various biological and biochemical purposes. To carry out the suspension culture, original equipment was constructed in collaboration with Sebastian Pieńkowski and Marek Trybała. These were a shaker with a large working area and a special apparatus suitable for the collection and washing of suspension samples.

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INTERSPECIFIC SOMATIC HYBRIDS DERIVED FROM POTATO FUSIONS: ANALYSIS OF THEIR GLYCOALKALOID CONTENT

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Introduction

Glycoalkaloids are naturally occurring secondary metabolites in plants. Steroidal glycoalkaloids are mainly found in the *Solanaceae* family, and therefore often called '*Solanum* glycoalkaloids'. Glycoalkaloids are glycosides which consist of two structural components: a hydrophobic C_{27}-carbon skeleton of cholestane (aglycone) and a hydrophilic carbohydrate side chain. Six-ring steroid aglycones are structurally divided into five groups (solanidanes, spirosolanes, epiminocholestanes, alkaloids with solanocapsine skeleton, and 3-aminospirostanes) (Ripperger and Schreiber, 1981). The major solanidane and spirosolane aglycones found in potato species are solanidine, demissidine, solasodine, tomatidenol and tomatidine (rev. by Laurila, 2004).

The increased glycoalkaloid concentration in potato tubers is one of the undesirable characteristics that diminishes their quality, because glycoalkaloids are potentially toxic to mammals and vertebrates (Friedman and McDonald, 1997). In cultivated potato (*Solanum tuberosum* L.) solanine and chaconine are the most common glycoalkaloids. The safety limit of α-solanine and α-chaconine for human consumption is 20 mg/100 g FW of tubers (Friedman and McDonald, 1997). In addition, at least 90 structurally different steroidal alkaloids have also been found in more than 350 *Solanum* species (rev. by Laurila, 2004). It must emphasised that the role of the glycoalkaloids naturally expressed in the *Solanum* species is to protect the host-plant from the attack by fungi, bacteria and insects (Väänänen *et al*., 2005).

Protoplast fusion is an alternative method to sexual crossing to transfer desired characters, such as resistance traits, from wild species to cultivated potato (Rokka *et al*., 1998). Most of the wild *Solanum* species are sexually incompatible with *S. tuberosum*, therefore somatic cell fusion has a high potential for utilization of the wild genetic resources in potato improvement. However, as a result of the fusion, many traits important for agronomy and tuber quality exhibit a wide variation in the somatic hybrids and their progenies. An important, but potentially undesirable trait which needs proper research is the glycoalkaloid content of the hybrids.

Materials and Methods

The plant material in the present study consisted of various interspecific somatic hybrids between tetraploid (2n=4x=48) and dihaploid (2n=2x=24) cultivated potato (*Solanum tuberosum*) (*tbr*) and two wild *Solanum* species; 2x *S. brevidens* (*brd*) CPC 2451

and 4x *S. acaule* (*acl*) PI 4726558 (7-8) including its anther-derived 2x lines. The parental species were also included into the study.
The glycoalkaloid contents were analysed using gas chromatography-mass spectrometry (GC-MS) (aglycones) (Laurila *et al*., 1999) and LC-ESI-MS (Väänänen *et al*., 2005). Glycoalkaloid aglycones were analysed from foliage samples, but entire glycoalkaloids with sugar moieties were determined also from tubers (Väänänen *et al*., 2005).

Results and Discussion

Our study comprised two genetically diverse wild *Solanum* species: the non-tuberous *S. brevidens* (E genome species) and the tuberous *S. acaule* (A genome sp.), which cannot be hybridized sexually with tetraploid *S. tuberosum* cultivars. The glycoalkaloid profiles of the hybrids differed considerably from those of cultivated potato.
Solanum tuberosum contained solanidine aglycone (α-solanine and α-chaconine) and the wild species *S. brevidens* expressed tomatidine aglycone (tomatine), and wild potato *S. acaule* had tomatidine (tomatine), demissidine (demissine) and solanidine aglycones (α-solanine and α-chaconine) (Laurila *et al*., 2001; Rokka *et al*., 2005; Väänänen *et al*., 2005). The genome constitution of the hybrid had a strong influence on the proportions of different glycoalkaloids (Laurila *et al*., 2001). Regardless of their low total tuber GA concentrations (approx. 9 mg/100 g FW), the somatic hybrids contained glycoalkaloids not detected in the parental species. A high proportion of spirotype glycoalkaloids consisting of 5,6-dihydrogenated aglycones, e.g. α-tomatine, and tomatidine bound with solatriose, and chacotriose, were found in the hybrids. In conclusion, the foliage of interspecific hybrids contained a higher variation in the structures of glycoalkaloids than did the tubers.

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EFFECT OF INDUCTION MEDIUM ON ANTHER CULTURE RESPONSE OF DURUM X BREAD WHEAT F_1 HYBRIDS

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Introduction

Durum wheat is considered as one of the most recalcitrant to anther culture crops (Ghaemi *et al*., 1993). Thus, the alternative approach of maize technique is employed. However, anther culture, due to its simplicity, could be more feasible if an effective protocol for durum wheat anther culture could be developed. Based on the assumption that the genes responsible for a good anther culture response of bread wheat are located on B genome chromosomes (Agache *et al.,* 1989), it is proposed that transfer of the genes responsible for anther culture response from bread to durum wheat might lead to a better response of durum wheat to anther culture. This could be done via crossing, selfing and backcrossing of the two species. Following such a procedure one could isolate durum wheat plants carrying the B genome chromosomes of the bread wheat (Tersi *et al*., 2002). This study was undertaken to investigate the effect of induction medium on anther culture response in durum X bread wheat F_1 hybrids.

Materials and Methods

Three durum wheat cultivars, produced at the Cereal Institute of Thessaloniki, Greece (Mexicalli E, Sifnos and Lemnos), exhibiting good yield and pasta quality (NAGREF-Cereal Institute, 1991), were crossed to two bread wheat cultivars (Acheloos and KVZ) exhibiting good response to anther culture(Zamani *et al.,* 2000).

Spikes of the aforementioned genotypes containing anthers in the mid (MU) to late uninucleate (LU) microspore developmental stage were used. The anthers were dissected under sterile conditions and placed on induction medium. Two different media were used: W_{14} (Ouyang *et al*., 1989) and solid potato-2 (Chuang *et al.,* 1978). Microspore derived structures were transferred to 190-2 regeneration medium (He and Ouyang, 1984). The plantlets obtained were transferred into Erlemayer flasks containing MS medium (Murashige and Skoog, 1962) without hormones and finally the well-differentiated green plants were transplanted into pots and kept in a growth chamber at 21°C /19°C day / night temperature regime and 16 hours illumination.

Results and discussion

A notable genotype effect was recorded in all examined traits (Table 1). Although the cross Lemnos X KVZ had a higher number of anthers responded and and it produced a high number of embyoids in both media, it did not produce any green plant on W14 and it gave the highest percentage of albino plants in potato-2. In contrast the cross (Mexicalli X Acheloos) produced the largest number of green plants and the lowest number of albino plants on W14. This genotypic effect on anther culture response is something common and there are many reports on that (Zamani *et al.,* 2000).

A notable differentiation between the examined induction media was recorded in all examined traits. Potato-2 was superior in all cases but it gave the highest percentage of albino plants. Despite this drawback, it could be concluded from the results of the pre-

sent study that potato-2 seems to be a more suitable medium for durum wheat anther culture. This is in agreement with previous reports (Ghaemi *et al.,* 1978).

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Table 1 Effect of induction medium on anther response, embryoid development and green plant and albino plant production, in four durum X read wheat F_1 hybrids

Cross	Number of anthers cultured		Anthers responded (%)		Number of embryoids / 100 anthers		Number of green plants /100 anthers		Number of albino plants/100 anthers	
	W14	Pt-2	W14	Pt-2	W14	Pt-2	W14	Pt-2	W14	Pt-2
Mexicalli X Acheloos	1701	1263	3.8 b	6.8 b	10.1 b	15.1 c	2.2 a	2.5 b	0.2 c	1.1 b
Sifnos X Acheloos	685	1403	4.5 b	7.5 b	15.9 a	18.0 b	0.3 b	5.3 a	13.6 a	1.3 b
Lemnos X KVZ	1700	1662	6.6 a	10.7 a	13.6 a	26.6 a	0.0	6.8 a	1.1 b	2.4 a
Lemnos X Acheloos	1881	2074	1.1 c	1.5 c	3.7 c	2.5 d	0.3 b	0.0	0.0	0.2 c
Total	5967	6402	3.8b	6.2a	9.8b	14.6a	0.8b	3.4a	1.9b	1.2a

THE EFFECT OF HEADSPACE RENEWAL IN A TEMPORARY IMMERSION BIOREACTOR ON PLANTAIN SHOOT PROLIFERATION AND QUALITY

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The positive effect of ventilation of the culture container on *in vitro* shoot proliferation and shoot quality has already been shown for different species. Our experiments clearly show that the headspace in a Temporary Immersion Bioreactor is renewed with every immersion and that this is one of the factors influencing the multiplication rate and shoot quality of plantain. The CO_2 and C_2H_4 concentration reached a maximum of 12% and 0.45 $\mu l.l^{-1}$ respectively in the control treatment on semi-solid medium compared to 5.7% CO_2 and 0.06 $\mu l.l^{-1}$ C_2H_4 in the Temporary Immersion Bioreactor. The O_2 concentration reached a minimum of 15.1% on semi-solid medium compared to only 19.3% in the Temporary Immersion Bioreactor. A higher number of shoots was produced in the latter system compared to the semi-solid medium (3.8 compared to 2.7), these shoots were taller (4.3 cm compared to 3.3 cm) and had more leaves (2.6 compared to 1.6). Moreover shoots produced on semi-solid medium showed distorted leaves. A typical day-night pattern in CO_2 and O_2 concentration was seen both in the Temporary Immersion Bioreactor and on semi-solid medium, indicating the photosynthetic activity in both systems.

EFFECT OF SOME FACTORS ON DEVELOPMENT AND MATURATION OF COMMON OAK (*Q. robur* L.) SOMATIC EMBRYOS

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Introduction

In the field of forest biotechnology the somatic embryogenesis has been reckoned a method of choice for genetic manipulations and mass propagation of superior genotypes (Wilhelm, 2000). Despite of the progress being made during the past years in studying different aspects of the somatic embryogenesis in *Quercus* spp, there is still a need for more profound knowledge about various factors affecting the integral efficiency of the process.

Results on effects of some factors on development and maturation of common oak (*Q. robur* L.) somatic embryos are presented in the study.

Materials and methods

Two common oak (*Q. robur* L.) embryogenic lines (1/2/98 and 12/2/98), maintained more than two years in culture, were used for realizing the experiments. The embryogenic status of the lines was maintained by regular monthly transfers on fresh hormone-free MS (Murashige and Skoog, 1962) medium supplemented with 250 $mg.l^{-1}$ L -glutamine and solidified with 2.5 $g.l^{-1}$ Phyagel™.

The effect of two ABA concentrations (0.1 and 1.0 $mg.l^{-1}$) and four exposure treatments (1, 2, 3 and 4 weeks) on development and maturation of somatic embryos in both the lines was studied in a factorial experiment. After the ABA exposure treatments the embryogenic clusters were transferred on the hormone-free variant of MS medium mentioned above. In a separate trial, the effect of two ABA concentrations (0.1 and 1.0 $mg.l^{-1}$) in combination with two types of carbohydrates (sucrose and maltose) applied in two concentrations (30 and 60 $g.l^{-1}$) on formation of cotyledonary-stage somatic embryos in only line 12/2/98 was evaluated within a similar scheme.

Plastic Petri dishes (60x10mm, Falcon™) were used for cultivation of explants. Three pieces of embryogenic tissue (150mg/dish) was placed into each vessel. Six dishes were allocated to each treatment.

The nutrient media were autoclaved at 121 °C for 20 min. Thermolable components were added to the media after filter sterilisation (Millipore®). The pH was preliminary adjusted to a value of 5.7. The cultures were cultivated in darkness at 23±1 °C.

The number of developed cotyledonary-stage somatic embryos was counted for estimation of putative treatment effects after 5-week period of time. The final data were subjected to analysis of variance (ANOVA) for testing the statistical significance of the treatment effects (the values were previously recalculated per 100 mg embryogenic tissue).

Results and discussion

The data analysis from the first experiment showed significant effect of both the genotype ($P<0.01$) and exposure time of the ABA treatment ($P<0.001$) on the process of embryo development and maturation. The embryogenic lines differed significantly ($P<0.001$) in their capacity of producing cotyledonary-stage somatic embryos. A significant interaction between the concentration and exposure time of the ABA treatment was recorded as well. There was no impact of the ABA concentration on the average

number of cotyledonary-stage embryos formed. The process of embryo development and maturation was significantly promoted by the increment of the exposure time of the ABA treatment, with relationship following a strong linear trend.

The results from the second experiment indicated a significant effect of both the carbohydrate type ($P<0.01$) and ABA concentration ($P<0.001$) on the number of the cotyledonary-stage somatic embryos formed. Significant interactions between the carbohydrate type from one hand and ABA and carbohydrate concentrations on the other hand, were also registered. The analytical data showed significantly greater efficiency of sucrose than maltose in respect to their abilities of promoting the process of somatic embryos development. The carbohydrate concentration did not affect the amount of cotyledonary-stage somatic embryos but the lower ABA dose (0.1 $mg.l^{-1}$) favoured in greater extent their formation.

Discussion

Sampling only two common oak embryogenic lines, no apparent interaction between the genotype and ABA concentration on common oak somatic embryo development was found. Nevertheless, such effect might be expected when working on broader genetic basis (bigger number of clonal lines), as reported by Merkle *et al*. (1989) in *Liriodendron tulipifera* L.

The maltose has been found to favour the maturation of somatic embryos in some coniferous species (Liao, Amerson, 1995; Nørgaard, 1997). On the contrary, the maltose appeared to be an inappropriate carbon source for supporting the process of development and maturation of common oak somatic embryos. The results presented are consistent with those announced by Carraway and Merkle (1997), who found a significantly bigger promotive potential of sucrose as compared with maltose and insignificant influence of the carbon source concentration on development and maturation of American chestnut (*C. dentata* (Marsh) Borkh.) somatic embryos.

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INDUCED ANDROGENESIS IN *Lycopersicon, Medicago, Capsicum* AND *Triticum*

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Methods for producing plants from haploid gametes have been, are and will be great interest to plant geneticist and breeders. They are of great importance especially in studies on the induction of mutations and also for the production of homozygous plants for very short time. Despite the considerable results obtained with anther cultures of tobacco, rice and potatoes research in tomato, alfalfa and pepper is yet insufficient.

The aim of this study was to identify factors influencing the androgenesis in anther cultures of tomato, alfalfa, pepper and wheat as well as to characterize the regenerated plants.

Material and Methods

Licopersicon: One hundred varietes and lines of *L. esculentum* L. and fifteen genotypes of *L. peruvitanum*, *L. chesmanii* var. *minor*, *Solanum penelii* were used as initial material. Two stages of pollen development – late meiosis and uninucleat microspore were tested. More than fifty variants of nutrient media after Nitsch (1969), Murashige and Skoog (1962), Gresshof and Doy (1972) in combination with different concentrations of phytohormones were tired out. Twelve different variants of pretreatment with temperature and gamma rays were tested. MS nutrient medium + IBA were used for rooting.

Medicago: Anthers of 10 alfalfa lines containing pollen in meiosis at uninuclear stage of development have been used as initial material. More than 30 variants of nutrient media after Murashige and Scoog (1962), Gamborg (1968), Blaydes (1966) with various concentrations kinetin, BAP, 2-ip, zeatin, IAA, NAA, 2,4-D were tested. Twenty-five different treatments with low temperatures and gamma rays have been tried in order to find out the best effect on callus induction and organogenesis.

Capsicum: Three basic nutrient media (Murashige and Scoog, 1962; Nitsch, 1969; Sibi *et al*., 1979) were used for *in vitro* androgenesis in pepper. Different modifications (14 variants), solid and liquid (Dumas de Vaulx, 1981), with phytohormons and carrot extract were tested. Modifications with or without activated charcoal were also used. Pretreatment wit temperature (35°C) and gamma rays (400 and 800 r) were tried out (Pundeva *et al*., 1990). Anthers in uninucleate stage of 5 pepper varieties and 2 interspecies hybrids were used as donor material.

Triticum: Anthers from 80 varieties and lines of *Triticum aestivum* L. and hybrids between *Triticum aestivum* x *Agropyron intermedium* were used as initial material. Haploid calli were induced from anthers at uninuclear stage of microspore development on Potato II modified medium. All regeneration procedures were after Mentewab *et al*. (1999). Embryos and calli are observed from anthers at least 8 days after inoculation. They are transferred until sterile conditions on the regeneration medium R9 (de Buyser and Henry, 1986).

Results and discussion

Our results suggest that the genotype, growing conditions of the donor plants, stage of microspore development, phytohormonal composition of the nutrient media and pretreatment of anthers with physical agents (temperature and gamma rays), alone or in combination, affected the frequency of organogenesis and regeneration in anther cultures of the studied species. The results showed that the best medium for androgenesis in tomato is MS + zeatin or 2-IP; in alfalfa - Blaydes + 2-IP; in pepper - MS + carrot extract; in wheat - MS + L-glutamine, potato extract, 2,4-D and kinetin. To stimulate androgenesis 25 different pretreatments (temperature and gamma rays) were tested. Hig-

hest rate of regeneration in tomato and alfalfa occurred at 4 ^{0}C/48 h. The most efficient radiation pretreatment for tomato was 4Gy alone or in combination with low temperature and for alfalfa – 1Gy combined with 4 ^{0}C. More then 10 000 plantlets have been obtained from tomato anther cultures, 5000 from alfalfa, 4 000 from wheat. They showed a great variability in morphological traits and chromosome number. Haploids, diploids, tetra- and mixoploids were observed between the produced regenerants.

Almost all the researchers studying haploid induction in anther or pollen cultures of tomatoes obtained only callus or, rarely, shoots that died at a very early stage of development (Debergh and Nitsch, 1973; Chlyah *et al*., 1990; Jaramillo and Summers, 1990; Summers *et al*., 1992). Some authors reported obtaining non-haploid plants of high ploidy level, i.e. 2n, 4n (Ancora *et al*., 1977; Zamir *et al*., 1980).

Haploids produced from anther cultures of *Medicago sativa* have not been reported until 1984 (Zagorska *et al.*, 1984). Saunders and Bingham (1972) obtained diploid plants from anther somatic tissue. The origin of the regenerants was proven by cytological analysis.

In our investigation R1, R2 progenies of tomato were tested for their resistance to *Clavibacter michiganense* subsp. *michiganense.* Some of regenerants were resistant to the pathogen. A biochemical analysis of fruits from R3 and R4 plants showed a higher content of dry matter, sugars and vitamin C in the regenerated plants obtained from the hybrids than in those from the cultivars and control plants. The results obtained suggest that induced androgenesis and gametoclonal variation may be used as an additional tool to create a large range of new forms. The application of the latter in breeding programs would accelerate the development of crop lines and varieties that would be more productive, disease-resistant, highly nutritive and flavour - acceptable.

The results from our investigations demonstrated that haploids are an ideal system for genetic manipulation of plants.

Acknowledgements

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ENDOGENOUS PHYTOHORMONES DURING NORWAY SPRUCE SOMATIC EMBRYOGENESIS

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Introduction

During somatic embryogenesis of any coniferous species, developmental events are regulated by exogenously applied plant growth regulators. Whereas usually we have enough information about the type of growth regulator, its concentration and timing; however, only limited knowledge is available on the patterns of endogenous phytohormones during the process.

Material and methods

Plant material: Embryogenic cultures were maintained as described elsewhere (see Fig. 1 for scheme) (Vágner *et al.,* 2005). AFO 541 cell line (originated from AFOCEL, Nangis, France) was used as basic experimental material. Other cell lines, which were induced in our lab in the past, were used for verification of results obtained. The phytohormonal patterns of developing somatic embryos were compared to the patterns of their zygotic counterparts.

ABA: ABA was analyzed by gas chromatography (GC HP 5890) (Prewein *et al.,* 2004), after previous extraction and purification of the samples (Vágner *et al.,* 1998).

IAA: Methanolic extracts were purified and IAA determined by the use of HPLC with fluorimetric detection (Eder *et al.,* 1988).

Cytokinins (CKs): Three methods were used for the determination of endogenous cytokinins: 1) HPLC partitioning and ELISA detection (Vágner *et al.,* 1998). 2) Liquid chromatography – mass spectrometry (LC-MS) was used for the detection of spectra and quantity of endogenous cytokinins (Zubko *et al.,* 2002; Veah *et al.,* 2003). The cytokinins were separated and quantified by HPLC linked to an ion trap mass spectrometer Finnigan MAT LCQ-MS^n equipped with electronspray interface (details of the method see in Veah *et al.,* 2003). 3) Immunolocalization using antibodies against zeatin riboside (ZR) and against isopentenyladenosine (iPA) was used (Feltlová and Macháčková, 2001; Vičánková *et al.,* 2004). The antibodies were produced in the laboratory of Prof. Strnad, IEB AS CR, Czech Republic. The specific detection was obtained for free bases of zeatin (Z) and isopentenyladenine (iP).

Results and discussion

The proliferation stage (under the external supply of BA and 2,4-D to the culture) is characterized by the relatively high level of the sum of endogenous cytokinins. The most abundant CK is iP, CK of zeatin type (Z, ZR) are relatively abundant. The levels of ABA and IAA remain low in this stage.

Exogenously added ABA replaces 2,4-D and BA and triggers the embryo development. During ***the first three weeks of development*** of somatic embryos, endogenous levels of CKs drop down. The level of endogenous ABA sharply increases (ABA is present in the nutrient medium during maturation see Fig. 1). Since the 3rd week, however, ABA is still present in the medium in constant concentration, endogenous level of ABA markedly decreases. The level of IAA sharply increases in the stage of development, in

which cotyledons emerge and elongate. Similar increase of IAA level was found in corresponding stage of zygotic embryos.

At the end of somatic embryo maturation (6 w since the start of ABA supply) levels of CKs remain low. In this stage, isopentenyladenosine (iPA) replaces iP to be the most abundant CK. The ratios of CK types are quite different compared to those of zygotic embryos. Like Zhang (2001), we also detected cytokinins, which we were not able to find in other samples from angiosperm plants. A complete identification of their chemical structures is still problematic, since they are present in the tissue in the extremely low concentrations. Z and iP were localized mainly in meristematic centers of ESM and in meristems of early somatic embryos. In fully developed somatic embryos, these CK were present mainly in the procambial part of embryo and in the root cap. At the end of embryo maturation, the level of IAA drops after a transient peak, and the level of ABA also gradually decreases.

During ***desiccation phase*** (3 w), somatic embryos laid on filter paper are exposed to high humidity treatment. So far, there is no external treatment by plant growth regulators. Surprisingly, a great transient increase of CKs level was found in mid desiccation stage (the increase remains marked if the levels of CK are computed on dry or fresh matter basis). The peak is mainly due to iP increase, however other CK also follow the trend. At the end of desiccation procedure, levels of CKs decreased and their concentration in embryos is similar to the start of desiccation. It seems that the increase of CKs in mid desiccation stage is not unique; similar increase was found in zygotic embryos after they finished their morphological development in the seeds. ABA slightly rises up at the start of desiccation. The increase is temporary, probably due to water stress in the consequence of manipulation at the start of desiccation. Thereafter ABA level tends to further decrease. Low level of ABA at the end of desiccation is necessary for subsequent successful germination. Level of IAA in this stage remains unchanged and low.

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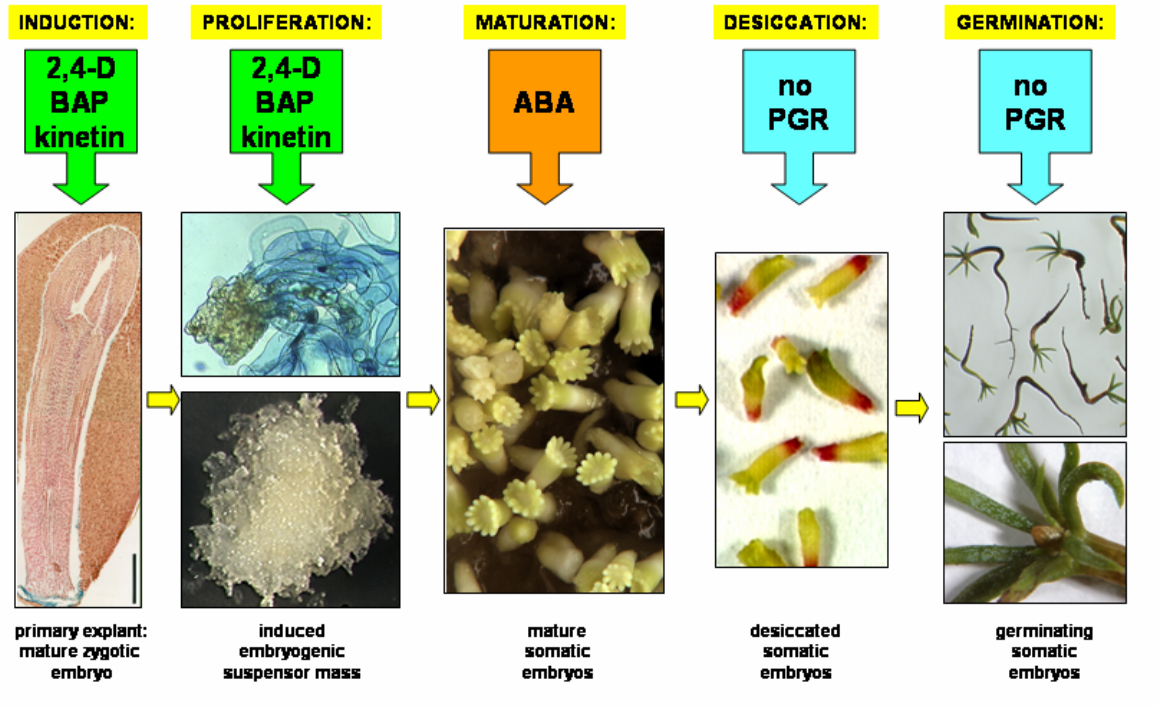

Figure 1 Exogenously supplied plant growth regulators during the somatic embryogenesis of Norway spruce (scheme)

SOMATIC EMBRYOGENESIS OF PEA (*Pisum sativum* L.) IN LIQUID MEDIA: TOWARDS THE DEVELOPMENT OF EMBRYOGENIC SUSPENSION CULTURE

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Introduction

Somatic embryogenesis was recently reported in about 60 leguminous species, including economically important grain legume crops, fodder legumes as well as leguminous trees; nevertheless, the formulation of efficient and reproducible embryogenic suspension culture protocols is limited to only several species, e.g. soybean, peanut, pigeonpea, cowpea, alfalfa, *Trifolium* spp. (Griga 1999; Lakshmanan & Taji 2000; Venkatachalam *et al*., 2003). Similar situation is also in pea - despite of some early (Jacobsen & Kysely 1984; Le Deunff *et al.,* 1992) and recent (Ochatt *et al*., 2002) attempts to induce somatic embryos in liquid medium, the routine protocol for complete plant regeneration from embryogenic suspension is still not available. Such protocol may represent an important tool for mass propagation, mutant selection and gene transfer technology (biolistics) in pea. Here we present some methodological data from our lab towards the development of pea embryogenic suspension.

Material and Methods

Experiments were performed with pea cultivars previously demonstrating high embryogenic competence on agar media (cvs. Citrina, Menhir, Oskar - Griga, 1998) and further with cv. Solara (Nielsen *et al.,* 1991) and cv. Frisson (Ochatt *et al.,* 2000). Three procedures for establishment of pea embryogenic suspension were designed and tested.

(1) Leaf explants of 7 day-old seedlings were cultured on C_N-medium (MS-medium supplemented with 2.69 μM NAA**,** 4.65 μM KIN, 1% agar; pH 5.8) in the dark at 25°C. 1-5 week-old cultures were transferred into liquid S_P-medium (MS-medium, 0.25 μM picloram, 0.44 μM BAP; pH=5.8) and agitated on the gyratory shaker (95-100 rpm) at 22°C 16 h/ 20°C 8 h.

(2) Apical meristems and leaf explants of 7 day-old seedlings were cultured on MS-medium with 87.5 mM sucrose, 0.8 % agar and 6.78 μM 2,4-D; pH 5.8 at 22°C 16 h/ 20°C 8 h (Anbazhagan & Ganapathi 1999). Ten day-old yellow-green calli were transferred into the liquid MS-medium supplemented with 87.5 mM sucrose and 4.52 μM 2,4-D (pH 5.8) and agitated on the gyratory shaker (85-90 rpm) at 22°C 16 h/ 20°C 8 h.

(3) Leaf, epicotyl, nodal and hypocotyl segments from pea embryo axis (Nielsen *et al.,* 1991) were used as starting material for verification of protocol proposed by Ochatt *et al.* (2002). For callus induction, explants were cultured on agar MS-medium with 0.41 μM picloram and 2.33 μM kinetin (MSM_1); after 6-8 d, explants were transferred onto liquid MS-medium with 0.41 μM picloram and 2.33 μM kinetin (MSM_2; gyratory shaker, 85-90 rpm, 22°C 16 h/ 20°C 8 h). In 10-14 d intervals, explants were transferred to sequence of liquid MS-media according to S. Ochatt (personal communication) for induction of pea embryogenic suspension - MSM_3 (10 mM NH_4Cl, 0.1 % CH and 4.52 μM 2,4-D), MSM_4 (MSB0), MSM_5 (5.37 μM NAA and 0.29 μM GA_3) and MSM_6 (2.89 μM GA_3).

Results

(1) The culture in liquid S_P-medium resulted in hard calli with green clumps of "globular embryo structures", which were not released onto liquid medium. In liquid media, this structures remained in globular stages and were overgrown by callus tissue.
(2) Anbazhagan & Ganapathi (1999) procedure for pigeonpea embryogenic culture applied in pea cultivars resulted in the production of green or yellow-green friable calli without somatic embryo formation in four cvs. tested (Fig. 1A); only cv. Menhir produced somatic embryos, which developed to the torpedo stage (Fig. 1B). Further development of the somatic embryos required transfer into agar MS-medium without hormones.
(3) The culture scheme proposed by S. Ochatt led to embryogenic callus formation in the embryogenic cultivars. The dark-green clusters of globular embryos appeared on the surface of green hard calli (Fig. 2), but they did not release onto liquid media, even though MSM_3-medium contained 0.1% CH, which contributes to the improvement of callus fragmentation (Loiseau *et al*. 1995).

Conclusions

Pea embryogenic response in liquid media was genotype-dependent (similarly as in agar media). Majority of approaches (tested in this study and previously in our lab) resulted in the formation of hard, compact calli which did not release single cells/cell clumps spontaneously into liquid medium. Obtained friable calli were non-embryogenic. Some of compact calli exhibited embryogenic capacity – formation of globular structures, which developed in connection with explant/callus. For further somatic embryo development and embryo conversion, it was necessary to transfer torpedo somatic embryos into the agar media. Future strategy for formulation of pea embryogenic suspension or repetitive embryogenesis in liquid media will be discussed.

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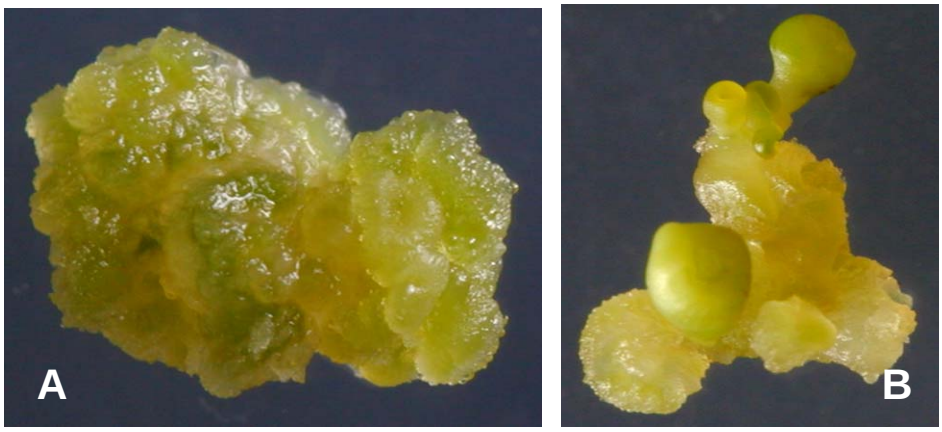

Figure 1 Non-mbryogenic callus of pea (*Pisum sativum* L.) cv. Citrina (**A**) and embryogenic callus of cv. Menhir (**B**) obtained in liquid culture MS-medium with 2,4-D according to the modified protocol for pigeonpea (*Cajanus cajan* L.) embryogenic suspension (Anbazhagan & Ganapathi, 1999)

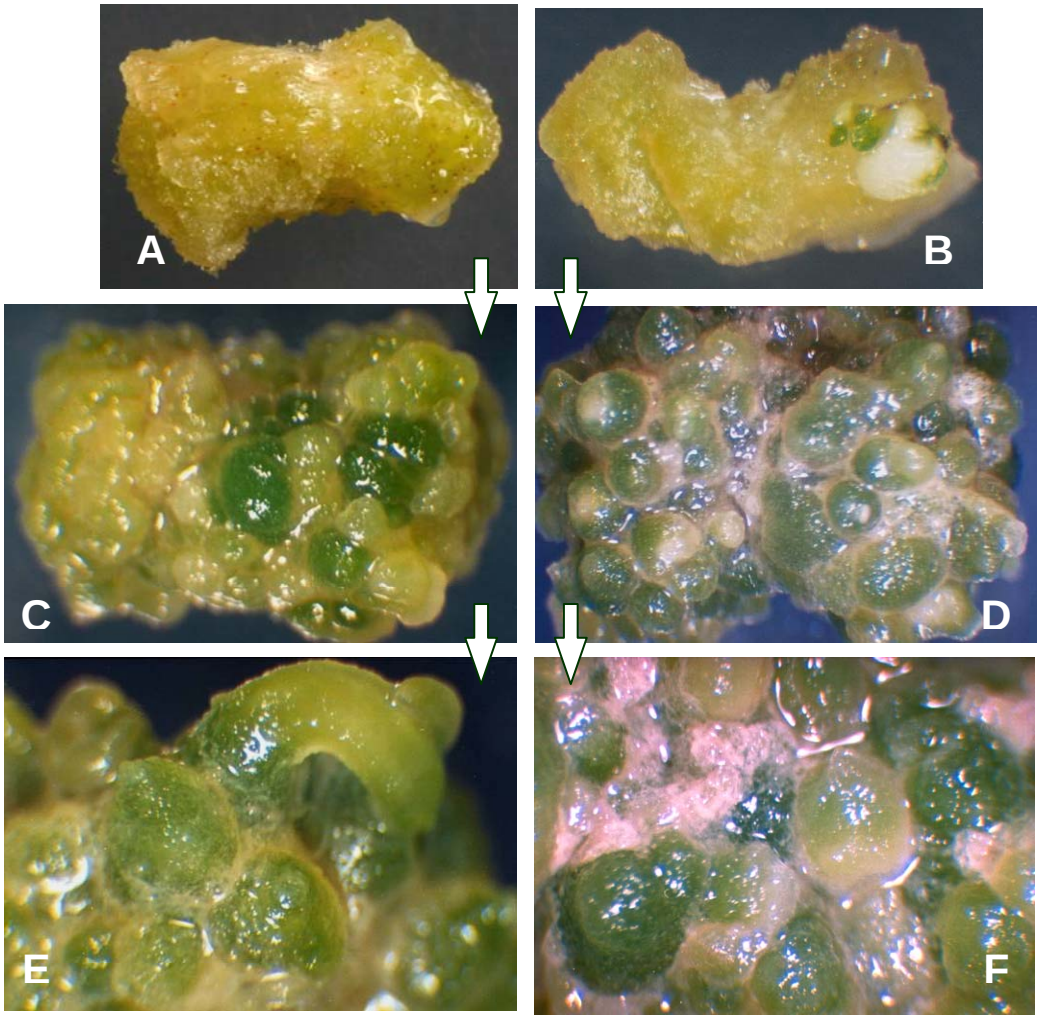

Figure 2 Embryogenic calli of pea (*Pisum sativum* L.) cv. Oskar obtained in liquid culture by modified sequence of media according to Ochatt *et al*. (2002 and personal communication). Left side – epicotyl-derived calli; right side – hypocotyl-derived calli; **A**, **B** – agar MSM_1; **C**, **D** – liquid MSM_4; **E**, **F** – liquid MSM_6

SOMATIC EMBRYOGENESIS IN CONIFERS: EMBRYO YIELD AND ENDOGENOUS CARBOHYDRATE DYNAMICS AS AFFECTED BY EXOGENOUS CARBOHYDRATE SUPPLY

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Introduction

Cultivation conditions play a crucial role in the efficiency of conifer somatic embryogenesis. Beside plant growth regulators having the primary directing effect on embryogenic cultures, types and concentrations of exogenous carbohydrates are also of great importance. Carbohydrates serve primarily as the source of carbon and energy, but other roles must not be neglected. The addition of carbohydrates to the medium decreases medium osmotic potential and leads to changes in osmotic status of the cells. Recently much evidence has been provided showing the importance of signalling role of carbohydrates (e.g., see review by Rook and Bevan, 2003). The relationship between the particular effects of carbohydrates, however, is not well understood and recent data clearly indicate it to be rather complicated. This hampers significantly the searching for the best exogenous carbohydrate support of somatic embryogenesis under *in vitro* conditions. The aim of the presented work was to study the effect of changes in carbohydrate supply during conifer somatic embryo development and assign the resulting embryo yields to the changes in endogenous carbohydrate content and spectrum.

Material and Methods

Picea abies embryogenic culture (line AFO 541, AFOCEL, France) grown on GD media (Gupta and Durzan, 1986), weekly subculturing, 25°C, dark. Proliferation medium: 5 μM 2,4-D, 2 μM kinetin, 2 μM BAP and 3% sucrose; maturation medium: 20 μM ABA, 3% sucrose or 1.57% glucose + 1.57% fructose. *Abies alba x Abies numidica* (line AN 72, SAS, Slovakia), grown DCR medium (Gupta and Durzan, 1985); subcultivation interval: two weeks, 25°C, dark. Proliferation medium: 1 $mg.l^{-1}$ BAP, 2% sucrose; maturation medium: 10 $mg.l^{-1}$ ABA, 10% PEG, 3% sucrose or maltose. Desiccation: isolated mature somatic embryos were subjected to high humidity desiccation treatment, 3 weeks, photoperiod 16h, temperature 20°C (±0.5°C). Carbohydrate content determination: HPLC analysis, refractometric detection, column IEX Pb form.

Results and Discussion

The yield of mature somatic embryos of *Picea abies* was negatively influenced by replacing sucrose with the mixture of glucose and fructose (Fig. 1). The difference was more pronounced when the cultures were grown on liquid media. The embryos matured on the media with sucrose were characterised by marked changes in endogenous carbohydrate content and spectrum with proceeding maturation. A decrease in total carbohydrate content was accompanied by the increase of sucrose : hexose content ratio which was found to be characteristic for well developing embryos (Lipavská *et al*, 2000). Accumulating sucrose can be the result of either enhanced sucrose uptake caused by the decrease in invertase activity (Konrádová *et al*., 2002) or by re-synthesis of sucrose from absorbed hexoses still prevailing in the cultivation medium (Kubeš and Lipavská, 2004). The analysis of endogenous carbohydrates in embryos grown on media with glucose and fructose revealed high content of sucrose, thus indicating the importance of sucrose re-synthesis (Fig. 2). The cultures of maturing somatic embryos of *Abies alba x Abies numudica* were grown on media supplemented either by 3% sucrose or maltose. The yield of embryos was significantly higher on the medium with maltose (Fig. 4). The analyses of endogenous carbohydrate status revealed the increase of su-

crose : hexose ratio (similarly as described for *Picea abies*) but also the presence of raffinose family oligosaccharides (RFO) in later phases of maturation (Fig. 3). RFO was found in *Picea abies* embryos only after desiccation or cold treatment (Lipavská and Konrádová, 2004). Surprisingly, in *Abies* the content of RFO was enhanced not only after desiccation treatment but also when the cultures were grown on maltose (Fig 3). Clearly, the amounts and spectra of endogenous carbohydrates do not simply reflect the carbohydrate supply from the medium, thus pointing to the necessity to consider besides nutritive roles also their osmotic as well as signalling ones.

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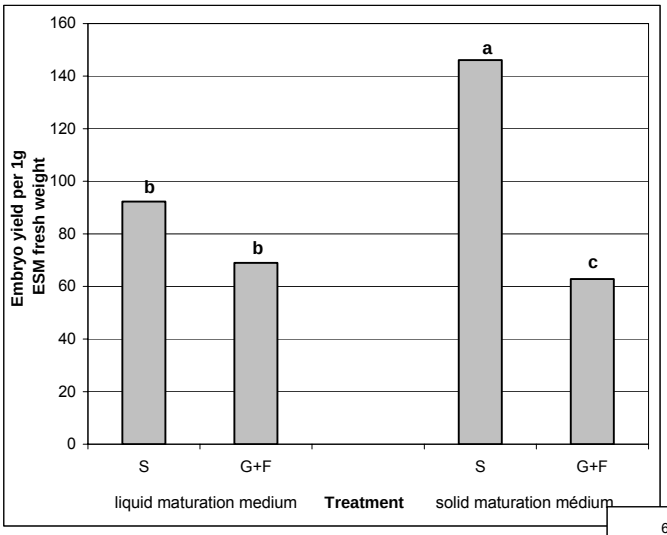


Figure 1 Yield of *Picea abies* somatic embryos as affected by the type of the sugar supplied from the medium

S… 3% sucrose; G+F … 1.57 % glucose + 1.57 % fructose

The yield is expressed as the number of embryos formed per 1 g of embryonal suspensor mass (ESM) at the start of maturation.

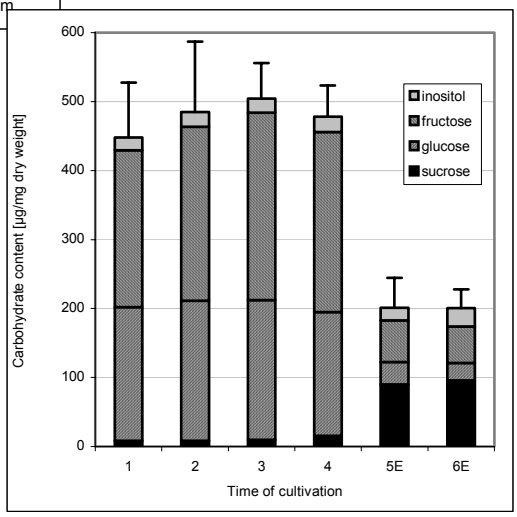
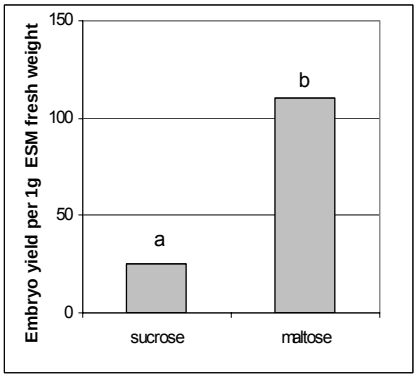


Figure 2 Carbohydrate content in *Picea abies* somatic embryos matured on the medium supplemented with 1.57% glucose + 1.57% fructose

1, 2, 3…. 1st, 2nd, 3rd… week of maturation - ESM

5E, 6E …5th, 6th week of maturation - isolated embryos

Figure 4 Yield of *Abies alba x Abies numidica* somatic embryos as affected by the type of the supplied sugar. 3% sucrose or 3% maltose, the yield is expressed as the number of embryos formed per 1 g of ESM at the start of maturation



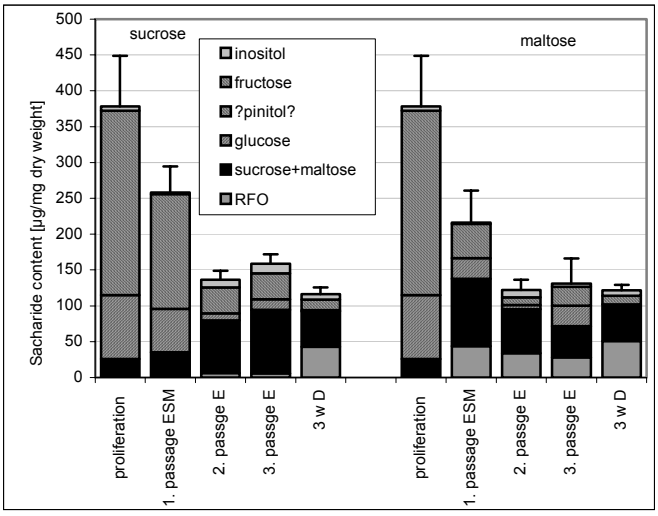


Figure 3 Carbohydrate content in *Abies alba x Abies numidica* somatic embryos matured on the medium supplemented with 3% sucrose or maltose

CRYOPRESERVATION IN QUALITY PLANT PRODUCTION

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Introduction

Cryopreservation has been considered an ideal means of long-term storage of plant genetic resources. Cryopreservation can prevent the stored materials from being exposed to the effects of weather, pests, diseases and other stresses that are risks of *ex situ* field collections or of *in vitro* conservation, and offer the greatest genetic stability of regenerated plants. Recently, it has been found that cryopreservation of shoot tips is able to eliminate plant viruses with even much higher rates of virus eradication than the conventional methods such as meristem culture (Brison *et al*., 1997; Wang *et al*., 2003). Furthermore, using cryopreservation as virus elimination method, the size of the shoot tips used can be larger than that in meristem culture (Brison *et al*., 1997; Wang *et al*., 2003).

Material and Methods

A project "Long-term storage of tested horticultural plants by cryopreservation" was initiated in our lab in 2003. The objective of the project was to establish protocols of long-term storage of some horticulturally important crops and at the same time to eliminate viruses such as raspberry bushy dwarf virus (RBDV). Encapsulation-vitrification (EnVi) (Tannoury *et al*., 1991) and encapsulation-dehydration (EnDe) (Fabre and Dereuddre, 1990) procedures were used. Also the droplet method (Schäfer-Menuhr *et al*., 1997) was tested for the cryopreservation of shoot tips and buds of raspberry, different Prunus cultivars (*Prunus cerasus* L., *Prunus domestica* L.) and hops (*Humulus lupulus* L.).

Results

Raspberry shoot tips of seven genotypes treated by EnVi protocol showed survival of 71% and regrowth of 68%. Shoot tips treated by EnDe protocol showed survival of 65% and regrowth of 50%. Droplet method was successfully used for cryopreservation of different genotypes of genies *Rubus*, *Prunus* and *Humulus*. Regenerated plants displayed no abnormal morphology or growth as compared to the original stock cultures. The results indicate EnVi as a simple and efficient method for long-term preservation of *R. idaeus* germplasm. The elimination of RBDV was however not successful, because the virus was present also in the meristem cells.

Discussion

The shoots that recovered and regenerated after cryopreservation were of good quality. This can depend either on the elimination of disturbing endophytic infections that are not seen in the routine tests performed to the quality plants or on the rejuvenation as only the young meristematic cells recover. This effect resembles the effects of meristem culture.

Acknowledgements
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ASPECTS OF SHOOT SEGMENT ENCAPSULATION IN *Dendranthema* AND *Rosa*

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Introduction

Creation of artificial seeds by encapsulation of somatic embryos or shoot tips and buds has been under discussion since first publication of this idea by Murashige (1977). Till now, many publications about encapsulation of shoot tips and buds can be found; however, most of encapsulated plant material was cultivated on a complete MS medium under sterile conditions. Piccioni and Standardi (1995) compared the conversion of encapsulated buds of six woody plants on a water agar and MS medium and found no or a reduced conversion on water agar. These results indicate problems in supplying the required nutrients only via the bead. Reports on conversion under unsterile conditions to meet really the demands of an artificial seed are scarce (Ganapathi *et al*., 1992; Pattniak *et al.*, 1995). The most important reason for that is certainly the encapsulation material. Mostly the plant material is encapsulated in Ca-alginate beads. Ca-alginate has advantages for encapsulation procedure and is well suitable for the plant material. The wet and sticky beads, however, cannot inhibit the loss of nutrients and water as well as the attacks of microbes.

The aim of our experiment was to investigate if it is possible to supply enough nutrients for the conversion process in the Ca-alginate beads. We compared the responses of Chrysanthemum and Rose shoot buds.

Material and Methods

Shoot cultures of *Dendranthema* x *grandiflorum* PS 27 and *Rosa* x *hydrida* cv. Kardinal were grown on modified MS media. Three to five weeks after subculture 4 to 5 mm long nodal segments were isolated and mixed within a 3% Na-alginate solution containing MS medium and 1 $mg.l^{-1}$ IAA (*Dendranthema*) or 2 $mg.l^{-1}$ IBA (*Rosa*). Droplets of 0.085 ml were dropped in 75 mM $CaCl_2$ and stirred for 30 min. After that the beads were transferred for conversion into 100ml Erlenmeyer flasks filled with 25 ml water agar. Cultures were maintained at 24°C and illuminated 16h/day with white light (PAR 35µmol m^{-2} s^{-1}, OSRAM L 58 W/30)

The conversion was recorded weekly as shoot and root formation. To investigate the nutrient content of the beads the nitrate content was measured daily during the first week with a nitrate selective electrode.

Per treatment 30 beads were used and each experiment was repeated at least twice. Statistical significance of results was checked with Chi-Square test and Fisher test ($p < 0.05$).

Results and Discussion

Without application of nutrients in *Rosa* there was no conversion, in *Dendranthema*, however, till 30% of encapsulated buds formed shoots. The application of MS medium improved the shoot development. Root formation was much more difficult than shoot formation in both cultures and application of auxin was a prerequisite for rooting. These results indicate that an external supply of nutrients and growth regulators is required for any developmental process in the encapsulated buds.

The question was if an appropriate supply with nutrients via the bead can be guarantied for a period of some weeks till plants are able for photoautotrophic growth. Measurement of nitrate content of the beads showed that already after one day of incubation of 25 ml water agar the nitrate content in the beads was reduced by 92%. The reduction of volume of water agar to 7 ml reduced the nitrate loss of the beads. This better nutrient supply resulted in a higher percentage of shoot formation of encapsulated rose segments. The root formation, however, was only slightly improved. The encapsulated *Dendranthema* segments developed even on 25 ml water agar to more that 90% and no improvement by the better nutrient supply on 7 ml water agar could be proved.
In the next experiment the bead volume was enlarged to have a better supply with nutrients. The increase in bead volume from 0.085 ml to 0.105 ml improved the root formation in encapsulated rose segments. Shoot formation was not affected. Maybe the higher amount of auxin available in the larger beads in the beginning of incubation resulted in better root formation. The amount of nutrients in total, however, was not enhanced up to a level necessary for better shoot development. In *Dendranthema* there was no effect of the increased bead volume on the conversion.
It seems that it is possible to supply the encapsulated nodal segments with an appropriate amount of nutrients via the beads if the plant material has not very high requirements like *Dendranthema*. In such cultures conversion on nutrient free or poor substrates under unsterile conditions can be possible regarding the aspect of nutrient supply even with Ca-alginate beads. In encapsulated rose segments the nutrient demand seems to be higher than offered with the beads. For such cultures the development of a seed coat is required to avoid nutrient losses.

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INVESTIGATIONS CONCERNING THE FORMATION OF SOMATIC EMBRYOS IN *Eleutherococcus sieboldianus* (Makino) Koidz. (*Araliaceae*)

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Within the frame of experiments to get organogenesis or embryogenesis of woody plant species, leaf explants of 3 different *Eleutherococcus* species (*E. gracilistylus*, *E. setchuenensis* and *E. sieboldianus*) were tested concerning their capability of regeneration. The aim of these investigations was to get plants free of contaminations which can be used for cultivation as medicinal plants and mycorrhizing experiments. It was looked for methods in vitro to achieve a vegetative propagation starting from explants of adult individuals because immature zygotic embryos were not available and seeds were not able to germinate. Nutrient media were tested, which were used successfully for induction or maintenance of somatic embryogenesis in other woody plant species. Cultures were kept on three different nutrient media in the dark for 11 months. Because of the best callus growth they were transferred afterwards onto the Linsmayer-Skoog medium with 2,4-D (2,4-dichlorphenoxy acetic acid).
The reduction of growth regulator concentration (0.5mg.l^{-1} 2,4-D) led in *E. gracilistylus* and *E. setchuenensis* to callus formation. Globular structures were observed in calli of *E. sieboldianus* after one year, which grew often out into roots.
Multiple subcultures on a nutrient medium with half the concentration of 2,4-D led to the formation of embryogenic callus and somatic embryos.
These somatic embryos started to grow, became green and developed a root after they were transferred to a cytokinin-containing nutrient medium in the light.
Endogenous bacteria were observed microscopically in and around the root hairs of older plantlets *in vitro*. This was for the first time that endogenous bacteria in tissue culture plants could be detected visually without any staining treatment. Investigations are underway to characterize these bacteria and their function within the plant.
The transfer of rooted somatic embryo derived plants into the soil was achieved by planting them into peat pellets or after a phase of hydroponic culture directly into pots.
There was no precocious flower formation of these plants observed during the first two years in the nursery, therefore we assume a rejuvenation of the plant material.
This is, according to our knowledge, for the first time that somatic embryogenesis in *Eleutherococcus sieboldianus* was achieved starting from leaves of adult plants.

TEMPERATE FRUIT TREES IN TEMPORARY IMMERSION: LAST IMPROVEMENTS

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Introduction

The interest in decreasing the cost production of micropropagated plants is stimulating the research to study new *in vitro* culture systems useful for automation of the entire process. Many papers reported an increasing of *in vitro* shoot proliferation and explants quality with the periodical immersion in a liquid culture media (Tisserat & Vandercook, 1985; Aitken-Crhristie & Davies, 1988). During the last 15 years several systems for the temporary immersion (TI) technique have been tested; recently Teisson & Alvard (1995) described a TI system for plant propagation called RITA®, that could be easily automatized. The RITA® system is successfully applied to the micropropagation of several species. The temporary immersion allows to reduce the production costs through a semi-automation of the system and to limit the contamination risk. Moreover the plants, being plunged into the medium for short period of time, do not lose the attitude for autotrophy (Monticelli *et al*., 2000; Gentile *et al*., 2000) therefore the transfer from in *vitro* to *in vivo* is less stressing. In our laboratory the TI technique has been applied to *in vitro* propagation of apple, peach, cherry, plum and other temperate small fruit in comparison with stationary liquid and solid media, in order to assess the influence on multiplication response, shoots quality and physiological state of the treated plants. In this paper we report an overview of the results obtained during the research activity of late years.

Materials and Methods

Plant materials. All the experiments were carried out the plantlets grown *in vitro*, using the media reported in Table 1.

Apple, peach, cherry, plum, small fruit shoots, grown *in vitro* for 2 years on solid media, were used. Plants were cultured for 60 days in: 1) solid condition (0.6% agar agar); 2) TI with an immersion period of 60' per day; 3) stationary liquid condition. For all the treatments the medium was renewed every 20 days. The culture conditions were temperature 24 °C ±1, photoperiod of 16 hours with light intensity of 25 μmol $m^{-2}s^{-1}$, provided by Osram fluora L58 W/77 lamps.

Temporary immersion system. The device was composed of two glass bottles connected with a silicone tube; one of the bottles was used to contain explants put on a glass beads layer (2 mm diameter) support, that guaranteed the isolation from the culture medium during the non immersion period. The other bottle contained the culture medium. The medium was transferred from one bottle to the other with the overpressure created by an air pump; the air insufflate in the bottle was sterilised with 0.2 μm sterile filter (Acro®50 - Pall Gelman) with hydrophobic (PTFE) membrane.

Biochemical analysis. Samples for the a- and b-chlorophyll analysis were prepared according to Arnon (1949). The pigment was determined spectrophotometrically (Spectrophotometer Hitachi 2000), measuring light absorbance by acetone extracts of chlo-

[1] Mr. Frattarelli has provided technical implementation of experimental plan and collection of data

rophylls (λ = 663nm and 645nm, respectively for chlorophylls a and b). The fructose determination (total and as sucrose) was based on the estimation of a chromophoric substance formed by the reaction of resorcinol and fructose according to a modified Roe's test (Davis et al., 1967). Fructose content was determined with a spectrophotome- ter (Hitachi 2000) at λ = 420nm.

Results and Discussion

The multiplication rate was observed after two subcultures (60 days).

With the temporary immersion technique the multiplication rate was much higher in apple, *Rubus* and *Myrtus*, than solid and liquid medium (Table 2). In particular, in *Rubus fruticosus* and *Myrtus communis* the multiplication rate was twofold respect to the solid medium system. Moreover, the plants showed an increased stem elongation. For the other species the multiplication rate did not differed significantly from the solid medium.

The plants cultured in TI never showed hyperhydricity (probably due to the frequent renewal of the atmosphere in the culture bottle) and apex necrosis. On the contrary, stationary liquid condition induced a decay of the plants, due to the hyperhydricity and necrosis, and a critical decreasing of the multiplication rate in most of cases. Hyperhydricity occurred in around 10% of the control, for all the genotypes, but never in the TI treatment.

The detrimental effects of stationary liquid culture were also evident on the photosynthetic pigment content. On the contrary, the TI stimulated the chlorophyll synthesis.

From the results here reported it was shown that in plants grown in TI the total amount of fructose significantly increased, mainly accumulated as sucrose, while in the plants grown in solid and stationary liquid conditions the free fructose prevailed. These results seem to support the hypothesis that the plants cultured in TI, being in contact with the sugars in the medium only for very short period during the culture, could partially restore the autotrophic ability and the capability to accumulate sugars.

Literature reports that the positive effects of TI are due to a better aeration and renewal of chemical components at each immersion, otherwise limited in solid condition by the agar matrix. In this way the risk of anoxia and vitrification, frequently observed in constant immersion as well as in solid culture, is significantly reduced (Etienne *et al.*, 1997). Preliminary experiments on the propagation of pomegranate *(Punica granatum)*, hawthorn *(Crataegus azarolus)* and service tree *(Sorbus domestica)*, gave similar results.

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Table 1 Multiplication media

		Apple	Peach	Cherry	Plum	*Rubus fruticosus*	*Arbutus unedo*	*Myrtus communis*
Macrosalts		MS	QL	MS	QL	MS	MS	MS
Microsalts		MS	MS	MS	QL	MS	MS	MS
Vitamins		MS*	MS	MS	MS	MS		MS
Thiamine-HCl	$mg.l^{-1}$	-	-	-	-	-	1.3	-
Myo-inositol	$mg.l^{-1}$	-	-	-	-	-	100	-
Calcium pantothenate	$mg.l^{-1}$	-	-	-	1	-	-	-
Biotin	$mg.l^{-1}$	-	-	-	0.1	-	-	-
Riboflavin	$mg.l^{-1}$	-	-	-	0.5	-	-	-
Sucrose	$g.l^{-1}$	30	30	30	30	30	30	20
BA	$mg.l^{-1}$	0.5	0.4	0.5	0.25	0.5		0.65
IBA	$mg.l^{-1}$	0.1	0.06	0.1	0.1	0.05	0.1	-
GA_3	$mg.l^{-1}$	-	0.03	-	-	0.1	-	0.1
Adenine sulphate.	$mg.l^{-1}$	-	3	-	-	3	-	-
2iP	$mg.l^{-1}$	-	-	-	-	-	0.01	-
chinetina	$mg.l^{-1}$	-	-	-	-	-	0.3	-
NAA	$mg.l^{-1}$	-	-	-	-	-	-	0.01

*Thiamine ten-fold concentrated ($1mg.l^{-1}$) MS: Murashige & Skoog, 1962; QL: Quoirin *et al*., 1977

Table 2 Effects of the culture system on multiplication rate

Cultural conditions	Plum	Cherry	Peach	Apple	*Rubus fruticosus*	*Arbutus unedo*	*Myrtus communis*
Solid	30	5	20	2	4	2	4
Liquid	3	2	3	3	3	3	2
Temporary immersion	28	4	14	8	8	3	9

INDUCTION OF SOMATIC EMBRYOGENESIS IN *Narcissus* L. 'CARLTON' OVARY CULTURE

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Introduction

Process of somatic embryogenesis in *Narcissus* genus were induced on bulb scale, leaf and scape (flower stem) explants of 'Golden Harvest' and 'St. Keverne' cultivars (Sage *et al.,* 2000) as well as on immature or mature zygotic embryos isolated from seeds of *N. confussus* (Selles *et al.,* 1999).

The aim of the presented experiment was to estimate the effect of growth regulators on embryogenic callus induction and somatic embryogenesis process using explants isolated from *Narcissus* L. 'Carlton' ovaries.

Material and Methods

As a plant material for *in vitro* culture ovaries of *Narcissus* L. 'Carlton' isolated from bulbs 12 cm in diameter were used. The bulbs were stored at temperature 18 °C or chilled for 12 weeks at 5 °C. Initial explants, 1-2-mm-thick slices of ovaries, were cultivated on the Murashige and Skoog 0,7% Difco bacto agar-solidified medium (1962) supplemented with auxin (A): 2,4-D or Picloram (10-100 μM) and cytokinin (C): BA (0.5-50 μM) in different proportion (A>C, A=C). The explants were orientated on medium the morphological side down.

The cultures were incubated in the dark at 20 °C and 90% relative humidity.

After 6 weeks of culture the percent of explants forming callus and intensity of callus formation were estimated. All experiments were conducted with a 5 replicates of 5-8 explants per Petri dish. Results of observation were calculated by analysis of variance and Duncan's test.

Results

Unchilled and chilled slices of ovaries swelled under the influence of growth regulators. On the cut surface of the initial explants the great number of long, transparent cells appeared first and after 4 weeks yellow, nodular callus formation in the swelled and wrinkled ovary walls were observed.

Effectiveness of callus induction was affected by the kind of auxin and the proportion of the one to cytokinin (Table 1). Under the influence of Picloram nodular callus has been initiated on the greater or comparable number of explants than under the influence of 2,4-D used at the same concentration and the same combination with BA. The most abundant caulogenesis was obtained on medium containing 100 μM of Picloram and 0.5 μM of BA.

The highest intensity of callus formation was observed on media with 25 μM of Picloram combinated with BA at concentration of 5 or 25 μM. 2,4-D caused single nodule of callus formation on the surface of the ovary slices (Table 1).

Effect of donor bulb treatment on effectiveness of callus induction and somatic embryos formation were proved. The number of ovary slices forming callus and intensity of callus formation were higher when donor bulbs were chilled at 5 °C than stored at 18 °C (Table 2).

Morphogenesis of nodular callus obtained under influence of high auxin concentrations were affected by cytokinin level in induction medium. On media containing high con-

centration of 2,4-D in combination with cytokinin in very low concentration (0.5 μM) roots developed. Nodular callus initiated under influence of BA at concentrations of 5-50 μM and auxin (Picloram or 2,4-D) at higher or the same concentration was capable to differentiation into somatic embryos.

Discussion

It is the first publication on the topic of somatic embryogenesis induction on *Narcissus* ovaries. Up to now *Narcissus* ovaries were used for adventitious shoot and bulb production (Seabrook *et al*., 1976; Hosoki and Asahira, 1980; Kozak i Dąbski, 1994). Hohe *et al.,* (1998) as well as Bach and Ptak (2001) took ovaries of bulbous and cormous plants as initial explants for somatic embryogenesis.

In the present investigation, the highest effectiveness of somatic embryogenesis induction on *Narcissus* ovary explants on media containing Picloram was observed. Similarly Bach (1992) in *Freesia* culture and Kim *et al.* (2001) in *Alstroemeria* culture stated that Picloram treatment is more effective than use of 2,4-D. Stimulation of *Manihot esculenta* Crantz embryogenic callus tissue formation under influence of high concentrations of Picloram Groll *et al.* (2001) obtained. Sage and Hammatt (2002) noted the most abundant nodular callus formation in *Narcissus* shoot culture on medium with 40 μM of Picloram and 0.5 μM of BA.

Results presented indicate the significant role of donor bulb treatment. Higher regenerative potential of explants originated from chilled bulbs could be caused by increasing the level of auxin in all tulip bulb organs at the end of the chilling period (Saniewski and Kawa-Miszczak, 1992).

It was observed that for nodular callus initiation the minimal induction concentration of cytokinin in medium is necessary. Somatic embryos were obtained on media containing 5-50 μM of BA in combination with high concentration of auxin (10-50 μM). At 0.5 μM of BA in the medium somatic embryos were not formed. Elevation of the initial auxin level and simultaneous lack of cytokinin caused the roots formation.

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Table 1 Callus induction on *Narcissus* L. 'Carlton' ovary explants under influence of different growth regulators

Proportion auxin to cytokinin	Growth regulators [μM]	Explants forming callus [%]	Intensity of callus formation	Morphogenesis (SE-somatic embryos, R-roots, - - no reaction)
A>C*	10 2,4-D + 0.5 BA	47.0 e-f**	1***	R
	25 2,4-D + 0.5 BA	42.0 d-f	1	R
	50 2,4-D + 0.5 BA	42.0 d-f	1	R
	100 2,4-D + 0.5 BA	22.0 a-c	1	R
	10 Picloram + 0.5 BA	63.0 g-h	2	-
	25 Picloram + 0.5 BA	71.0 h-i	2	-
	50 Picloram + 0.5 BA	81.0 i-j	2	-
	100 Picloram+ 0.5 BA	95.0 j	1	-
	10 2,4-D + 5.0 BA	10.0 a-b	1	SE
	25 2,4-D + 5.0 BA	11.3 a-b	1	SE
	50 2,4-D + 5.0 BA	5.8 a	1	SE
	10 Picloram + 5.0 BA	23.1 b-c	2	SE
	25 Picloram + 5.0 BA	26.5 b-d	3	SE
	50 Picloram + 5.0 BA	12.1 a-b	2	SE
A=C	10 2,4-D + 10 BA	21.5 a-c	1	SE
	25 2,4-D + 25 BA	31.7 c-e	1	SE
	50 2,4-D + 50 BA	13.0 a-b	1	SE
	10 Picloram + 10 BA	33.0 c-e	2	SE
	25 Picloram + 25 BA	54.6 f-g	3	SE
	50 Picloram + 50 BA	39.7 d-f	2	SE

* Proportion of growth regulators (A – auxin, C – cytokinin)
** Means followed by the same letters do not differ significantly
*** Intensity of callus formation: high (3), medium (2), low (1)

Table 2 The effect of bulb temperature treatment on callus induction in *Narcissus* L. 'Carlton' ovary cultures

Treatment of donor bulbs	Explants forming callus [%]	Intensity of callus formation	Direction of morphogenesis (SE-somatic embryos, R-roots)
unchilled	29.2 a*	2	SE
chilled	45.3 b	3	SE, R

*see table 1

ULTRASTRUCTURAL CHANGES DURING SOMATIC EMBRYOGENESIS IN COTYLEDONS OF *EUCALYPTUS GLOBULUS* LABILL

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Abstract
Induction of somatic embryogenesis, considered a highly reliable micropropagation system, leads to anatomical, histological and cytological changes in explants/embryos. In Eucalyptus, a recalcitrant genus concerning somatic embryogenesis, there is very limited information concerning the histological and cytological characterisation of the somatic embryogenesis process. Bandyopathyay and Hamill (2000) compared ultra-structural aspects of sporadically formed somatic embryos and zygotic embryos of *Eucalyptus nitens*. However, in this genus, the whole process from induction to conversion was never followed histologically and cytologically terms.
Recently, Pinto *et al.* (2002) described for the first time, a reliable protocol for somatic embryogenesis and conversion in *E. globulus* and *a* protocol for repetitive somatic embryogenesis was also tested (Pinto *et al*., 2004a). These somatic embryos were shown to be stable at ploidy level (Pinto *et al.*, 2004b) allowing a multiplicative potential for clonal mass propagation.
Once the system has been optimised, this will be an excellent tool for induction, expression, and conversion of somatic embryos to plantlets. The objective of this work is to describe the changes observed in *E. globulus* explants during the induction, expression and developmental stages of somatic embryos. Changes in lipid and starch contents will be discussed as well as, organell evolution, vascular differentiation and morphometric studies.

Acknowledgements
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PLANT REGENERATION IN ANTHER CULTURE OF YELLOW BUCK *(Aesculus flava Marshall.)*

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Introduction

Aesculus flava is, as most of trees, characterised by a long reproductive cycle and a high level of heterozygosity. This makes genetic improvement by classical breeding difficult. The production of homozygous (doubled-haploid) plants *via* androgenesis *in vitro* can contribute to more efficient breeding. The ability to rapidly produce large numbers of high quality haploid embryos from anther cultures of *Aesculus flava* Marshall. allows this culture system to be utilized as a model system for androgenic or somatic embryo induction, morphogenesis, maturation and germination. Not only can large, uniform embryo populations be generated, but these are for the most part free of genetic anomalies since embryos of tree species develop directly from microspores without an intermediate callus phase (Radojević 1991; Germana *et al*., 1994; Capuana and Deberg, 1997; Radojević *et al*. 1998; Ćalić *et al*., 2003/4).

Material and Methods

Completely closed flower buds (4-12 mm long) used in the experiments were obtained from an *Aesculus flava* L. tree about 5 years old, growing in the Botanical Garden "Jevremovac" of the Belgrade University. The selected buds from three segments (A-female; B-bisexual and C-male flowers) of inflorescence was surface sterilized with 95% ethanol and 70% ethanol for about 5 min, followed by three rinses in sterile distilled water. Induction of androgenic embryos of *Aesculus flava* was achieved on solid MS medium contained Murashige and Skoog's (1962) mineral salts, 2% sucrose, 0.7% agar, and was supplemented with the following [$mg.dm^{-3}$]: panthotehenic acid 10.0, nicotinic acid 5.0, vitamin B_1 2.0, adenine sulphate 2.0, myo-inositol 100, casein-hydrolysate 200, 2,4-dichlorophenoxyacetic acid (2,4-D) 1.0 and kinetin (Kin) 1.0. The MS_1 medium for embryo multiplication consisted reduced concentration of 2,4-D (0.01 $mg.dm^{-3}$) and the same concentration of Kin (1.0 $mg.dm^{-3}$).

Anthers were inoculated in each culture tube containing 8 cm^3 of the MS induction medium. Embryo development and multiplication of androgenic embryos from anther culture proceeded on MS_1. After medium for multiplication embryos were cultured on media without hormone. Filter sterilized L-glutamine (Glu) was added 400 $mg.l^{-1}$ to improve embryo maturation in later stages of their development.

Results

The development of androgenic embryos was asynchronous, so that embryos at their globular, heart-shaped, torpedo-like and cotyledon stages were observed in the same culture, on MS_1 medium (Figs. 1-2). Androgenic embryos with 2 and 3 cotyledons of *A. flava* were cultured on solid MS hormone free medium for embryo germination. Secondary embryos appeared on the radiculi of androgenic embryos grown on the MS hormone-free medium. Some embryos formed only epicotyl (Fig. 3), with the absence roots on hormone-free medium. The largest number of green embryos per anther formed from segment C (6.58 ±

0.56), while between segments A (4.51 ± 0.50) and B (4.66 ± 0.51) hadn't significant difference. These results are in agreement with previous reports on the induction in anther culture of horse chestnut (Radojević *et al*., 2000). The goal of the work was mass production of haploid androgenic embryos from anther culture and induction of secondary somatic embryogenesis and plantlets formation of *Aesculus flava* in order to develop efficient *in vitro* regeneration methods to be used in genetic transformation experiments applicable in the pharmaceutical industry.

Discussion

Establishment of anther culture in woody plants is generally rather complicated. This is the first reported successful plant regeneration in anther culture of yellow buck. The described protocol for an efficient haploid induction in anther culture of *A. flava* can be used in genetic manipulation to secondary metabolism.

Acknowledgements

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Figure 1-2 Embryos in globular, heath-like, torpedo and cotyledonary stage on MS_1 medium

Figure 3 Embryos formed only epicotyl with the absence of roots on hormone-free medium

PLANT REGENERATION PATHWAY IN FLAX ANTHER CULTURE

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Introduction

Haploid plant production via culturing flax anthers with microspores at appropriate stage (Obert *et al*., 2004) belongs to the alternative biotechnological approaches. However, there are some problems in flax anther culture and therefore its further application in breeding programmes is constricted (Chen & Dribnenki, 2004). In this contribution we aim to characterize the pathway of plant regeneration and the origin of plants regenerated from microspore derived calli using isozymes.

Material and Methods

The anther culture was prepared as described previously (Obert *et al*., 2004). The anthers of oil flax genotype AC Emerson with microspores at the late uninucleate stage (Fig. 1) were at 8 ºC (7d.) and were cultured on induction media: liquid IMN6 and semisolid IMP2.3 (Bartošová & Preťová, 2003). The cultures were cultivated in the dark for 7 days and at the 16/8 hrs. (day/night) photoperiod for following 21 days. The induced calli of microspore origin were transferred to MS (Murashige and Skoog, 1962) medium containing 2,4-D (1 $mg.l^{-1}$) for 14 days and after that to regeneration medium according to Nichterlein (2003). Induced shoots were cultivated on rooting MS medium (2% sucrose). The ploidy level of regenerants was examined using squashes on root tip cells according to Tomašková (1974).

The true leaves of plants were collected to analyse the protein spectra of chosen enzymes, acid phosphatase - ACP and peroxidase - PRX. The tissue of leaves (0.1-0.5g) was frozen in liquid N_2, homogenised with the extraction buffer (0.25M TRIS; 0.05M EDTA; 5mM cystein-HCl; pH=6.8) of proper volume (250-500µl) and the mixture was centrifuged for 30 min. at 12 000 rpm (4 ºC). The content of proteins was analyzed in supernatant spectrophotometrically according to Bradford (1976) using ELISA reader (Elx800, Bio-Tek, Insruments, Inc.). Supernatant was stored at –20 ºC or used directly for electrophoresis. Enzymes, ACP and PRX, were separated using 10% native discontinuous polyacrylamide gel electrophoresis (Laemmli, 1970). For both gels 7 mA were used within 4 hrs. To detect ACP the gels were stained in 100 ml cold acetate buffer (pH=5.2) containing 0.03 g Fast Green GBC and 0.05 g 1-Naphthyl Phosphate for one hour. To visualise PRX the gels were stained in 100 ml acetate buffer (pH=4.6) supplemented with saturated Benzidine R. (0.2%) and 1.7 ml H_2O_2 (10%). Stained gels were kept in 7% acetic acid. The isozyme spectra from true leaves of 16 donor plants from field conditions and spectra of true leaves excised from 16 seedlings grown *in vitro* after 1 subcultivation on MS medium (2% sucrose) were used as control samples.

Results

After 4 weeks in culture the ratio of induced calli in anther culture (Fig. 2) of used genotype AC Emerson was 2.6%. Semiliquid medium IMP2.3 appeared more effective for callus induction in anther culture than the liquid IMN6 medium. The colour and also the quality of calli varied in terms of cultivation. At the beginning of culturing we observed friable light green calli, but later also dark green compact calli with granular surface were present. Differentiation of shoots from calli was observed after 6 months of subculturing. The frequency of regeneration rate was 27.3% and some microspore de-

rived plants (5%) were haploids (Fig. 3). The analysis of isozyme spectra in true leaves showed segregation of alleles in two loci of PRX and ACP that proved homozygozity of doubled haploid regenerants (Fig. 4). Expectedly somaclonal variation appeared in all regenerants. The epigenetic modifications occured in expression of PRX in all *in vitro* seedlings. The intravarietal variability was also detected among field plants in ACP (12.5%). The average number of alleles/locus increased (2-3) in *in vitro* seedlings (ACP, PRX) and regenerants (PRX).

Discussion

Several protocols for flax anther culture has already been published (Chen *et al*., 2002; Nichterlein, 2003; Rutkowska-Krause, 2003; Obert *et al*., 2004b). However, the indirect regeneration pathway from flax anther culture is preferentially organogenesis though the microspore origin of regenerated plants was proven (Chen *et al*., 1998).

In this article we described isozyme markers that identified the gametic origin of regenerants originated from anther culture. However the found polymorphism in enzyme expression between donor material and regenerants was low, because the starting material were stable breeding lines.

Acknowledgement

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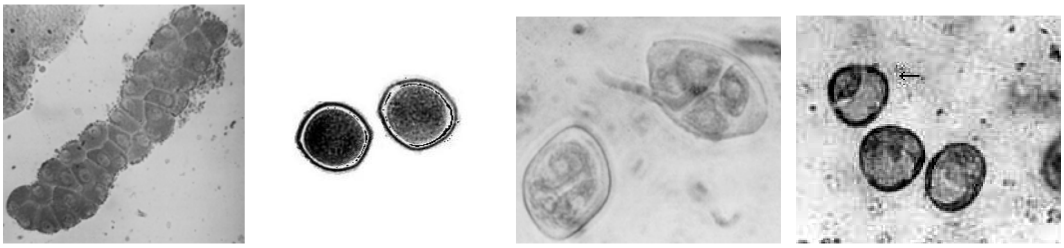
Figure 1 Stages of microspore development: microspore mother cells (a, b), tetrads (c), late uninucleate microspores (d)



(a) (b) (c) (d)

Figure 2 Responsive anthers in the culture

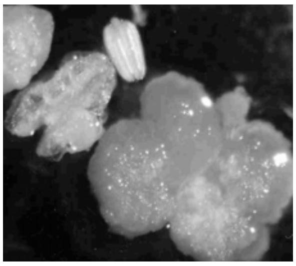


Figure 3 Haploid set of chromosomes in root tip cells

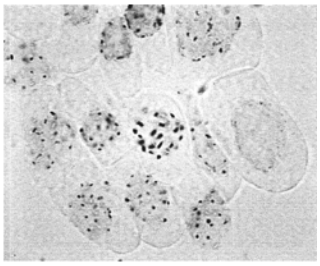
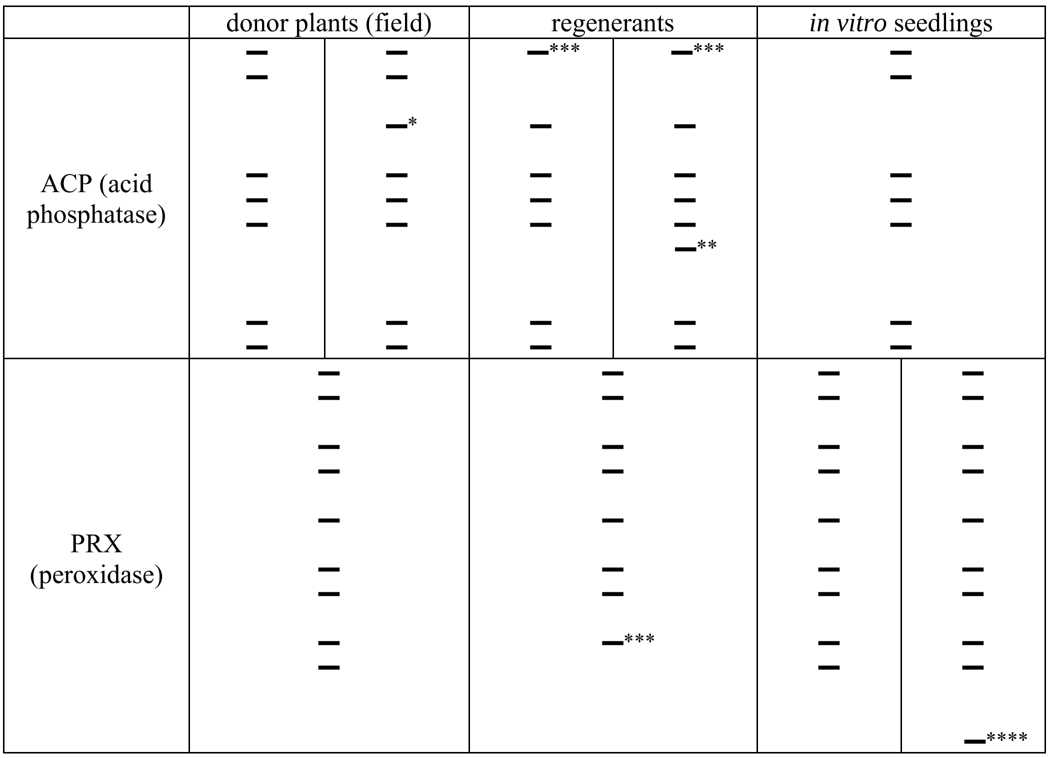


Figure 4 Isozyme spectra of AC Emerson



* intravarietal variability, ** somaclonal variability, *** homozygozity, **** new isozyme

SOMATIC EMBRYOGENESIS IN SOME HYBRID FIRS FROM IMMATURE ZYGOTIC EMBRYOS AFTER ARTIFFICIAL POLLINATION

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Introduction

Embryogenic cultures of hybrid firs have been derived from immature (*A. alba* x *A.alba*, *A. alba* x *A. nordmanniana*, Gajdošová *et al*., 1995; *A. alba* x *A. cephalonica*, *A. alba* x *A. numidica*, Salajová *et al.,* 1996; *A. cilicica* x *A. nordmanniana*, Vooková and Kormuťák, 2003) and mature (*A. alba* x *A.* cephalonica, Salaj and Salaj, 2003/2004) zygotic embryos. After maturation treatment a cotyledonary embryos developed and germinated in a small plantlets.

The present study aimes at initiation of the somatic embryogenesis from immature seeds of incompatible crossings of firs where zygotic embryos abort usually several weeks after pollination.

Material and Methods

Artificial pollination experiment was carried out in Arboretum Mlyňany, Slovakia, using one mother tree of *Abies concolor* Lindl. - Gord., *A. nordmanniana* (Stev.) Spach., *A. pinsapo* Boissier, *A. alba* Miller and one father tree *A. weitchii* Carr., *A. alba*, A. *pinsapo, A. concolor.* Female flowers of mother tree were isolated before opening of their scales using paper bags as isolators. Artificial pollination of female flowers were performed at stage of their maximal receptivity at the beginning of May using freshly collected pollen of father tree. The isolators were removed from female flowers after pollination.

The cones containing immature seeds were collected at regular intervals during the periode of Jun – August 1999. Immature seeds were surface-sterilized for 10 min in 10% H_2O_2. Endosperms containing embryos were plated on SH initiation medium (Schenk and Hildebrandt, 1972) with 5 or 10 µM benzylaminopurine (BAP) and 2% sucrose. The medium was solidified with 0.3% Phytagel. All media components were autoclaved at 121 °C for 20 min.The cultures were kept in the dark at 21-23 ^{0}C.

Maturation of somatic embryos

Modified MS (Murashige and Skoog, 1962) medium was used for maturation of somatic embryos. This medium contained 1/2 strength MS macro, original micro elements and FeEDTA, modified vitamins: 5.5 µM nicotic acid, 3 µM thiamine HCl, 4.9 µM pyridoxin HCl, 13.3 µM glycine, 0.01% *myo*-inositol, and 4% maltose, 10% PEG-4000, L-glutamine and casein hydrolysate in concentration 0.5%, (±) cis-trans- abscisic acid (ABA) in concentration 40 µM was co-autoclaved together with other substances of media The experiment consisted of 5 replicate plastic plates (∅ 60 mm) per cell line, each containing embryogenic tissue of approximately 300 mg.

Results and Discussion

The number of explants available at the date of collection was limited by the number of developing megagametophytes in a cone (Table 1). During early collection (16/6), only megagametophytes of *A. concolor* x *A. weitchii, A. concolor* x *A. alba* and *A. alba* x *A. weitchii* were developed. Later when embryo was not present any more in developing seeds and megagametophytes degenerated meanwhile, the seeds were too hard to remove their coats.

After 4-8 weeks of cultivation on medium with BAP, white embryogenic tissue, typical for conifer was induced from mikropylar ends of the megagametophyte. Induction was rather rare and occured with the frequencies of 0.64-1.6% in *A. nordmanniana* x *A.concolor,* 0.69-3.82 % in *A. nordmanniana* x *A. weitchii, 5*.55% in *A. concolor* x *A. weitchii,* 0.64-1.60% explants of *A. nordmanniana* x *A. concolor* as well as 1.23% in *A. pinsapo* x *A. weitchii*. The initiation of embryogenic tissue from other hybrid firs explants was not achieved at all (Table 1). It seems that higher concentration (10 μM) of BAP in induction medium was more effective.

Initiation of embryogenic tissue from seeds of compatibile crossing was higher. In *A. cilicica x A. nordmanniana* ranged from 3 to 27.6% (Vooková and Kormuťák, 2003). In hybrid firs, *Abies alba* x *Abies cephalonica* and *Abies alba* x *Abies numidica*, Salajová *et al*. (1996) obtained as high as 40% frequency rate of somatic embryogenesis induction. Similarly, a 38% induction frequency was also obtained in *Abies alba* x *Abies nordmanniana* hybrid fir, in A. alba x A. alba 24 % (Gajdošová *et al*., 1995).

Time of maturation treatment was 8-10 weeks depending on genotype (cell line) rather than on differences between species. Maturation up to cotyledonary stage of development was achieve only in *A. nordmanniana* x *A. weitchii* somatic embryos (Table 2). Development of somatic embryos was observed also in two cell lines of *A. concolor* x *A. weitchii* but they formed only globule shaped embryos. This cell lines represented group B with undeveloped somatic embryos (Mo *et al*., 1996). Probably their development could be stimulated by prematuration treatment or optimizing of maturation process. Plantlet regeneration was obtained by germination of mature desiccated embryos on ½ SH medium with 1% sucrose and 1% charcoal.

Further experiments are necessary to verify the hybrid nature of the embryogenic tissue using DNA markers.

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Table 1 Initiation (in %) of embryogenic tissue of hybrid firs from immature zygotic

embryos during Jun-August 1999. (NE) = number of explants

Hybrid	Jun 16	(NE)	Jun 28	(NE)	July 8	(NE)	July 16	(NE)	August 6	(NE)
A. concolor x *A. weitchii*	5.55	(54)	0	(9)	0	(14)	0	(10)	0	(16)
A. concolor x *A. alba*	0	(90)	0	(1)	0	(0)	0	(0)	0	(1)
A. concolor x *A. pinsapo*	-	-	0	(13)	0	(2)	0	(0)	0	(4)
A.nordmanniana x *A. weitchii*	-	-	3.82	(366)	1.24	(161)	0.69	(145)	-	-
A.nordmanniana x *A. concolor*	-	-	1.60	(250)	0.64	(156)	0	(135)	0	(3)
A. pinsapo x *A. weitchii*	-	-	-	-	1.23	(162)	0	(185)	0	(30)
A. pinsapo x *A. concolor*	-	-	-	-	0	(157)	0	(157)	0	(4)
A. alba x *A. weitchii*	0	(41)	-	-	-	-	-	-	-	-

Table 2 Response of embryogenic cell lines on maturation treatment
SE = somatic embryos

Hybrid	Number of induced cell lines	Number of cell lines forming cotyledonary SE (in %)	
A. concolor x *A. weitchii*	3	0	0
A.nordmanniana x *A. weitchii*	16	10	62
A. nordmanniana x *A. concolor*	4	0	0
A. pinsapo x *A. weitchii*	3	0	0

SOMATIC EMBRYOGENESIS OF CHESTNUT: APPLICATIONS TO GENETIC TRANSFORMATION AND CRYOPRESERVATION

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Introduction

Somatic embryogenesis is an important biotechnological tool that demonstrates significant benefits when applied to forest tree species; clonal propagation, cryostorage of valuable germoplasm and genetic transformation are among the most promising of its applications. One of the most important goals in chestnut is to increase resistance to fungal diseases by transferring genes such as quitinases or glucanases. However, an efficient plant regeneration system is necessary to achieve transformed plants. This report describes the induction of somatic embryogenesis from leaf explants of *C. sativa*, and evaluates the capacity of these embryogenic lines for use in cryopreservation and transformation experiments.

Material and Methods

Somatic embryogenic cultures were initiated when leaf explants excised from stock shoot multiplication cultures of *C. sativa* were cultured on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of NAA (5.37; 10.74; 20 μM) in combination with BA (2.22; 4.44; 8.87 μM). Leaf explants were then transferred to the same medium with 0.54 μM NAA and 0.44 μM BA for 4 weeks, and were subsequently transferred to PGR-free basal medium. Embryogenic cultures were maintained by secondary embryogenesis in proliferation medium (Corredoira *et al*., 2003).

For histological study, leaves that developed embryogenic cultures were fixed in formaldehyde : acetic acid:50% ethanol (1:1:18 v/v), dehydrated in an ethanol-n-butanol series, embedded in paraffin wax and sectioned at 8-10 μm. The sections were stained either with safranin-fast green or periodic acid-Shiff's (PAS) reaction in combination with naphthol blue black.

For transformation, somatic embryo clumps at globular-early cotyledonary stages initiated from leaf explants were co-cultured at different intervals with *Agrobacterium tumefaciens* strains EHA105pUbiGUSINT, EHA105p35SGUSINT and C58C1pBI121.

For cryopreservation, explants consisting of small clumps (6–8 mg) of two to three somatic embryos in globular or early torpedo stages were cryopreserved according to the vitrification procedure described by Mártinez *et al*. (2003).

Results and Discussion

Induction of embryogenic cultures

The use of leaf explants excised from shoot cultures to initiate the embryogenic systems offers advantages over the zygotic embryo tissues, as clonal material may be a suitable source of explants for inducing somatic embryogenesis from selected, mature genotypes. The best results were obtained when leaf explants were initially cultured with 5.37 μM NAA and 4.44 μM BA, with an induction frequency of 1%, a lower value than those obtained from zygotic embryos (Vieitez *et al*., 1990; Carraway and Merkle, 1997), which could be expected in a more differentiated tissue, such as that of leaves.

The anatomical study performed on cultured leaf explants showed that they yielded callus tissue comprising parenchymatic cells with vascular elements. Certain zones in the periphery of this callus exhibited a gradual disruption of tissue integrity, which gave

rise to a friable callus area formed by expanded parenchymatic cells and large intercellular spaces that took on a disaggregating appearance. Within this zone, clumps of small densely cytoplasmic cells were differentiated, having a large centrally positioned nucleous with prominent nucleoli, and accumulation of starch grains. These characteristics correspond to those displayed by embryogenic cells, whereas the occurrence of embryogenic cell clumps undergoing a series of divisions with a common thick cell wall indicates a probable unicellular origin. Only a small number of these cell clumps continued to develop nodular embryogenic masses, which emerged on the disgregating callus surface, and were generally formed of small vacuolated cells and zones of meristematic cells at the periphery; neither vascular elements nor starch grains were observed in these nodular masses. Somatic embryos at different developmental stages, including the cotyledonary stage, were differentiated from the meristematic areas of the nodular embryogenic masses, which were attached to the callus during initiation, but became detached at later stages of development. Embryogenic masses seem to be of unicellular origin, although somatic embryos later originated from these masses appear to be of either unicellular or multicellular origin.

Transformation of embryogenic cultures

The development of a reliable and reproducible genetic transformation protocol for European chestnut was obtained in this study. A transformation efficiency of 25% was recorded when somatic embryos at the globular to early torpedo stages were co-cultured for 4 days with *A. tumefaciens* strain EHA105 harbouring the pUbiGUSINT plasmid containing marker genes. Transformation was verified by a histochemical β-glucuronidase (GUS) assay, PCR and Southern blot analyses for the *uidA* (GUS) and *npt*II (neomycin phosphotransferase II) genes, and germination and plant recovery was achieved from transformed somatic embryos. The definition of a transformation protocol using marker genes opens up the possibility of applying biotechnological tools to the development of blight- and/or ink-resistant trees.

Cryopreservation of embryogenic cultures

Chestnut embryogenic cultures are generally maintained by repetitive embryogenesis. Cryopreservation may be a reliable alternative for facilitating the management of embryogenic lines and limit the risks of somaclonal variation and contamination, as well as to reduce labour and supply costs. In the present work, an embryogenesis resumption level of 68% was obtained by first preculturing 6-8 mg clumps of globular or heart-shaped somatic embryos on medium containing 0.3M sucrose for three days, followed by 60 min application of PVS2 vitrification solution (Sakai *et al*., 1990) before direct immersion in liquid nitrogen.

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IMPROVING SOMATIC EMBRYOGENESIS SYSTEMS IN MATURE *Quercus robur* TREES

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Introduction

Somatic embryogenesis has been shown to be a plant tissue culture method with potential applications for large-scale clonal propagation, genetic transformation, cryopreservation and use in studies of embryo development (Akhtar and Jain, 2000). The integration of this technology into oak improvement programs could be very useful, especially if the embryogenic system is initiated from mature trees. In a previous report we obtained somatic embryo induction and plant regeneration from leaf tissues of several hundred-year-old oak trees (Toribio *et al*., 2004). The resulting embryogenic lines being proliferated and maintained by repetitive embryogenesis which may result in a risk of somaclonal variation and contamination, as well as a great effort in labour and supply costs. Cryopreservation appears to be the safest and most cost-effective method for long-term conservation of these embryogenic lines. Moreover, the genetic stability of regenerants compared to the plant of origin is of great importance for the maintenance of selected genotypes.

Induction of somatic embryogenesis may be influenced by the physiological or developmental stage of the donor tree from which the initial explants are taken (Bonga, 2004). In the present study, we investigate whether plant collections from the source tree, made at different periods throughout the year, may influence the embryogenic capacity for a specific oak genotype. In addition, we evaluate the ability of six embryogenic lines (originated from six mature oak trees) to withstand cryostorage in liquid nitrogen (LN), and the genetic stability of these lines using RAPD markers.

Material and Methods

Branch segments (approximately 25 cm long and 1-5 cm in diameter) from a specific oak tree (CR-0) growing in a selected stand were collected in February, March, May and November, and forced to flush in a growth chamber. The newly sprouting epicormic shoots were used as source of expanding leaves (1.0-1.5 cm) which were collected, sterilized and cultured following a multistage treatment procedure (Toribio *et al.*, 2004). Flushing capacity of branch segments and somatic embryogenesis induction in explants from different collection dates were evaluated.

Six embryogenic lines originated from leaf explants of different mature trees were used for the cryopreservation experiments. These lines are maintained by secondary embryogenesis in proliferation medium (Sánchez *et al*., 2003). Harvested 4-6 mg clumps of globular heart-stage somatic embryos were cryopreserved using the vitrification procedure described by Martínez *et al*., (2003), with the application of PVS2 vitrification solution (Sakai *et al*., 1990) for 60-90 min prior to rapid plunging in LN. Recovery was assessed in terms of percentage of clumps that showed resumption of embryogenesis 8 weeks after thawing.

Genomic DNA was extracted from individual somatic embryos of 13 embryogenic lines derived from different leaves of three oak genotypes and from leaf tissues of the same oak trees, using the DNeasy Plant Mini Kit (Quiagen). Amplifications were carried out in a Hybaid Omnigene Thermocycler using 25 ng of genomic DNA and 40 arbitrary 10-

base primers included in the Kits A and S from Operon Technologies Inc. The PCR conditions were as previously described by Sánchez *et al*. (2003). All amplifications were repeated two o three times to assess reproductibility, and only bands appearing in at least two replicates were deemed valid.

Results and Discussion

After 8-10 days in the growth chamber, high flushing rates (79-93%) occurred in branch segments of all collections. Flushing capacity, expressed in terms of flushing frequency and shoot number produced per segment, was related to branch diameter, with higher values being obtained in 3-4 cm diameter branch segments (Table 1). This seems to be a suitable system for providing expanding leaf explants at different times of the year regardless of weather collection is made out of the dormant season or not. Cultured leaf explants initially responded with callus formation which ranged between 40% (material collected in November) and 88% (May collection). Even though somatic embryogenesis induction rates were low, somatic embryos were initiated in leaf explants from all collections, with the highest value achieved in November material (Table 2). Somatic embryos always originated from callus tissue previously formed on the explants, but the callus formation frequency was not related to the embryogenic capacity, as the best embryo induction rate (4.1%) was achieved in material exhibiting the lowest callus formation (November collection).

Embryo recovery levels in excess of 50% were achieved for the six embryogenic lines following vitrification and cryopreservation, with values ranging between 57% (line B-18) and 92% (line B-13). The PVS2 treatment affording the highest embryogenesis levels among cryopreserved embryos was 60 min for four lines, and 90 min for the two other lines, although no significant differences were obtained between the two PVS2 application periods.

RAPD analyses were employed to evaluate the possible somaclonal variation among different somatic embryogenic lines originated from different leaves of the same tree. RAPD profiles from somatic embryos were also compared with those obtained from their respective mother plant. Initially, there were no signs of polymorphism among somatic embryos of the same line, and among lines of the same genotype. Similarly, the analysis showed the absence of polymorphism between the embryogenic lines and the leaves of the donor trees from which the somatic embryos were originated. These results suggest that this embryogenic system did not induce changes in the DNA sequence.

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Table 1 Flushing capacity of branch segments (25 cm in length) taken from a mature Quercus robur tree on different collection dates. Branch segments, ranging from 0.5 to > 4 cm in diameter, were placed in a growth chamber to force flushing of epicornic shoots. %: percentage of branch segments producing shoots. N: flushed shoot number per branch segment.

Collection date	Branch segment diameter									
	0-1 cm		1-2 cm		2-3 cm		3-4 cm		>4 cm	
	%	N	%	N	%	N	%	N	%	N
February	100	3.5±0.6	80	8.5±3.1	100	13.2±6.1	100	24.0±12.6	-	-
March	67	2.5±1.2	100	6.1±0.8	100	23.8±3.1	100	43.0± 0	-	-
May	40	2.0±0.6	81.6	4.3±0.4	100	12.4±1.8	100	23.8±1.4	100	28.0±2.6
November	73.3	2.6±0.6	89.5	7.6±1.4	100	11.3±2.6	-	-	100	7.0±0

Table 2 Callus formation and somatic embryogenesis percentages from cultured leaf explants excised from newly sprouting epicornic shoots produced in branch segments taken on different collection dates.

Collection date	Callus %	No. of embryogenic leaf explants/total explanted leaves	Somatic embryogenesis %
February	50.2	10/648	1.5
March	54.4	7/557	1.3
May	69.4	4/551	0.73
November	39.6	25/618	4.1

ANALYSIS OF PROTEINS FROM EMBRYOGENIC AND NON-EMBRYOGENIC CELL LINES OF *CYCLAMEN PERSICUM* MILL.

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Introduction

Cyclamen (*Cyclamen persicum* Mill.) is an important pot plant and bedding plant in southern and western Europe. The normal propagation of Cyclamen is currently through F_1-hybrid seeds, which are relatively expensive. Vegetative propagation has so far been performed on a small scale, as there have been many obstacles. Somatic embryogenesis in vitro offers a possibility for vegetative propagation of *Cyclamen persicum* Mill. Plant tissue cultures are in addition useful for studying mechanisms of plant development. In particular, cellular and molecular analyses of somatic embryogenesis can provide information about the earliest stages of plant development. Differences in protein syntesis pattern have been observed between embryogenic and non-embryogenic calluses obtained from cultures of different species. Presently, there is no information describing biochemical changes during Cyclamen somatic embryogenesis.

This study was initiated with the long-term objective of learning more about the factors regulating gene expression in embryogenic (E) and non-embryogenic (NE) growth in Cyclamen. The different appearance and regeneration potential of E and NE calli implies that there are differences in gene expression between the two callus lines. The specific objective of this study was to determine if there were differences in the proteins present in E and NE Cyclamen cultures, that might be used as molecular markers, indicating altered states of gene expression and embryogenesis.

Material and methods

Embryogenic (E) and non-embryogenic (NE) cell lines of *Cyclamen* were obtained as callus cultures *in vitro* from Institute for Vegetable and Ornamental Crops, Kuehnhausen, Germany. The cultures were initiated in March 1991 starting from unpollinated ovules of flower buds (about 2.5 cm in length) from one individual plant of *Cyclamen persicum* 'Giganteum' Mill. cv. 'Purple Flamed' (genotype 3738), as described by Schwenkel & Winkelmann (1998).

The inoculum, of size fraction 100-1000 µm, was separated into liquid growth medium, with and without auxin (2 mg l^{-1} 2,4-D). The media used were based on MS medium (Murashige and Skoog 1962) with the modifications described by Schwenkel and Winkelmann (1998). The cultures were grown at 24°C in the dark. Cell samples were withdrawn every day for one week. Proteins from three cell preparations (loosely attached to the cell wall, bound to or part of the wall, or total protein from washed cells) were separated by 1-D SDS-PAGE (Laemmli 1970, BioRad minigel system). Protein patterns were compared to find changes associated with entry into the embryogenic state. 2-D electrophoresis (Multiphor II, Amersham Biosciences) were used to characterize the two cell lines.

Results

A visual difference in the callus and suspension cultures is that the embryogenic (E) cell line contains high levels of phenolics, which gives them a brownish appearance, whilst the non-embryogenic (NE) cultures are yellow and show no signs of phenolics (Figure 1). When the surface of the callus cultures were examined at high magnification under scanning electron microscope (JEOL JSM-5310, 1.3kV), no differences in cell shape were seen.

SDS-PAGE gave in general a lot of protein-bands. Isolation and protein characterization of separate bands was impossible. However, samples from cell walls gave interesting bands in the NE culture around 22, 35, 40 and 50 kDa (results not shown). These bands did not appear in the E culture. No clear differences between induced and non-induced cells could be seen. 2-D electrophoresis indicated many differences between the two lines E and NE (Figure 2).

Discussion

Many authors have described the meristematic, organised surface of embryogenic tissue versus the elongated, unorganized non-embryogenic tissue. It is sayd that this seems to be a universal feature, whether the plants are dicots or monocots, herbaceous or woody (Hvoslef-Eide and Corke 1997). In the case of the herbaceous dicot *Cyclamen persicum* Mill., this physical appearance do not appear. Anyway, some regions of the E cell line are more meristematic in appearance. The visible colour difference between the cell lines, is genetically determined. Cyclamen cultures were grown in darkness, and therefore the light did not have any influence on the colour difference.

The cell lines show differences at a biochemical level, in addition to their physical appearance, as suggested. Interesting proteins about 30 and 50 kDa have been observed by others as well (Hvoslef-Eide and Corke 1997). The reason why inductive and non-inductive conditions did not give different protein patterns after SDS-PAGE, is probably the closeness of bands. 2-D electrophoresis of the same samples will be performed to separate the proteins at a higher level. 2-D electrophoresis was used to characterize the cell lines, and the interesting spots will as soon as possible be analysed by MALDI-ToF and Q-ToF.

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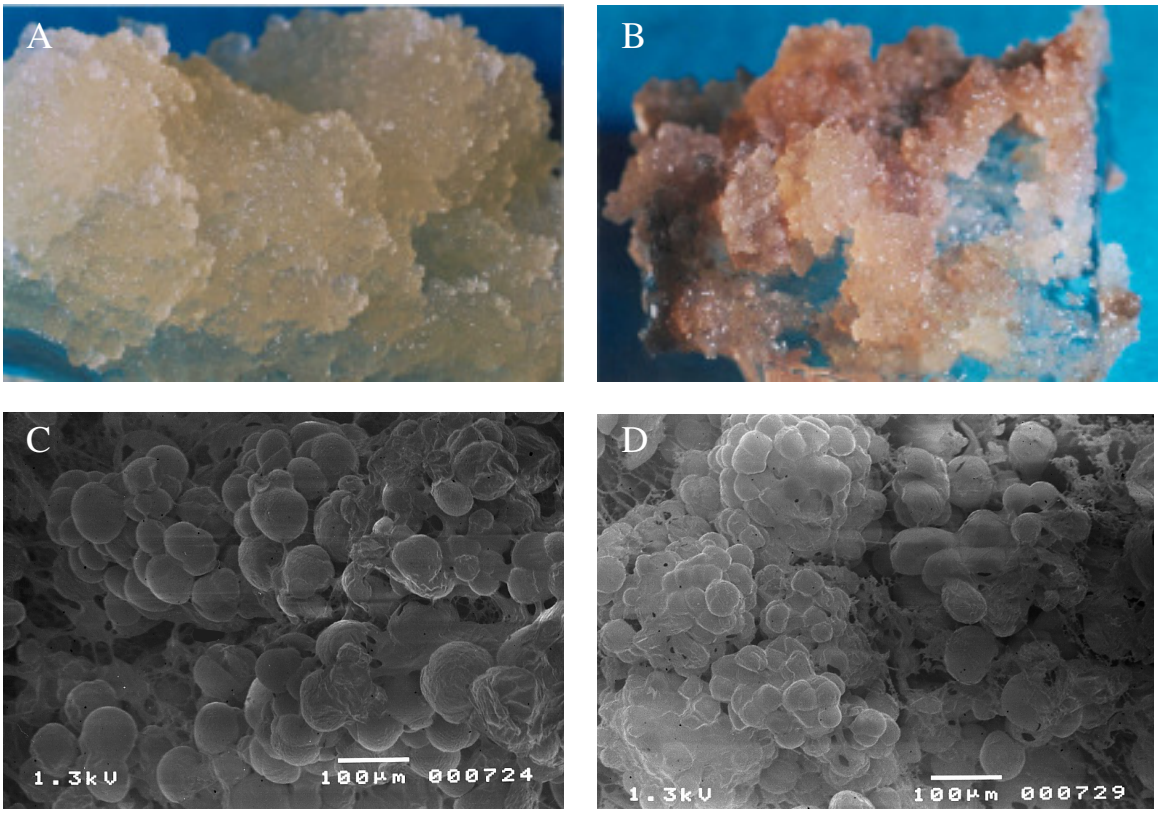

Figure 1
Callus of an non-embryogenic (NE) (A, C) and embryogenic (E) (B, D) cell line, cultured in darkness for 4 weeks on 2,4-D-containing MS-medium. The calluses were harvested fresh from the medium, without any preparation before scanning microscopy (C, D = 150x).

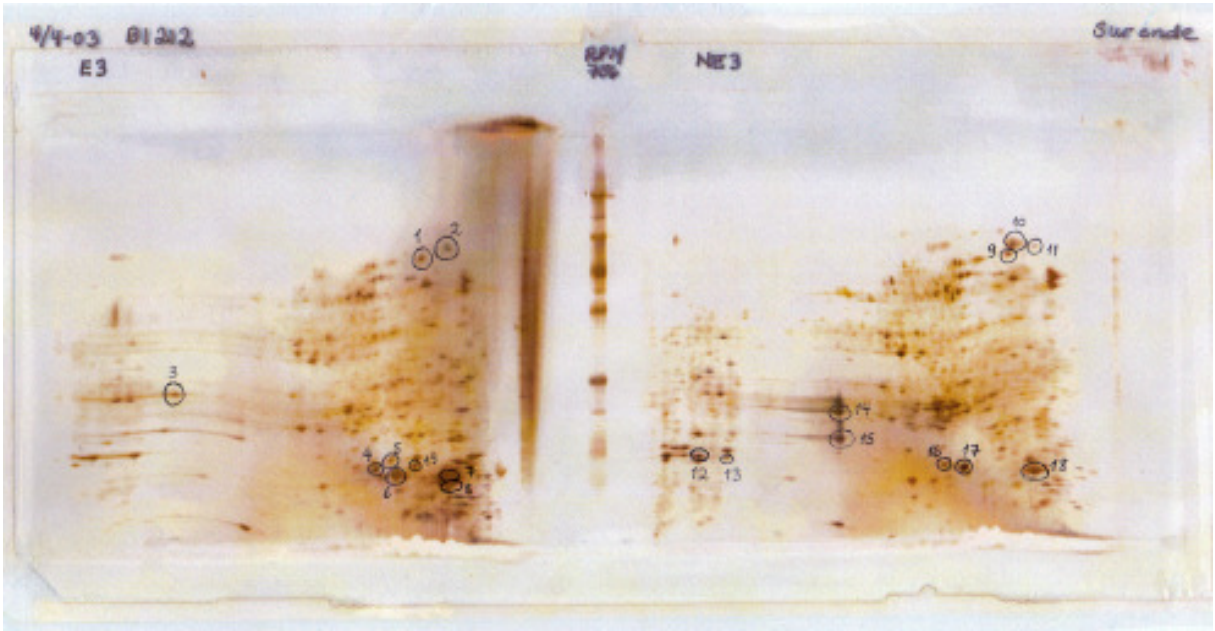

Figure 2
Proteins from embryogenic (E) and non-embryogenic (NE) Cyclamen cultures separated by 2-D electrophoresis. Circles 1-18 represent interesting spots. Marker is RPN756 (Amersham Biosciences).

SESSION III:
ASSESSMENT OF PERFORMANCE: PHYSIOLOGICAL HEALTH AND (EPI-)GENETIC STABILITY

ARE THERE ADEQUATE METHODS FOR ASSESSING SOMACLONAL VARIATION IN TISSUE CULTURE-PROPAGATED PLANTS?

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Tissue culture may lead to abnormal plants. The changes occur especially in plants produced through adventitious regeneration; multiplication from existing meristems appears to be relatively 'safe'. The frequency of changes depends on the species (and the genotype), the tissue from which the adventitious plantlets have been regenerated, and the medium composition.
Two classes of abnormalities may be distinguished: genetic changes and epigenetic changes. Genetic changes include polyploidy, aneuploidy, (point) mutations, and new insertions of (retro)transposons. Genetic changes behave as Mendelian traits in crosses. Epigenetic changes do not involve changes of the primary DNA sequence, but are the result of alterations in DNA methylation, of changes in histone modifications, or a combination of these epigenetic mechanisms that modify gene expression. They are in theory temporary (plants 'revert' to normal phenotype), but are sometimes nevertheless taken over into the progeny as well. Typical for epigenetic changes due to adventitious regeneration, next to reversal, is that the same change often occurs at a higher frequency (whereas genetic changes occur, in principle, at random).
De Klerk (1990) and Vazquez (2001) noted that the distinction between genetic and epigenetic changes is an oversimplification. Molecular evidence is now accumulating showing that epigenetic changes may lead to many genetic abnormalities (aneuploidy, new transposon insertions). A human example: the RB1 protein recruits chromatin-modifying enzymes to specific promoters. Loss of RB1 leads to global loss of a repressive chromatin state, but also to loss of DNA methylation, and to aneuploidy and tetraploidy in cell lines (Gonzalo *et al.*, 2005).
Here, I focus on the possibilities of detection of changes at the molecular level. First, I will infer some hypotheses about the changes and formulate when changes can be considered to have been detected. Then I will discuss the power of various molecular methods that are being used to measure variation. Finally, I will indicate what type of measurements may be used in the future.

Characteristics of epigenetic changes

Epigenetic mechanisms include DNA methylation and histone modifications. These two systems interact, and probably reinforce one another. In this way, the possibilities for transcription can be enhanced (acetylation of the lysine on position 9 of histone 3 [H3K9]) or strongly decreased (methylation of cytosines in the DNA, methylation of H3K9). This regulatory system has several functions. On the one hand, it appears to form a defence mechanism against the activity of retrotransposons; it also contributes to sustain the centromere through the centromeric repeats. Silencing of repeats is thought to occur in all cells, regardless of their location or developmental state. It is a step in the formation of heterochromatin. On the other hand, the epigenetic mechanisms regulate gene expression in a developmental way: genes may be methylated and switched off in one tissue, but free of methylation and active in other tissues. The clearest examples of this type of epigenetic regulation are from mammalian embryogenesis: imprinting (activity of a gene in early embryo development depends on whether it is inherited from the father or the mother), X-inactivation (to achieve the proper gene dosage, of the two X chromosomes in females is heavily methylated and transcription is switched off), and reprogramming (of genome-wide DNA methylation patterns in the gastrula stage). Imprinting has also been found to occur during plant endosperm development.
During plant development, the epigenetic 'imprints' lead to a transient 'memory' of the developmental program and influence the cell's reactions and its future development. The imprints may also block certain alternative developmental pathways. Adventitious regeneration uses the ability of plants to redirect development, and must begin with erasing at least part of these marks, to regain totipotency. This process is called dedifferentiation. One hypothesis that is consistent with this notion, is that differences in regeneration capacity are the result of diffe-

rences in how fast and how easily epigenetic marks can be erased or reprogrammed across species or across genotypes within a species.
It has been hypothesised that cell type, developmental age, and physical age may affect the speed of reprogramming, the resistance to reprogramming, and the accuracy of the process. In this view, epigenetic changes in adventitiously regenerated plants are due to incorrect, perhaps incomplete, resetting of the epigenetic developmental program – much like what causes abnormalities in cloned mammals. Unfortunately, the dedifferentiation process is not understood, and although there is some idea about what may induce dedifferentiation, epigenetic changes during dedifferentiation in plants have not been the subject of [intense] study, e.g. by looking at the expressions of genes typical for the donor tissue or of the new developmental stages.

Present methods for assessment

Current molecular marker methods employed (such as RFLPs, RAPDs, AFLPs, ISSRs) have originally been developed for the purpose of finding genetic differences between genotypes – which are plenty. In maize it was even found that whole genes may be missing from one plant to another. Microsatellite markers make use of the very fact that the number of repeats mutates much more than an average stretch of DNA, thus increasing the chance of finding multiple variants within a population. Statistics are quite different, however, if differences have to be found between two nearly identical individuals, i.e. individuals originating from a single mother plant via vegetative propagation. It is well known, e.g. for identification purposes, that all known mutants of a variety have the same fingerprint, regardless of the molecular marker method applied. When plants are treated with EMS or a mutating chemical, lots of mutations arise in the DNA, but even these are difficult to detect with molecular marker methods (Bouman and De Klerk, 2001, and see also below).
Therefore, a major issue is to obtain sufficient sensitivity to find the changes that occur. Changes can be identified either by obtaining a level of detection above the background level of a given technique, and by ascertaining that changes are real by independent controls. The error frequency of molecular markers methods is at least 0.8% (in AFLPs, as determined by comparison of duplicates or clones; error rate in the other methods mentioned is probably the same or higher), which means that if a single difference is found, it should be repeated or replicated before one can be sure it is real – but unique changes are difficult to reproduce properly. As a consequence, obtaining a few changes in many gels of many plants does not produce compelling evidence.
RFLPs and AFLPs can also be used to find DNA methylation differences, by using restriction enzymes that are sensitive to DNA methylation. Unfortunately, the statistics against detection of changes remain the same, even if we use optimistic assumptions. This becomes clear when we use Arabidopsis as an example. Arabidopsis has a small genome of 125 Mb (haploid size). Any AFLP band can be eliminated by a change in the restriction site plus the selective bases, maximally 16 bases per band, plus any insertion or deletion, but these are far less frequent. Ignoring insertions and deletions, but with an optimistic number of, say, 250 bands per primer pair, 4 kb would be sampled per primer combination. A very intensive analysis would perhaps use 100 primer combinations. Assuming no overlap between bands, that means that in total 4.10^5 bases could be tested, or 0.32% of the genome. For tomato (almost one billion (10^9) nucleotides) it would be less than 0.1%, for wheat (16 billion) less than 0.01%. As a consequence, we cannot expect to see much, if any, variation between regenerated plants with such molecular marker methods, even if they demonstrate clear morphological changes. Worse, the fact that we do not find any change in the portion of the genome that is assessed, does not mean that the other 99.68% (or 99.9% or 99.99%) of the genome is unchanged as well. Thus, the absence of differences in marker profiles tells us nothing conclusive!
This is especially true since the effect of genetic and epigenetic changes on the physiology and morphology of a plant is far from linear: many changes may have no effect at all, while one change that affects the function or expression of one gene, may have a profound effect. This is one of the reasons why variation is easily detectable in the phenotype and not at the DNA level. Two other reasons are: the phenotype integrates the effect of the expression of thousands of

genes; and comparing many identical plants under identical conditions greatly enhances our ability to visually detect off-types in morphology and development. Thus, at present tests at the phenotypic level are still more conclusive than tests at the DNA level.

Prospects

Alternative strategies for detection of mutations have been developed to enable the genome-wide production or selection of targeted mutations. Tilling (McCallum *et al*., 2000), for instance, in which two genomes are cut and hybridised to each other, identifies the base pair mismatches that are the results of mutations in one of the genomes by denaturing HPLC or by the mismatch cleavage endonuclease CEL I (Till *et al*., 2003). Using this technique to detect mutations in natural populations was termed ecotilling (Comai *et al*., 2004). In this way, rare differences can be visualised. Finding a particular mutation, however, requires an elaborate scheme of pools of thousands of plants; a similar study aimed at finding mutations induced by tissue culture would probably require the screening of thousands of regenerants, too.

Measurement of overall DNA methylation or methylation in arbitrary sequences will suffer from the fact that many changes are random, at least in any particular plant. As a consequence, even if changes are found, they will not correlate with morphological or physiological abnormalities in particular regenerants. This has indeed been found repeatedly (for instance, Jaligot *et al*., 2002). An alternative is to attempt to correlate the degree of changes to the degree of abnormalities, but for this one would have to measure a large number of sites in the genome.

The repeated occurrence of changes may help since (i) these may be more easily found, and (ii) the correlation of observed changes in DNA and histone modifications with the phenotypic changes can be assessed. In cancer research, this appears to work very well (aberrantly methylated CpG islands in the genome serve as biomarker for several types of cancer, Esteller and Herman, 2002). However, the progression of a cancer goes through several specific stages, including an initial step of massive DNA demethylation, and changes induced by adventitious regeneration are not so predictable. An alternative approach is to make use of epi-alleles (Kakutani, 2002) with an effect on morphology as a model system in which repeated changes are easily observed and studied.

The methods to be used should be able to assess epigenetic status, either DNA methylation or histone modifications. This field of science is quickly developing (Fraga and Esteller, 2002). For DNA methylation detection, various methods and protocols exist, mainly using bisulfite treatment of DNA changes, which changes cytosines but not methylated cytosines into uridines, and/or methylation-sensitive restriction enzymes; e.g. Xiong and Laird (1997), Gonzalgo and Jones (1997), etc. Some use antibodies against 5-methylcytosine (Barton *et al.*, 2001). Histone modifications are studied using chromatin immunoprecipitation (ChIP) of associated DNA followed by PCR or cDNA microarray hybridisation (Shi *et al*., 2003; Roh *et al.*, 2004; Matarazzo *et al.*, 2004), and many sites can be probed simultaneously.

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THE USE OF NATURAL VARIATION IN *Arabidopsis* FOR FUNCTIONAL ANALYSIS

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The functional analysis of *Arabidopsis* genes has been largely based on the phenotypic characterisation of mutants selected by forward and reverse genetics in a very limited number of laboratory 'wild-type' genotypes. As an alternative to induced mutants, an important source of genetic variation can be found among naturally occurring populations of *Arabidopsis* called. Considerable variation has been found for potentially adaptive traits such as resistance (measured as plant survival or damage) to biotic and abiotic stresses, physiological developmental and biochemical traits. The study of natural variation requires quantitative trait loci (QTL) analysis. For efficient QTL analysis sets of recombinant inbred lines have been developed, which show segregation for a large number of traits including those important in crop plants. The variation and genetic analysis of traits such as sugar, starch, phytate and mineral accumulation, plant growth, enzyme activities, seed dormancy and seed quality has been analysed. The feasibility to clone individual QTL by a combination of map based cloning, using near isogenic introgression lines has been demonstrated by the isolation of a gene conferring daylength insensitivity, which appears to encode a cryptochrome 2 protein with increases protein stability. In case of seed dormancy the combination of fine mapping of a single QTL and a mutant approaches allowed the isolation of gene controlling seed dormancy. QTL variation for enzyme activities often co-locates with structural genes but also potential regulatory loci have been found.

SOMACLONAL VARIATION RELATED TO METHYLATION PROFILES

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Introduction

This study is a further investigation of the somaclonal variation phenomenon. The cytometric investigation of plantlets regenerated from potato internodes revealed that all the phenotypically non conform plants had in their nuclei abnormal contents of DNA (1). Moreover a few apparently normal looking regenerants presented such nuclei with aberrant DNA content. Most plantlets had cells with a higher content in DNA than the normal tetraploid cell. In fact most of them were aneuploid.

The phenotypical differences between the plantlets were numerous: dark green, light green, reddish, smaller, more hairy, rounder or crinkled leaves, stems with bluish flavonoids instead of purple, histological modification of the shape of the epidermis cells and the stomata, accumulation of secondary metabolites (2). No relation could be established between the type of cytometric profile and the type of observed phenotypic abnormalities. But apparently, there is a relationship between the aberrant DNA content and the expression of the genes controlling the plant metabolism.

From these observations a question has arisen. What was the mechanism modulating the gene expression in somaclonal variants? Were there some differences in cytosine methylation pattern?

This study was undertaken in view to examine the methylation status of the regenerated plantlets.

Materials and Methods

Internode segments from *in vitro* plantlets of the potato variety Bintje were used to regenerate shoots. The genomic DNA from 100 mg of the leaves of each regenerated plantlet was extracted using the Qiagen DNeasy plant mini kit. The DNA from each plantlets was digested using, in a first reaction, the restriction enzymes EcoRI and HpaII and simultaneously, in another digestion, EcoRI and MspI, following the method developed by Mingliang *et al.* (3). The isoschizomers HpaII and MspI have different sensitivity to cytosine methylation. Appropriate ligation fragments were attached to the restriction fragments. A classical AFLP's preamplification and amplification has been performed as described by Vos *et al.* (1995). The EcoRI primer was labelled with IRD-700.

Results and Discussion

The DNA methylation profiles revealed plenty of different bands for the HpaII restriction fragments and the MspI restriction fragments. This fact indicates that many restriction sites harbour methylated cytosines. But the profiles reveal very few differences between the plantlets, despite their great variety of phenotypes. These different bands appear at very precise sites. When different bands appear, they are all located at the same sites, thus indicating the same loci for cytosine methylation. This indicates that the modification in the cytosine methylation is not random but only a few precise loci are concerned. For these differencially methylated cytosine loci, all the present alleles are concerned (4 for the tetraploids, more for the hyperploids). But for a few plantlets some new restriction fragments appear for both of the restriction enzymes HpaII and MspI. These means that a new restriction site appeared, that some sequences have been modi-

fied. These bands are weaker than those indicating differential methylation. It is presumably because only one of the multiple allele is concerned.

Conclusion

The conclusion which was drawn from these facts is that abnormal DNA content of the cells nuclei are just one of the phenomena. There are other modifications in the genome of somaclonal variants:

- epigenetic modification with differential cytosine methylation, therefore activation or inactivation of gene expression
- mutation in the nucleotide sequences with apparition of new restriction sites.

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TRANSITION FROM THE *IN-VITRO* TO THE *EX-VITRO* ENVIRONMENT; STRESS AND PERFORMANCE

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Introduction

Transfer of tissue-cultured plants to *ex vitro* conditions is relatively little-researched. In particular, the effect of tissue culture conditions on performance after planting has hardly been studied. This presentation deals with this topic.

Plants cultured *in vitro* have specific features because of the *in-vitro* conditions. A major negative feature for performance after explanting is the poor water retention capacity (WRC) of the leaves. This is caused by malfunctioning stomata and not so much by a poor cuticle (Santamaria and Kerstiens, 1994; De Klerk and Wijnhoven, 2005). This shortcoming has been dealt with in various ways. (1) Usually, after transplanting the microcuttings are first kept at high humidity and slowly adapted to ambient humidity during ca. 10 days. After weaning, the WRC has improved but is still far less than the WRC of leaves that have developed *ex vitro* (De Klerk, 2000). (2) To facilitate replenishment of water excessively lost because of the poor stomata, microcuttings are often allowed to root *in vitro*. (3) It has also been attempted to reduce humidity during tissue culture so that weaning occurs to some extent *in vitro*. At the same time, *ex vitro* rooting is desirable as it involves less labour. We have studied the functioning of *in-vitro* produced roots of apple microcuttings and performance of apple microcuttings after *in-vitro* and *ex-vitro* rooting.

Another negative feature of microplants is related to the accumulation of ethylene in the headspace. It is well-known that auxin induces ethylene synthesis (Imaseki, 1999), thus in particular during *in-vitro* rooting this senescence-inducing hormone may accumulate. We studied the effect of removal of ethylene by $KMnO_4$.

When the proper precautions are taken, the transition from *in vitro* to *ex vitro* still involves a high level of stress (Debergh, 1991). Plants protect themselves from stress among others by the accumulation of protective compounds. This has been reported in many papers (Smirnoff, 1998). Such compounds sprayed over plants or 'applied' by transgenic incorporation of biosynthetic genes result in increased resistance towards various types of stress. In tissue culture, plants may be prepared for the initially very stressful period after exflasking by application of naturally-occurring protective compounds during the final period *in vitro*.

Materials and methods

The experiments were performed with *Malus* 'Jork 9', and with *Rosa hybrida* 'Madelon'. The procedures for propagation, rooting and culture *ex vitro* have been described before (De Klerk *et al.*, 1995; De Klerk, 2000; Van Telgen *et al.*, 1992).

Results and Discussion

Poor water retention capacity (WRC) and the need for in vitro rooting

Leaves from tissue culture plants have poor functioning stomata. Water lost because of this may be replenished more easily when shoots with a root system are transplanted. Therefore, microcuttings are usually rooted *in vitro* before transplanting. It has been suggested that these roots do not function. It is difficult to show that they function properly, but we observed that the roots resume growth directly after planting (Fig. 1). In a

more detailed study, it was shown that growth recommenced during the first 24 h after transplanting and that the growth rate of the roots (more than 0.5 cm per day) remained constant during the first week. In addition, *in vitro* rooted shoots perform far better than ex vitro rooted ones (Fig. 2). In the experiment shown in Figure 2 various durations of *in vitro* rooting and various periods of culture in the glasshouse are being compared. Interestingly, best growth was obtained after the longest rooting period *in vitro*. (Compare the 10 weeks after propagation values as this value includes the 4 weeks *in vitro* rooting + 6 weeks in the glasshouse).

These data suggest that *in vitro* produced roots are vital and relevant for performance. It is unlikely though that the section of the root that has developed in tissue culture is essential for water uptake as the root hairs have no optimal contact with the soil. It is more likely that the increased performance is related to the section that has developed in soil after transplanting. As noted above, a root growth more than 0.5 cm per day. When the shoots are rooted *ex vitro*, the new roots emerge from the stems after ca. 1 week (De Klerk *et al*., 1995) and start to function only after that. Thus, during the first week water uptake is though the cut surface of the stem only.

Detrimental effect of the gases in the headspace of tissue culture containers

The gaseous atmosphere in tissue culture is only incidentally examined. It is characterized by high humidity (which is the major or only cause of the poor WRC), low CO_2 during the light period and low O_2 during the dark period. Also organic gases may accumulate. One of these gases is ethylene which is considered to be the senescence-inducing plant growth regulator. Organic gases including ethylene may be removed by porous grains coated with $KMnO_4$. We observed strong effects in rose and apple but only slight effects in gerbera. Data with rose are shown in Figure 3.

Protectants

Tissue culture enables easy application of protective compounds so that they may accumulate within the tissues prior to exflasking. We have studied addition of putrescine, proline and betaine. Each was supplied at a range of concentrations. The optimal concentration for each is shown in Figure 4. Putrescine gave the best results

Conclusions

The conditions during tissue culture may have a very large effect on performance after transplanting. Increased growth in the glasshouse may be obtained by minimising negative features (such as senescence induced by ethylene) and by preparing the shoots for growth in the glasshouse allowing them to form a root system *in vitro* and allowing them to accumulate high levels of protective compounds *in vitro*.

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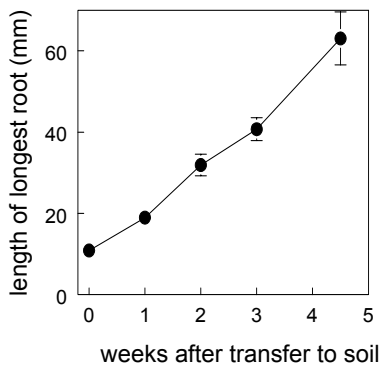

Figure 1 Length of the longest root after transfer to soil of apple microcuttings rooted under standard conditions.

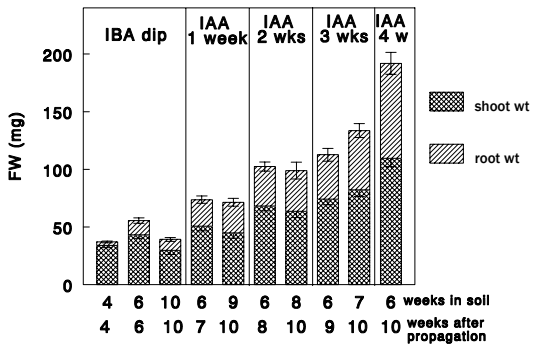

Figure 2 Fresh weight of apple shoots after 6 weeks in soil. The shoots had been rooted *ex vitro* (after a 1h dip in an optimal KIBA concentration) or *in vitro* (for 1, 2, 3 or 4 weeks under standard conditions). FW was determined after various periods in soil. Both the period in soil, and the period of the rooting treatment + period of growth in soil (= weeks after propagation) are shown.

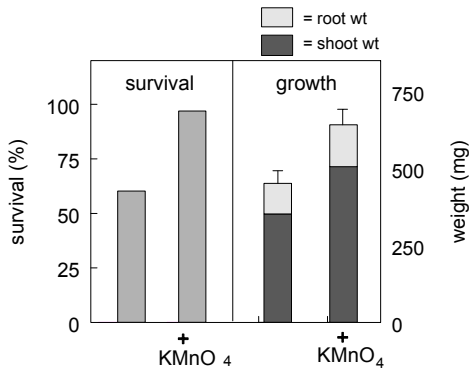

Figure 3 Survival and growth of surviving microcuttings of rose rooted *in vitro* with 10 µM IAA. Ethylene was allowed to accumulate in the headspace or removed by grains coated with $KMnO_4$. The grains were in a small lid on top of the medium.

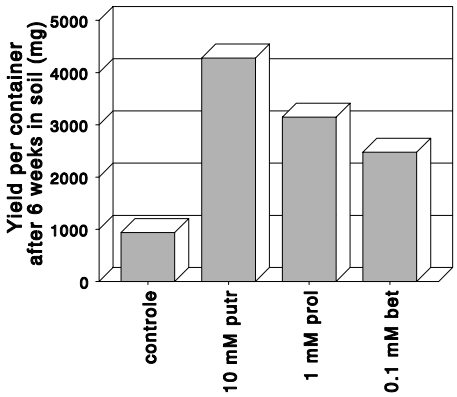

Figure 4 Yield per container (= 10 rose microcuttings) after rooting *in vitro* in the presence of $KMnO_4$. During the rooting treatment a range of concentrations of putrescine, proline and betaine were given. In the graph, the optimal concentrations are shown.

RETROELEMENTS STABILITY AND METHYLATION LEVEL ALTERATION IN LONG TERM *IN VITRO* SHOOT CULTURE OF PEA (*Pisum sativum* L.)

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Introduction

Somaclonal variation is often detected during or after tissue culture. Recently, two mechanisms were proposed to be at least in part responsible for this event. They could be even related to each other. One is epigenetic, concerning of alternation in methylation pattern, the other is related to re-arrangements of highly abundant temporary mobile DNA elements such as transposons and retrotransposons. Somaclonal variation resulting in regenerants abnormalities could be partly circumvented by culture procedure alternation, however there is no fundamental understanding of the cause or of methods to prevent or diagnose it. Consequences of altered methylation were reported to be consistent with alternation of gene expression. Furthermore, substantial data show that various retroelements are activated under stress conditions, in particular, *in vitro* cultivation (see review by Grandbastien, 1998). The hypomethylation and retrotransposon activation phenomenon are related. Hypermethylation on the other hand could be a mechanism to protect genome from such possibly deleterious changes (Martienssen and Colot, 2001).

Multiple shoot culture of dry-seed pea cv. Bohatýr (established via cytokinin stimulation of proliferation of isolated shoot apical meristems and axillary meristems of cotyledons and primary scales) was developed in our laboratory twenty years ago (Griga *et al*., 1984, 1986).

Previously we have examined the ploidy level and checked several molecular markers in particular repetetive microsatellite and retroelement sequences (Griga *et al.,* 2004). The present study examine in more details possible effect on repetetive sequences particularly on retrotransposons and genome-wide changes in methylation caused by prolong tissue culture in pea.

Material and Methods

Plant material: As a control, seeds of cv. Bohatyr were germinated and shoots ***(Co)*** were harvested from 20 individual plants. The same procedure and number of plants were sampled from individual shoots from independent shoot clumps of long term *in vitro* culture ***(In)***. As example of likely unstable culture, callus samples were taken ***(Ca)*** and also *in vitro* samples treated with 5 azacytidine to induce hypomethylation (***Aza).***

***Methylation level measurement Genomic DNA hydrolysis and HPCE analysis*:**

Genomic DNA was hydrolyzed and further analyzed on uncoated fused silica capillary (Waters Chromatography, S.A. 600 x 0,075 μm I.D. Effective length 570 mm) in a capillary electrophoresis system (Capillary Ion Analyzer, Waters Chromatography S.A.) connected to a Millenium data processing station (Waters Chromatography S.A.) as described in Fraga *et al.* (2000, 2002).

DNA analyses: In case of **IRAP** (inter-retrotransposon amplification polymorphism) analysis, either *Ogre* (Y299398) or *Cyclop* (J000640) were used as in Smýkal *et al*.

(2004). **SSAP (**sequence- specific amplification polymorphism**)** was performed as described originally by Ellis *et al*. 1998. **MSAP** (methylation sensitive amplification polymorphism) was performed according to protocol and primer combinations described in Portis *et al*. (2004).

Results and discussion

Growth characteristics of long-term culture: Multiple-shoot culture consisting of clumps of shoots was maintained on MSB agar medium with 20 μM BAP and 0.01 μM NAA since 1983 to 2004 (16 h photoperiod, temperature 20±2^{o}C, subculture usually every 4 weeks). When requested, the isolated shoots could be rooted (MSB with 1μM NAA). No evident morphological alterations were recorded in tissue culture level.

Retrotransposon and methylation analyses: We have chosen two different fingerprinting methods to assess the level of genetic variability/stability of this material set with specific focus on retroelements and methylation status. Previous testing of repetitive microsatellite sequences did not show any detectable variation (Griga *et al.,* 2004). The retrotransposone-based IRAP with *gypsy* type elements have displayed very low intra-accession variability (genetic similarity coefficient 0.95-1) when 10 pea varieties were tested (Smýkal *et al*., 2004). No significant differences were found in any samples of germinated seed controls (*Co*), nor between *in vitro* culture (*In*) demonstrating high uniformity and stability of the material (Fig. 2). The differences were, however, found when callus (Ca) or 5-azacytidine (Aza) treated samples were analyzed. As this method can visualize only high copy retroelements sufficiently close to be amplified another more global method was employed. SSAP combines the advantage of AFLP based on restriction and retroelements for primer binding. In such a way retroelement adjacent regions can be visualized and any new inzertion detected. We have analyzed *PDR-1* element of *Ty1-copia* type and *cyclop* of *Ty3-gypsy* type. The results of this analysis will be shown and discussed.

The most dramatic differences were found when global 5 mdC methylation level was measured, with significantly higher level in *in vitro* culture samples. This difference was further visualized by MSAP with *Hpa/Msp* enzyme combinations (Fig.1). Methylation level could be set back to normal level by 2 weeks of 5-azacytidine treatment. Possible consequences of higher methylation to morphogenic capacity (problematic rooting and further growth *ex vitro*, occasional morphological abnormalities) will be discussed.

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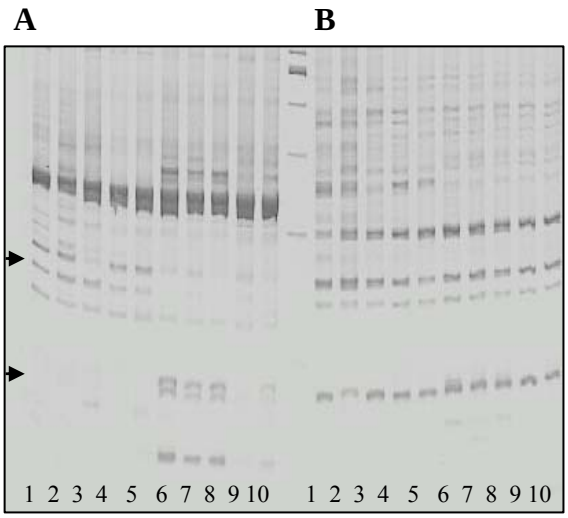

Figure 1
MSAP analysis performed on *Hpa II* - methylation sensitive ***(A***) or *Msp I* - insensitive ***(B)*** digested DNA samples

Samples 1-5 are control seedlings,
6-10 are from long term *in vitro* culture

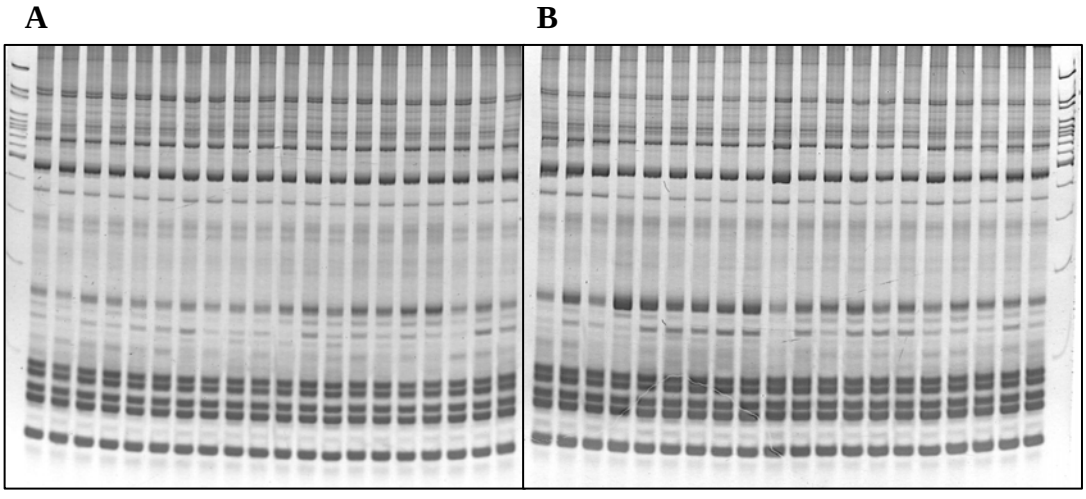

Figure 2 IRAP PCR analysis performed with *Cyclop* retrotransposon on 20 individual samples from control seedlings *(A)*, *in vitro* long term culture *(B)*

GENES CONTROLLING *IN VITRO* SHOOT ORGANOGENESIS AND CELL ELONGATION IN HIGHER PLANTS

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Introduction

Intensive efforts in recent years have resulted in a considerable increase in the spectrum of plant species for which totipotent cell culture systems have been established. This rapidly expending ability to regenerate fertile plants from cultured cells introduces new possibilities for genetic experiments with higher plants. However, despite the many successful applications of plant tissue culture in agriculture and horticulture, differentiation and especially plant regeneration, remain a major problem with a number of crop species. Relatively few crop species are capable of predictable regeneration. Even in those instances where regeneration has been reported, e.g. *Nicotiana*, *Petunia* and *Arabidopsis*, through empirical selection of explants and media, problems are often encountered with particular cultivars within a species. Hence, these problems constitute a serious block to crop improvement (Sangwan *et al*., 1997).

Plant growth and morphogenesis are controlled by complex interactions of multiple plant hormones, interactions in which both the relative and absolute amounts of these substances are important. Coupled with the question of totipotency and dedifferentiation is the nature of cell activation, which starts a cell on its altered pathway toward dedifferentiation, is critical. We, however, have little information on these processes. How totipotency and diffentiation are regulated at the molecular level? Therefore, isolation and characterisation of genes controlling these processes would greatly help in dissecting the molecular events involved. In recent years, Arabidopsis has emerged as a model species for molecular genetic dissection of several complex plant biological processes. A large number of interesting mutations affecting plant growth and development have been characterized. (for a review see Andersen and Roberts, 1998).

We established a T-DNA insertion bank in *Arabidopsis* based on *Agrobacterium*-mediated zygotic embryo transformation protocol (Sangwan *et al.,* 1991) and looked for regeneration competence mutants in F2 plants on a basal medium (Murashige and Skoog, 1962) without phytohormones. Two mutants identified in this work, among the several thousands families analysed, carried a recessive nuclear mutation which we have designated high shoot organogenic competency (*hoc*) (Catterou *et al.,* 2002) and *bul1*-defective in the process of cell elongation (Catterou *et al*., 2001 a and b). We will discuss these two mutants during this cost meeting.

Material and Methods

The *hoc* mutant was isolated from several independent transgenic lines of *Arabidopsis thaliana*. Ecotype C24 harbours T-DNA insertions which were generated in our laboratory by employing the *Agrobacterium*-mediated *in vitro* transformation technique of *Arabidospis thaliana* (Sangwan *et al.,* 1991) with the promotorless vector pPCV6NF-Lux and pPCV6NF-GUS carrying hygromycin resistance gene (Catterou *et al.,* 2002). For *hoc* after selfing (F1 plants), the F2 seeds were aseptically cultured on a germinating medium (MS/2, Murashige and Skoog, 1962) and also in the greenhouse. Prior to the experiments, this mutant was backcrossed into wild-type 2 times. Due to the high fertility of this mutant, seeds of homozygous plants were used as stock seeds in all experiments. For *bul1* mutant, 25 heterozygous plants from the T-DNA lines were grown to flower and, after selfing, most of the F2 population segregated to give *bul1* mutant plants. Plants from these progenies were used throughout this study.The *Petunia* mutant of dumpy phenotype; *trapu* has been isolated as detailed before (Dubois *et al.,* 1996).

Results and Discussions

The *hoc* mutant was isolated from F2 seedlings derived by selfing of one T-DNA transformed plant of *Arabidopsis thaliana* (ecotype C24) tested for organogenic capacity of isolated root explants on medium without phytohormone. In contrast to the wild-type, one transgenic plant, R347, reproducibly, regenerated shoots from isolated roots. A mutant, with an abnormal phenotype, was observed in the line and named *hoc* (for: high shoot organogenic capacity). This *hoc* mutant had an increased leaf number with several leaf rosettes leading to a typical "bushy" appearance. In greenhouse, the bushy phenotype was particularly evident and shows an increased number of fertile inflorescences. Location of the *HOC* locus on the genome map was determined. Analyses of 371 F2 plants with *hoc* phenotype revealed the HOC locus to be located near the CAPS markers GAPB, at 61.78±0.57 cM on chromosome I. When excised roots from mutants and wild-type roots began to change from whitish to yellowish / brownish, after 15 days of culture followed by necrosis and dead after one month of culture. Mutant root, in contrast, turned green, and numerous green nodule-like structures appeared at the junction between the primary and the secondary

roots. Several shoots, with *hoc* phenotype, developed at the excision point and at the green nodule-like structures on mutant's roots.

Genetic and molecular analysis of bul1

We have also isolated and characterized an extremely dwarf mutant of *Arabidopsis* named *bul1-1(*Catterou *et al.,* 2001a and b). *The bul1-1 phenotype was found to be linked to SSLP marker nga172 on chromosome III (only one recombinant out of 125 mutant F2 plants examined).* The extreme phenotype of the *bul1-1* mutant was due to a recessive nuclear mutation. As is the case of *dwf7* and other BR mutants, hypocotyls were restored completely to wild-type length with BL treatment, and sterols analysis confirmed that *bul1-1* is defective in the Δ7-sterol-C5 (6) desaturation step of the sterol pathway leading to brassinosteroid synthesis. Consequently, *bull1* is a sterol-defective mutant and also a BR-defective mutant. The drastic *bul1-1* phenotype suggested that *bul1-1* is a null mutation of the DWF7/STE gene. Similarly, we found that the Petunia tra1 gene controls cell elonation and plant development, and mediates response to cytokinins (Dubois *et al*., 1996).

Effects of BR on bul1-1 morphology

In order to visualize the effect of BR treatment on our bul1-1 mutants, 22(S), 23(S)-homobrassinolide (homoBL) was tested on the bul1-1 mutant. Hypocotyl and petiole elongation was observed in the presence of homoBL (between 0 and 0.1μM). The beneficial effect was best observed in the hypocotyl where approximately a 2-fold elongation was seen at 0.1μM homoBL. Similarly, at the same concentration, a drastic elongation of bul1-1 petiole was induced which led to a modification of the mutant phenotype. At higher BR concentrations, mutant petioles continued to elongate (reaching nearly 90% of the wild type grown in the absence of BR), but in a non-ordered way (twisting of the petiole was frequently observed).

hoc, a shoot regenerating mutant versus others Araidopsis mutant

Considering the classical findings of Skoog and Miller (1957), that high CK and low auxin levels favour shoot formation in tobacco tissue cultures, the high regeneration capacity of the *hoc* mutant suggests that it is impaired in CK or auxin metabolism. This leads to an altered CK/auxin ratio in favour of CK. The *hoc* mutant of Arabidopsis *thaliana*, described in the present paper, has an increased level of endogenous CK and a number of developmental abnormalities. These include reduced apical dominance, slower growth of shoots, release of axillary's branching, retarded flowering, increase in leaf numbers, delayed senescence and capacity to regenerate shoots without phytohormones, which would be expected of a mutant overproducing CK. The *hoc* mutant showed the phenotype of the "CK syndrome", i.e. phenotype observed in *ipt* transgenic plants, however, the effects of elevated endogenous CK in *hoc* on morphogenesis and plant development were not the same as in *ipt* transgenic plants. Among the several roles of CK in morphogenesis, the analysis of *hoc* mutant also indicated that CK can lead cells to develop an organogenetic programme and can effect ethylene production, cell radial expansion, floral induction, and photomorphogenesis.

Interestingly, Chua *et al.* (Banno *et al*., 2001) have isolated an « enhancer of shoot regeneration (ESR1) gene that confers cytokinin-independent shoot formation when over expressed in transgenic Arabidopsis root explants. ESR1 over expression appears to be specific for shoot formation. It does not affect induction of calli or formation of roots, but it does appear to act synergistically with cytokinin, greatly increasing the efficiency of shoot formation when used in combination with cytokinin. Thus, the identification of proteins that interact with ESR1 and HOC and the genes under ESR1/HOC regulation should enable us to determine the chain of events that culminates in shoot regeneration

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TRANSCRIPTION OF PHOTOOXIDATIVE STRESS GENES OF *IN VITRO* GRAPEVINE AT TRANSFER TO *EX VITRO*

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Introduction

When plants are subjected to high intensities, photon absorption can exceed the rate of utilization, leading to an imbalance in energy flow. Some developmental processes are associated with the transfer of new structures from dark or low light conditions to higher environmental irradiances (Karpinski *et al.*, 1999). Reactive Oxygen Species (ROS) production in excess causes oxidative damage to cellular components. Cellular antioxidant system provides protection against ROS produced during both normal cellular metabolic activity and stress conditions. When *in vitro* propagated grapevine is transferred to *ex vitro* under high light intensities symptoms of photoinhibition appear (Carvalho *et al.*, 2001). Therefore, this transition phase is proving to be a suitable model to study the response to photooxidative stress.

Superoxide radical and hydrogen peroxide are ROS that are generated when plant tissues are exposed to environmental stresses, namely light stress. The use of ROS-specific tracer dyes in conjunction with high resolution imaging provides the opportunity to identify sites of photooxidative stress and ROS accumulation in leaves. In parallel with this insight into ROS production, an array of 380 primer combinations designed from *Arabidopsis thaliana* ESTs that respond to light in excess was used to scan grapevine, providing an overview on early excess light stress, on the functioning of the anti-oxidative system and on the signalling pathways involved.

Material and methods

Vitis vinifera L., var.Touriga Nacional was propagated *in vitro* under PDF 50 ± 5 µmol $m^{-2}.s^{-1}$ and transplanted to *ex vitro* under PFD four fold higher (Carvalho *et al*,. 2001). Leaves (*in vitro*, 0, after 24h and 48h) were collected in the middle of the light period.

Total RNA was extracted (Gévaudant *et al.*, 1999) and reverse transcribed using random hexamers and Superscript II RNase H- reverse Transcriptase (Invitrogen, Carlsbad, CA). Primer pairs for amplification of the array of 380 light responsive genes were deduced from genomic sequences of *Arabidopsis thaliana*. Real-time PCR was performed in a reaction mixture of cDNA, gene-specific primers and master mix DyNAmo SYBR Green qPCR Kit (Finnzymes, MJ Bioworks) using an Opticon2 Real Time PCR. Relative amounts were calculated and normalized with respect to *Act2* mRNA level (An *et al*., 1996). Data were arranged in increasing levels of transcription (from –5 to 5, range 20%) and were analysed with the software of "cluster analysis" Expression Profile data CLUSTering and analysis (http://ep.ebi.ac.uk/EP/EPCLUST/). The parameters of hierarchical clustering selected were “euclidean distance squared“ and “average linkage“.

To detect ROS accumulation, leaves were infiltrated with ROS specific dyes. Nitroblue tetrazolium (NBT), 6 mM, to detect superoxide through the production of a dark blue insoluble formazan compound and 3-3’-diaminobenzidine (DAB), 5 mM at pH 3.8, to detect hydrogen peroxide since it forms a deep brown polymerization product. Chloro-

phyll was removed with 80% ethanol, at 60 ºC (Fryer *et al*., 2002). Pictures were taken with a stereomicroscope or a digital camera.

Results

An accumulation of ROS was detected at the 48th hour of analysis. In stressed leaves superoxide radical showed a uniform distribution while hydrogen peroxide presented a preferential accumulation in the cells surrounding the stomata (Fig. 1).

Two hundred primer pairs out of the 380 amplified grapevine cDNA. Cluster analysis divided genes into eight groups, two of which were most relevant due to high levels of up-regulation (shown in Fig. 2). The first group, genes with high transcription levels at both moments, 24 and 48h, included 50% stress-related, 17% signalling, 8% protein-fate and 8% transcription: The second group, genes with high transcription levels at 48h, consisted of 19% stress-related, 25% signalling, 6% protein-fate, 6% transcription and 25% metabolism.

Discussion

ROS imaging presents evidence that grapevine suffers from oxidative stress and the study of transcriptome reveals mainly an activation of defence mechanisms and signalling pathways, and, to a smaller extent, of metabolic adjustments, protein fate and cell wall modifications. Our work also puts in evidence that genes responding to light stress are, to a large extent, conserved between plant species, since 53% of scanned *A. thaliana* ESTs showed homology with *V. vinifera*.

Acknowledgements

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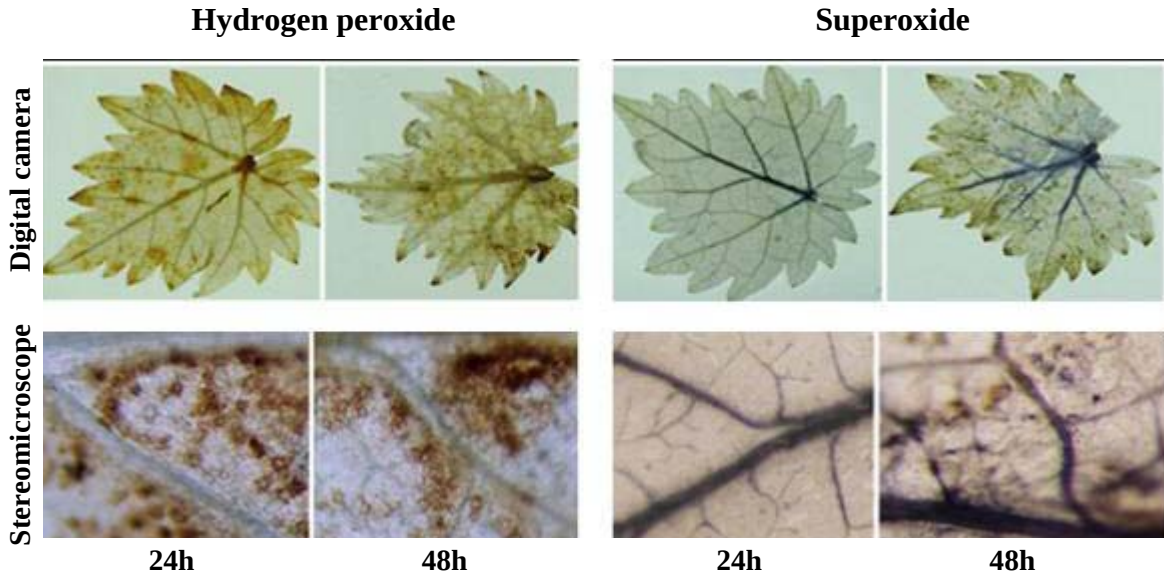

Figure 1 Imaging of hydrogen peroxide and superoxide ion 24 and 48h after transition to *ex vitro*

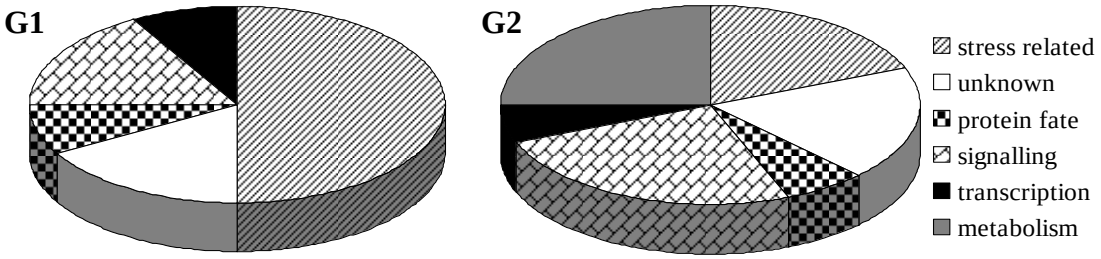

Figure 2 The two most significant groups obtained by cluster analysis of the data. G1 shows up-regulation from 24h; G2 shows the same level of up-regulation at 48h

VIABLE PROTOPLASTS RELEASE NEWLY SYNTHESISED DNA

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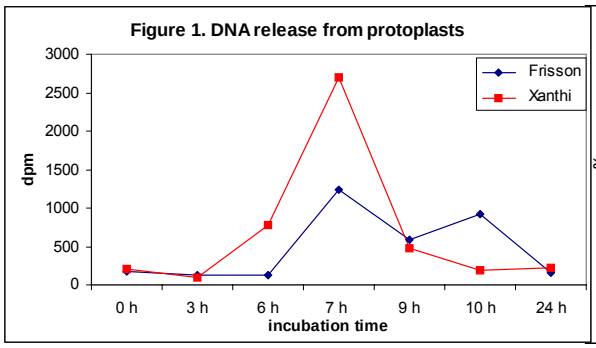
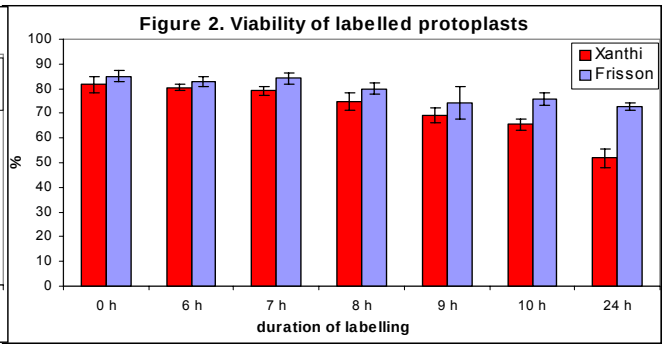
Spontaneous release of newly synthesised DNA from animal cells into the milieu has been well documented (1, 2). This low molecular weight DNA, which is associated with a lipoprotein, is not due to release from dead or dying cells (*2*). It can freely enter other cells, where it is expressed (2, 4). Thus, a DNA fraction from tumour cells can stimulate DNA synthesis in non-dividing cells, whilst that from non-dividing cells can inhibit DNA synthesis in tumour cells (3).

DNA has been shown to readily enter plants and, though that entering via the roots is broken down by DNAse (5), entry into shoots permits its free circulation and entry into nuclei, mitochondria and plastids. Its nuclear incorporation results in expression in adult tissues and passage to the F1 generation, where it can be expressed (4). This mobility of DNA between plant cells in the intact plant has led to the proposition that plant cells may behave as do animal cells with respect to the release of newly synthesised DNA, which may act as a messenger between cells. This may partly explain the mechanism by which plant cells *in vitro* are affected by the use of conditioned medium to rescue cells that would otherwise die (6).

Here, we sought to determine if plant cells release such a newly synthesised DNA. Hence, fresh, highly viable (85-99%) protoplasts of *Pisum sativum* cv Frisson (7) and *Nicotiana tabacum* cv Xanthi were incubated at 1 x 10^6 protoplasts ml^{-1} for 90 minutes in the presence of 1.25 mCi [H^3]-thymidine (72 Ci $mmol^{-1}$) per 5ml of protoplasts. Thrice-washed protoplasts were incubated in fresh medium in the absence of [H^3]-thymidine for various periods up to 24h and, after removal, were tested for viability. The supernatant DNA was precipitated from the medium and prepared for scintillation counting.

No radioactivity was observed above the levels of the 0h samples at 3h and 6h in Frisson, but at 7h, 9h and 10h there were significant levels of labelled material in the supernatant. In Xanthi, significant levels were noted already at 6h. In contrast, no labelled material was found at 24h with either species (Fig. 1). The maximum release of newly synthesised DNA occurred between 6h and 9h, when protoplast viability was still high (Fig. 2). The lack of H^3-DNA in the supernatant at 24h could be explained through uptake by the protoplasts, as formerly shown for mammalian cells *in vitro* (2).





Thus, we have demonstrated that protoplasts release material behaving as [^{3}H]-DNA, *in vitro*. We aimed also to separate this DNA from the protoplast cytosolic fraction which, in animal cells, has been shown to contain a high proportion of such released DNA (*8*). Highly viable protoplasts of Frisson and Xanthi were prepared and pelleted prior to re-suspending in distilled water (osmotic shock) followed by vortexing for 2 min. The preparation was centrifuged at 10^3 g for 10min and the supernatant respun at 10^5 g for 1h. Two ml of the supernatant were loaded at room temperature onto an agarose gel column (48 cm long, 2.5 cm diameter) prepared using Biogel A1.5 beads (exclusion limit 1.5 x 10^6 Da), equilibrated with phosphate buffered saline (PBS) pH 7.4, and eluted with the same buffer at approximately 30 ml.h^{-1}. Fractions were collected and the DNA identified by U-V absorbance at 260 nm. The 260 nm/280 nm ratio showed values implying that there was some protein in the DNA preparations, as would be expected if this DNA was also complexed with lipoglycoproteins, as reported for DNA released from animal cells (2). Thus, we consider that plant cells, similarly to animal cells, can spontaneously release a newly synthesised DNA fraction into the milieu, which is available for incorporation into other plant cells, possibly as a messenger.

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TRANSFORMATION OF *Gypsophila*

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Introduction

Gypsophila is a member of the Caryophyllaceae, which contains over 300 species, native to Asia and Europe. *Gypsophila paniculata* is the only species that is used as a cut flower. As one of the major contributors to the flower market, gypsophila is an important target for the breeding of new varieties with novel characteristics. Yet, classical breeding is strongly hampered by the essentially complete sterility of gypsophila. As a consequence, a very limited number of gypsophila varieties are sold worldwide on the flower market (Shillo, 1985; Ahroni *et al*., 1997).

New tools for the introduction of foreign genes into plants and the growing knowledge and technology related to gene identification and isolation have enabled the specific alteration of single traits in an otherwise successful cultivar. To allow molecular breeding of gypsophila, we developed a system for efficient transient transformation and regeneration/selection of stably transformed plants. It should be noted that low transformation efficiency is a very serious bottleneck in the production of plants with novel traits of interest, since a large number of transgenes must be generated and screened to enable selection of the target genotype (Zuker *et al*., 1998).

Materials and Methods

Plant material and Media composition

Unrooted cuttings of gypsophila were obtained from Danziger "DAN" Flower Farm (Moshav Mishmar Hashiva, Israel). Stem cuttings were used to prepare stem explants (Ahroni *et al*., 1997). Murashige and Skoog basal medium (MS; Murashige, Skoog, 1962) with sucrose (30 $g.l^{-1}$) and solidified with agar (8 $g.l^{-1}$) (basic medium), was supplemented with growth regulators and antibiotics for cocultivation with *Agrobacterium*, regeneration and selection of adventitious shoots, and elongation and rooting of transgenic plants. For cocultivation of stem explants with *Agrobacterium*, the basic medium was supplemented with 0.1 $mg.l^{-1}$ α-naphthalene acetic acid (NAA), 0.5 $mg.l^{-1}$ 6-benzylaminopurine (BAP) and 100 μM acetosyringone. For shoot regeneration and two-step selection of transformants, the basic medium was supplemented with 0.1 $mg.l^{-1}$ NAA and 3 $mg.l^{-1}$ TDZ (T3, first selection cycle), or with 0.1 $mg.l^{-1}$ NAA and 1 BAP (B1, second selection cycle). Both media were also supplemented with 300 $mg.l^{-1}$ carbenicillin and, unless otherwise stated, 70 $mg.l^{-1}$ (for T3) or 100 $mg.l^{-1}$ kanamycin (for B1). Elongation and rooting of transgenic shoots, following the second selection cycle, were performed on the basic medium containing 0.1 $mg.l^{-1}$ NAA, 0.1 $mg.l^{-1}$ gibberellic acid (GA), 200 $mg.l^{-1}$ carbenicillin and 70 mg. l^{-1} kanamycin.

Bacteria

Agrobacterium tumefaciens strain AGLO (Lazo *et al*., 1991) carrying pCGN7001 (Comai *et al*., 1990) or pAM (Zuker *et al*., 2001) was used for stable transformation of gypsophila. Bacteria were prepared according to Zuker *et al*. (1999).

Results and Discussion

To allow effective selection of transgenic plants on kanamycin, stable transformation of cv. Arbel was performed with pCGN7001 which carries 35S-driven *nptII*. Inoculation of explants with bacteria at an OD_{550} of 0.1-0.2 was optimal, allowing to control bacterial growth with no adverse effect on the further tissue culture and regeneration of plantlets following transfer to the regeneration/selection T3 medium. After ca. 1 month in culture following inoculation, adventitious shoot clusters, regenerated directly from sectioned stem explants, were easily scorable (Figure 1b). To minimize generation of putatively chimeric transgenic plants (Figure 1c) a second selection/regeneration cycle was performed. Leaves were excised from shoot clusters and cultured on B1 medium. Leaves originating from individual clusters were cultured separately for each cluster to eliminate the possibility of generating transgenes representing a single transformation event. After ca. 2-3 weeks of the second-selection cycle, 50% of the independent clusters yielded scorable shoots. These adventitious shoots (ca. 4 shoots per leaf) regenerated directly from the basal part of the leaves (Figure 1d). In almost all of these shoots, histochemical assay revealed GUS expression throughout the tissues, with no observable chimerism (Figure 1e, f). To assess the overall efficiency of the two cycles of selection, only one GUS-expressing shoot per individual cluster was counted, even though 10-15 GUS-expressing shoots were usually generated from leaves of each cluster. Based on this consideration, which allows an estimation of independent transformation events, the overall yield of the procedure was ca. 5 GUS-expressing shoots generated per 100 *Agrobacterium*-inoculated stem explants.

Flowering of ca. 30 independent transgenic lines grown in the greenhouse was normal (Figure 1g). The molecular analysis of transgenic plants is shown in Figure 2 *nptII* and *uidA* PCR amplification yielded a DNA fragment of the expected size (0.8 and 0.5 kb, respectively) in all analyzed kanamycin-resistant GUS-expressing plants and not in controls. The applicability of the transformation procedure was also assessed with another binary vector pAM. Transgenic plants resistant to kanamycin were generated and grown in the greenhouse. PCR (Figure 3) analyses of the plants, yielding expected DNA fragments.

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Figure 1 Transformation and regeneration of transgenic gypsophila plants. (**a**) Stem explants expressing GUS 5 days after inoculation with EHA105/pKIWI105 (**b**) Shoot regeneration from a stem explant. (**c**) The chimeric pattern of GUS expression following transformation with AGLO/pCGN7001 and the first selection cycle. (**d**) Second selection cycle of adventitious shoots. Shoots developed from the leaf area which showed resistance to kanamycin. (**e**, **f**) Solid, non-chimeric GUS expression in adventitious shoots regenerated from leaves following the second selection cycle. (**g**) Transgenic plant.

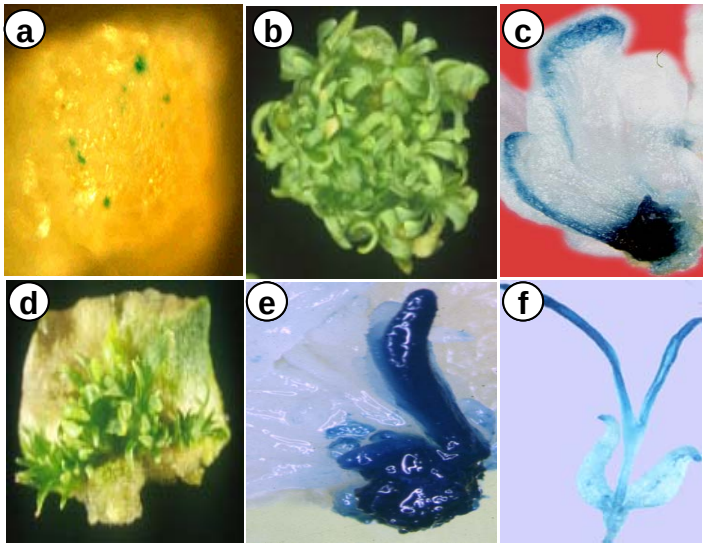


Figure 2 PCR analysis of independent GUS-expressing kanamycin-resistant transgenic clones (1-4) and untransformed (C) cv. Arbel gypsophila plants. (P) Control plasmid pKIWI105

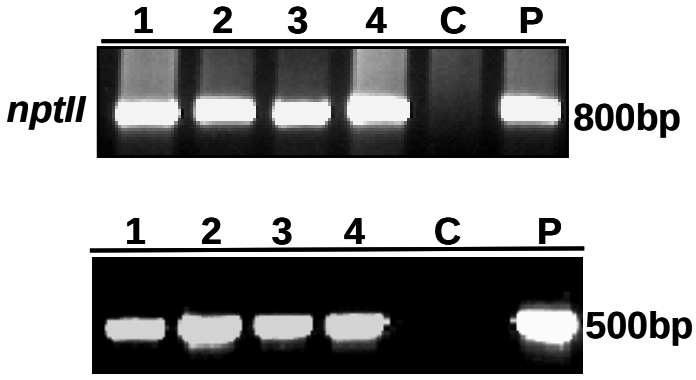
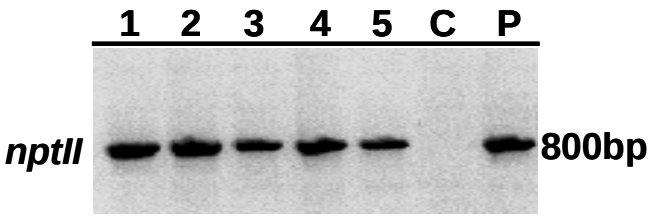


Figure 3 PCR analysis of independent kanamycin-resistant pAM transgenic clones (1-5) and untransformed (C) cv. Arbel gypsophila plants. (P) Control plasmid pAM



GENETIC TRANSFORMATION OF ALFALFA FOR IMPROVEMENT OF NUTRITIONAL VALUE

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Introduction

Alfalfa (*Medicago sativa* L.) is the most important forage crop in the world. As the plant is cross-fertilizing autotetraploid, relying on bees for pollination, it responds more slowly to selection than a diploid species. There is a scope for improvement of the crop in most of the agronomic characteristics, as well as nutritional quality traits and disease resistance. In addition to conventional breeding programmes, other genetic techniques are being developed to improve the characteristics of currently available cultivars. In recent years, the use of *in vitro* cell cultures and incorporation of genes from other (unrelated) species into alfalfa genome through genetic transformation are regarded as the most promising methods for alfalfa improvement. Different methods have been developed to deliver the foreign DNA into alfalfa cells, however the use of *Agrobacterium tumefaciens* as a vector is the most efficient one.

There is a substantial interest to improve the nutritional quality of alfalfa, since it is well established, that the S-containing essential amino acids (EAA) methionine (M) and cysteine (C) each constitute only about 1 % or less of crude protein content of alfalfa stems (Frame *et al*., 1998). One approach to achieve higher EAA content in alfalfa, is to incorporate genes coding for high M+C proteins, such as sunflower albumin 8 (Tabe *et al*., 1995) and maize zeins (Bellucci *et al*., 1997), or genes for modification of M biosynthetic pathway (Galili *et al*., 2000; Avraham *et al*., 2005).

Our aims were i) to establish an efficient *in vitro* regeneration system through induction of somatic embryogenesis in alfalfa, ii) to obtain a set of highly regenerable lines from different cultivars of alfalfa, and iii) to introduce a gene *Ov* from japanese quail (*Coturnix coturnix*) coding for high M+C containing protein into the genomes of selected higly regenerable alfalfa genotypes.

Material and Methods

Development of an efficient in vitro regeneration system for alfalfa. To induce somatic embryogenesis in alfalfa, we adapted and modified a method of Mezentsev (1981) for regenaration of alfalfa in *in vitro* culture. We tested several types of seedling - (root segments, hypocotyl segments, cotyledons, petiole segments and young leaflets) and mature plant-derived explants (stem segments, petiole segments and leaflets) for ability to regenerate through somatic embryogenesis.

Selection and breeding of highly regenerable alfalfa genotypes. To select highly regenerable (by our definition <80% embryogenic calli/explant and <4 somatic embryos/callus) genotypes, we tested 13 cultivars of alfalfa for presence of embryogenic genotypes. In cultivar Lucia we performed recurrent selection for improving the regeneration ability of the progeny of regenerable genotypes of alfalfa.

Genetic transformation of selected highly regenerable genotypes of alfalfa. We used an *Agrobacterium tumefaciens*-mediated genetic transformation method to introduce the gene *Ov* into the genome of alfalfa with the aim to improve the nutritional quality of the plant. The *Ov* gene was isolated from Japanese quail (*Coturnix coturnix*) (Mucha *et al*., 1991) and coded for a protein containing high (5.7%) concentration of M+C. The gene was inserted into a plasmid vectors pPDE1001 and the *A. tumefaciens* strain AGL1. Petiole segments and leaflets of the highly regenerable alfalfa lines Rg9/I-14-22 and Rg11/I-10-68 (Faragó *et al*., 1997) were co-cultured with *agrobacteria* and selected on kanamycin containing media.

Results and discussion

We developed a four-step protocol for regeneration of complete alfalfa plantlets in *in vitro* culture. In the first step, a medium GMK containing a combination of three plant growth regulators, kinetin (37.2

μM), NAA (2.7 μM), and 2,4-D (36.2 μM) is used to induce embryogenic calli. Somatic embryo development is induced by a short treatment on the medium B5m containing BAP (0.56 μM). For maturation of somatic embryos, embryogenic calli are transfered to a growth regulator free medium GMR. Conversion of well developed somatic embryos is induced on a medium designated MS0.25 containing IBA (1.23 μM). Of the several different explant types used, the petiole segments (seedling- and mature plant-derived) and hypocotyl segments (seedling-derived) proved to be the most useful.

Five Slovak and 8 Czech cultivars of alfalfa were screened for the presence of embryogenic genotypes by the improved regeneration protocol and using petiole-derived explants. Seven cultivars (Jarka, Magda, Niva, Palava, Viktoria, Syntéza 1/2 a Syntéza 1/4) did not respond regenerating somatic embryos, while the other 6 cultivars contained 5,0 (Zuzana) to 13.3 % (Syntéza 1/1) embryogenic genotypes. The recurrent selection for embryogenic capacity increased the frequencies of embryogenic genotypes in some progenies of hybrids up to 83.3 %. Two highly embryogenic lines, Rg9/I-14-22 and Rg11/I-10-68, were selected for further work to transform alfalfa.

In two individual experiments, 754 explants of both the selected lines were inoculated and consecutively co-cultured with *A. tumefaciens* AGL1. Noninoculated petiole, and leaf segment explants were used as controls. The results of experiments are presented in table 1. Petiole segments proved to be more suitable explant sources for use in genetic transformation (9% Kan^R embryogenic callus formation on selection medium) in accordance with their higher regeneration ability compared with leaf segments (1% Kan^R calli). Also, with line Rg9/I-14-22 we were able to obtain slightly more kanamycin resistant (Kan^R) embryogenic calli (9.0%), than with the line Rg11/I-10-68 (6.7%). That means, probability of recieving transformed alfalfa plants seems to be determined by the regeneration capacity of plant tissues *in vitro.* In total, 116 putatively transformed Kan^R alfalfa lines were obtained and maintained as shoot cultures on MS0.25 medium supplemented with 50 $mg.l^{-1}$ kanamycin.

The Kan^R plants producing profound root system on kanamycin media were tested preliminarily for the expression of the *npt*II gene using a leaf bleach assay. NPTII ELISA assay was also performed with selected lines. PCR analysis was performed to show the presence of the transgenes in the plant genome (results not shown).

Preliminary HPLC analyses of amino acid content showed that 15 of the 32 transgenic lines analysed contained higher protein-bound M+C than the control non-transgenic lines (Figure 1).

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Table 1 Summary of *A. tumefaciens* mediated genetic transformation experiments with two regenerative lines (Rg9/I/14-22 and Rg11/I-10-68) of alfalfa cv. Lucia. Two types of explants (leaves and petiole segments) were used to cocultivation with the AGL1 strain of *A. tumefaciens* carrying the binary plasmid pPDE1001Ov. Different inoculation solutions and modes of inoculation were applied before cocultivation of explants with the bacteria

Genotype	Explant	Cocultivation	Inoculation solution	Vacuum infiltr.	No. explt.[e]	No. (%) calli[f]	No. (%) EC[g]	No. regs.[h]
Rg9/I-14-22	Leaves	Pozit. contr.[a]	GMK-L[c]	VI-[d]	36	16 (44)	8 (22)	ND[i]
		Negat. contr.[a]	GMK-L	VI-	18	5 (28)	0 (0)	ND
		Cocultured[b]	GMK-L	VI-	109	36 (33)	1 (1)	12
	Petioles	Pozit. contr.	GMK-L	VI-	48	35 (73)	35 (73)	ND
		Negat. contr.	GMK-L	VI-	24	5 (21)	0 (0)	ND
		Cocultured	GMK-L	VI-	144	35 (24)	13 (9)	73
		Cocultured	GMK-L	VI+[d]	135	17 (13)	6 (4)	5
Rg11/I-10-68		Cocultured	H_2O[c]	VI-	60	13 (22)	1 (2)	0
		Cocultured	NaCl[c]	VI-	60	14 (23)	1 (2)	15
		Cocultured	GMK-L	VI-	60	16 (27)	4 (7)	0
		Cocultured	Sa[c]	VI-	60	18 (30)	3 (5)	0

[a] The pozitive controls were cultured on non-selective media without antibiotics; the negative controls were cultured on selective media amended by 50 $mg.l^{-1}$ kanamycin, 500 $mg.l^{-1}$ cefotaxime and 500 $mg.l^{1}$ carbenicillin

[b] Cocultivation of explants with *A. tumefaciens* was carried out on non-selective GMK medium

[c] Alfalfa leaf- and petiole-derived explants were inoculated in a liquid GMK medium (GMK-L); in redistilled water (H_2O); 0.85% (w/v) NaCl solution or GMK-L supplemented with 10% (w/v) sucrose and 100 μM acetosyringone (SA)

[d] Inoculation of explants in bacterial suspension was (+) or was not (-) under application of vacuum infiltration

[e] Number of explants initially plated

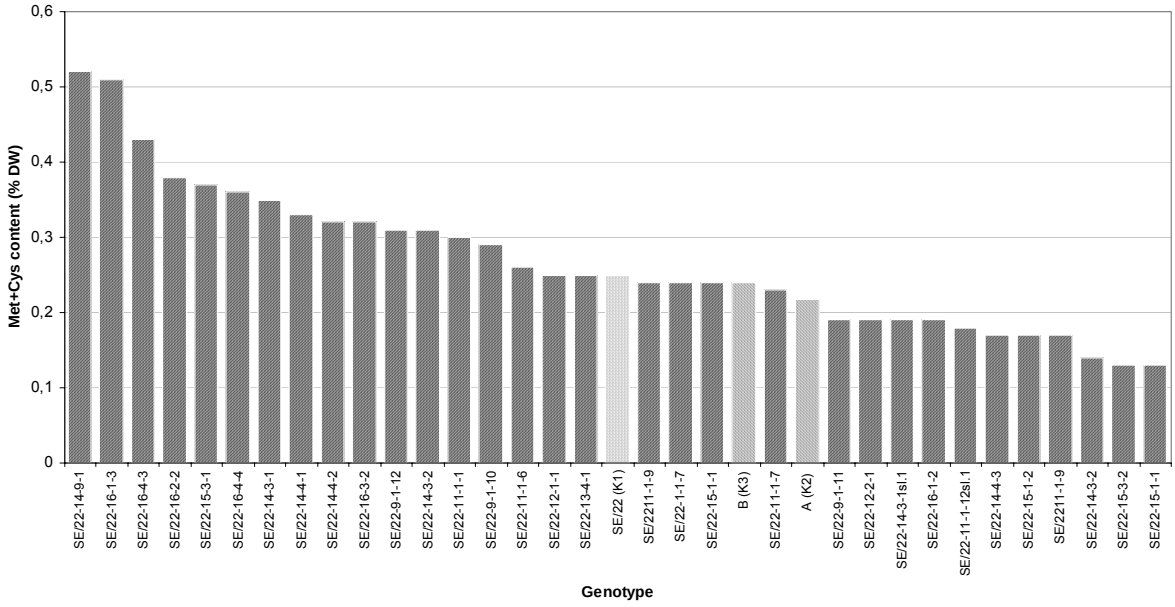
[f] Number of explants forming calli; the number in parentheses is the percentage of callus forming explants

[g] Number of embryogenic calli; the number in parentheses is the percentage of embryogenic explants

[h] Number of kanamycin resistant shoots/lines derived from embryogenic calli by conversion of cotyledonary somatic embryos on MS0.25 medium containing half the concentrations of each of the antibiotika

[i] Not defined

Figure 1 Protein-bound amino acid analysis in 32 transgenic and 3 non-transgenic (control, K1 = non-transgenic Rg9/I-14-22; K2 and K3 = non-transgenic seed-derived plants of cultivar Lucia) lines of alfalfa



EVALUATION OF THE DAO1 GENE AS A SELECTABLE MARKER IN THE TRANSFORMATION OF THE APPLE ROOTSTOCK M26

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Introduction

Objective

The aim of this study was to evaluate the *dao1* gene, coding for D-amino acid oxidase, as a selectable marker in the transformation of the apple rootstock M26.

Materials and methods

Regeneration

Three to six weeks old in vitro propagated shoots of apple rootstock M26 were used in the experiments. Two to four of the youngest unfolded leaves were excised and wounded perpendicularly to the midrib with a scalpel and placed on the callus induction medium (CIM), containing basal MS supplemented with 30 $g.l^{-1}$ sucrose, 2.5 $g.l^{-1}$ gelrite, 10 µM TDZ (thidiazuron), 2.7 µM NAA (naphthalene acetic acid) and D-alanine at pH 5.5. The concentrations of D-alanine were: 0, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM. Fifty leaves, placed in 5 Petri dishes, were used per treatment. The Petri dishes were placed in dark. After 3 weeks the leaves were transferred to the shoot induction medium (SIM) which contained the same components except for the replacements of TDZ by 22 µM BAP (benzylaminopurine) and the reduction of NAA to 1.1 µM. When shoots appeared the explants were transferred to light.

Transformation

In the experiments the *Agrobacterium tumefaciens* strain C58C1 (pGV3101) harbouring the binary vector pPCV702:dao1 was used. The binary vector contained the *dao1* gene controlled by the CaMV 35S promoter and the *nptII* gene driven by the *nos* promoter.
Nine transformation experiments were carried out. Selection with kanamycin (25 or 50 $mg.l^{-1}$) was used in 3 experiments (TK1-3) with a total of 993 explants. D-alanine (1.2, 1.5, 1.8 or 2.2 mM) was used in 6 experiments (TA1-6) and a total of 1724 explants. The concentration of the selective agents was adjusted during the experiment depending on the sensitivity or the appearance of the explants to selective agents (Table 2).
DNA isolation and PCR analysis
DNA and PCR analysis was carried out according to standard methods.

Results

Regeneration

After 2 weeks in dark the explants grown on medium containing up to 0.5 mM D-alanine developed calli at the cut leaf surfaces. One week later shoots were formed. Explants exposed to higher concentrations of D-alanine formed calli after 3 weeks (1.0 mM) and 5 weeks (1.5-3.0 mM). These explants formed calli and shoots simultaneously. Concentrations of 1.5-3.0 mM D-alanine were associated with a relatively low regeneration percentage and a low number of shoots per explant (Table 1).

Transformation experiments

None of the excised shoots from experiments TA1, TA2, TA4 and TA5 survived the selection. In experiment TA3, selection with 1.2 and 1.5 mM showed similar results in regeneration percentage and shoot yield. The most resistant shoots against D-alanine originated from this transformation occasion and 13 shoots were tested with PCR (Table

3). The first 2 experiments with kanamycin selection did not give rise to shoots even though the kanamycin concentrations were lowered from 50 $mg.l^{-1}$ to 25 $mg.l^{-1}$. In the last attempt one shoot was produced and survived selection (Table 3).

PCR analysis

A total of 16 shoots were analysed with PCR. The *dao1* gene was verified in 7 clones (Table 3). The transformation frequency for selection with D-alanine was 0.35% (calculated as the number of independent transformants, displaying *dao1* acivity, in percent of the initial number of explants). One of the clones was selected with kanamycin.

Table 1 Regeneration of leaf explants on medium containing different concentrations of D-alanine

D-alanine (mM)	Callus formation (%)	Shoot formation (%)	Shoot no. per explant*
0	96a	96a	7.8 ± 4.8a
0.01	98a	96a	5.7 ± 3.8bc
0.05	100a	98a	6.1 ± 3.6b
0.1	100a	98a	4.8 ± 2.6cd
0.5	100a	96a	4.3 ± 2.7d
1.0	64b	70b	2.4 ± 2.5e
1.5	38c	36c	0.6 ± 1.1f
2.0	22d	28c	0.7 ± 1.2f
2.5	14de	22c	0.4 ± 0.8f
3.0	4e	22c	0.3 ± 0.6f

*mean ± standard deviation

Table 2 Summary of medium and selective agent in the transfers of the experiments TK3, TA3 and TA6. The transfers to fresh medium were performed every two weeks. CI (callus Induction Medium), I) SI (Shoot Induction Medium) and SM (Shoot Multiplication Medium)

	Transfer 1		Transfer 2		Transfer 3		Transfer 4		Transfer 5		Transfer 6	
	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala
TK3	CI	50	CI	25	CI	25	SI	25	SI	25	SI	25
TA3	CI	1.2	CI	1.2	SI	1.2	SM	1.5	SM	1.5	SM	1.5
TA3	CI	1.5	CI	1.5	SI	1.2	SM	1.5	SM	1.5	SM	1.5
TA3	CI	1.8	CI	1.8	SI	1.2	SI	1.5	SM	1.5	SM	1.5
TA6	CI	1.2	CI	1.2	SI	1.2	SI	1.2	SM	1.2	SM	1.2

	Transfer 7		Transfer 8		Transfer 9		Transfer 10		Transfer 11	
	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala	med	kan/ D-ala
TK3	SI	25	SM	25	SM	25	SM	25		
TA3	SM	1.5	SM	3.0	SM	4.0	SM	4.0	SM	5.0
TA3	SM	1.5	SM	3.0	SM	4.0	SM	4.0	SM	5.0
TA3	SM	1.5	SM	3.0	SM	4.0	SM	4.0	SM	5.0
TA6	SM	1.2	SM	4.0						

Table 3 Results from the transformation experiments TA3, TA6 and TK3

Transformation experiment	D-alanine (mM)	Explants with shoots (%)	No. of excised shoots/explant	No. of shoots tested with PCR	No. of shoots verified with dao 1
TA3	1.2	19a	14.1 ± 9.3a	8	3
TA3	1.5	10ab	13.3 ± 5.2a	4	2
TA3	1.8	0.9b	40b	1	-
TA6	1.2	3.3	5.6 ± 3.8	2	1
TK3	(25 $mg.l^{-1}$ kan)	0.3	1	1	1

THE USE OF CRE/LOX REKOMBINATION SYSTEM TO DERIVE MARKER FREE TRANSGENIC TOBACCO PLANTS

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Introduction

To avoid the negative effect of selectable marker genes on environment, several approaches have been reported (Yoder and Goldsbrough, 1994). These also include site specific Cre/lox recombination system. In an autoexcision strategy the *cre* gene is a part of the same T-DNA as the selectable marker gene and both are flanked by a single pair of *lox*P sites in relative direct orientation. A CRE-mediated intramolecular recombination between *lox*P sites will result in the excision of the intervening DNA. Together with the self-excision of the *cre* gene the selectable gene will be also deleted. When the expression of *cre* gene is controlled by tissue-specific promoter the auto-excision will occur at fixed stage in plant development.

In this work, a plant binary vector containing Cre/lox system with *cre* recombinase gene under the control of embryo specific cruciferin promoter was prepared and used for genetic transformation of tobacco via *Agrobacterium tumefaciens* with aim to receive auto-excision of *cre* recombinase and *nptII* gene during the embryogenesis.

Material and Methods

Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) was transformed by the leaf discs transformation procedure (Horsch *et al*., 1985). *Agrobacterium tumefaciens* LBA 4404 carried binary vector pEV2 containing GUS gene unit and self-excising cassette consisting of intron-containing recombinase gene (cre^{INT}) fused to seed specific promoter and nptII expression unit. The histochemical assay for detection of β-glucuronidase (GUS) activity was carried out as described by Jefferson (1987) with 5-bromo-chloro-3-indolyl glucuronide (X-gluc) as a substrate. For PCR analysis of transgenic tobacco plants, DNA was isolated from leaf material by urea-phenol extraction procedure (Chen *et al*., 1992). Two combinations of primers were used. The primers 5´-GTTCCTGATTAACCACAAACC-3´and 5´-CCAGTGGCGAAATATTCC-3´ amplifying a 425 bp internal fragment of *gus* gene. The primers detecting a 550 bp fragment of the *nptII* gene were 5´-GGGACCACCTATGATGTGG-3´ and 5´-CTCCCAATCAGGCTTGATCC-3´. PCR reactions were performed on apparatus Primus 25 (MWG-BIOTECH AG). Samples were first heated at 94 ^{o}C for 4 min followed by 35 cycles of 94 ^{o}C for 45 sec, 58 ^{o}C (for *nptII*) or 55 ^{o}C (for *gus*) for 45 sec and 72 ^{o}C for 2 min. Cycling was followed with the ending step of 72 ^{o}C for 5 min.

Results and Discussion

Genetic transformation of tobacco plants was performed using strain *A. tumefaciens* LBA 4404 harbouring plant binary vector pEV2. For the selection of transformed tissue, kanamycin (50 mg.l^{-1}) was used as a selectable marker. Leaf explants produced shoots with 56.8% efficiency. During regeneration the problems with rooting under selection pressure were observed, indicating a possible escapes or premature activation of cruciferin promoter resulting in removing of *nptII* gene. Finally, those shoots were rooted in

the absence of kanamycin. In total, 97 independent putative transgenic plants were obtained. Histochemical analysis of GUS gene expression confirmed 74 (76.3%) analysed plants contained an active *gus* gene. Plants without dectable GUS activity (23.7%) were omitted from further analysis. Genomic DNA from the GUS positive plants was subjected to PCR analysis for the presence of the transgenes. All of them gave the predicted DNA fragment of 0.425 kb indicating the presence of gus gene. In case of PCR reaction with primers corresponding to the *nptII* gene, only 34 out of 74 (45.9%) GUS positive plants gave predicted 0.55 kb DNA fragment. Two possible explanations could account for the absence of *nptII* gene in analysed GUS positive transgenic plants. The first is the transcriptional readthrough from strong doubled CaMV promoter driving the GUS gene. Another option is that cruciferin promoter is not so tightly tissue-specific as we hoped for. Both *cre* recombinase and *nptII* genes would be removed from the plant genome during *in vitro* regeneration. Premature transcription of tissue specific promoters in *in vitro* conditions was also reported by others (Trindade *et al*., 2003). More experiments are required to evaluate the influence of *in vitro* growth conditions (different hormones, sucrose, long day, etc.) on ineligible activation of seed specific cruciferin promoter. All transgenic plants will be transferred to greenhouse for analyses of selfed progeny.

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Proteomics of cold acclimation and photoperiod in woody plants: differential in-gel electrophoresis (2-D DiGE) of soluble proteins extracted from peach bark tissue.

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Proteomics, along with genomics and metabolomics are providing valuable insights into the regulation of complex processes in plants such as stress tolerance. In particular, proteomics allows one to develop an understanding of the relationship between gene and protein expression, the impact of posttranslational regulation of proteins on stress tolerance, as well as the impact of ectopic gene expression on plant metabolism. In the present study we discuss the benefits of using DiGE technology to study the proteomics of cold acclimation and photoperiod in peach trees.

One-year-old, seedlings of peach, cv. 'Canadian Harmony' on Tennessee Natural rootstock, were grown in a glasshouse in the spring under ambient light and temperature conditions. Photoperiod and temperature experiments were conducted as follows. Fourteen trees were placed within a Conviron CMP4030 growth chamber at either 5ºC or 25ºC, with 8h light/ 16h dark cycles (short day, SD); long day (LD) trees were under the same light/ dark regime but with a 10 min night break. PPFD was approximately 500 mmol photons.m^{-2}.sec^{-1}. Trees were subjected to these photoperiods and temperatures for 3 and 5 weeks.

Total soluble proteins were extracted from bark tissues with a TCA/acetone precipitation. Extracted proteins were then subjected to differential in-gel electrophoresis (2-D DiGE). This technique allows one to mix and run 3 samples in the same bidimensional electrophoresis gel. The labeled samples can be multiplexed (i.e. separately visualized using 3 excitation wavelengths) during the capture of images. Multiplexing increases the throughput, reproducibility, and accuracy of protein expression studies. The images are then merged, and differences between them can be determined using image analysis software. The dyes are purported to have a linear response to variation in protein concentration over five orders of magnitude, offer sub-nanogram sensitivity, and are compatible with mass spectrometry (MS) analysis. An internal standard, consisting of a pool of the different samples studied, can be used to match protein patterns across gels and bring a higher statistical confidence and level of reliability to 2-D electrophoresis analyses.

In the present experiment, 5 batches of triplicated gels were used to carry out the total analysis of 9 labeled samples in addition to the internal standard (Table 1).

Table I: Experimental design for 2-D DiGE comparison of the 9 samples.

Batch #	Cy2	Cy3	Cy5
1	Internal standard	Control	SD 5°C 3 weeks
2	Internal standard	SD 25°C 3weeks	SD 5°C 5 weeks
3	Internal standard	SD 25°C 5 weeks	LD 5°C 3 weeks
4	Internal standard	LD 5°C 5 weeks	-
5	Internal standard	LD 25°C 3 weeks	LD 25°C 5 weeks

The extracted soluble proteins were quantified and labeled with fluorescent dyes (Cy2, Cy3 and Cy5, Figure 1a) and run on pH 4 to 7 isoelectrofocusing gels, followed by a 12% PAGE. Images were analyzed with DeCyder Differential Analysis Software (AmershamBiosciences; Figure 1b). Comparisons were made in protein expression between SD, LD, 5 C, and 25 C. Additional comparisons were made within a specific treatment at different time points (3 and 5 weeks). An example of comparing expression of a single protein spot in two different samples is provided in Fig. 1.

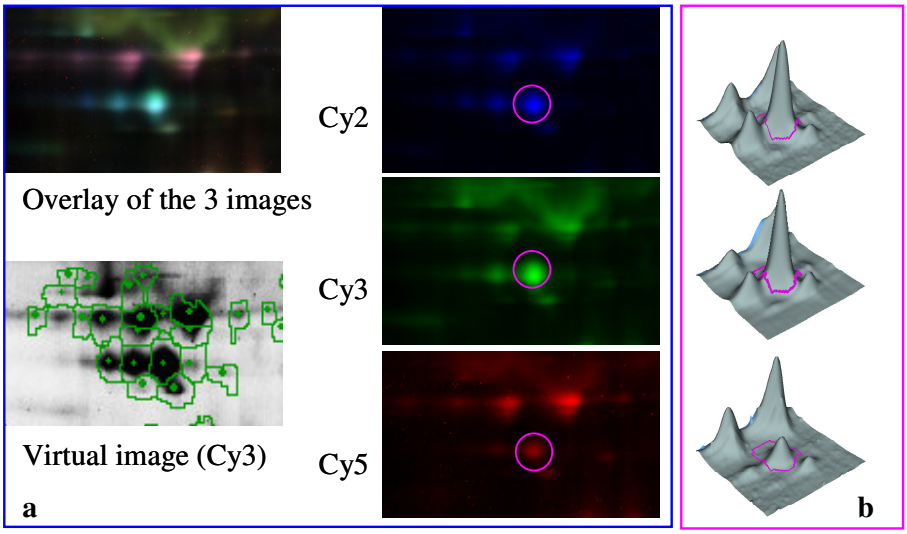

Figure 1: a:2-D DiGE comparison of 2 samples on a portion of a single gel. Internal standard is labeled with Cy2; sample SD 25°C 5 weeks labeled with Cy3 and sample LD 5°C 3 weeks labeled with Cy5; b: 3-dimensional views of the circled spot are presented in the rectangle on the far right.

Computerized analysis of 2-D gels revealed changes in abundance of more than 13% of the spots (109/824). Forty-one of these proteins have been identified by mass spectrometry. Among these proteins, some are related to PR proteins (e.g. Major allergen Pru av 1), chaperonins (e.g. HSP70), glycolysis (e.g. 2,3-bisphosphoglycerate-independent phosphoglycerate mutase), photosynthetic pathway (e.g. PS II oxygen evolving complex protein 1), amino acid metabolism (e.g. cysteine synthase), energy metabolism (e.g. ATP synthase beta unit), cell structure (e.g. beta tubulin), protein metabolism (e.g. 20S proteasome alpha subunit B) or plant hormone response (e.g. auxin-binding protein 20), etc. The overall results will be discussed in detail as they relate to photoperiod and temperature conditions over time.

COMPARISONS OF PROTEIN PATTERNS AS SHOWN BY 2-D ELECTRO-PHO RESIS IN EMBRYOGENIC CULTURES OF Cyclamen persicum - EFFECTS OF ENVIRONMENTAL CONDITIONS

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Introduction

The use of antibodies towards embryogeneis specific proteins gives the opportunity for early prediction of embryogensis in suspensions, as well as locating the proteins in the cells by immunofluorescence. It would also provide a tool to develop magnetic beads that could be coupled to the antibodies and maybe be able to separate embryogenic cells from non-embryogenic. At the Norwegian University of Life Sciences we were collaborating with John Innes Centre, Norwich, UK for the development of such antibodies. We showed that within 24 h after removal of auxin, the protein profiles in birch changed dramatically (Hvoslef-Eide & Corke, 1997) and that more proteins disappeared than the number that actually did arise. We have found a similar situation in *Cyclamen persicum.*

Material and Methods

Callus cultures of *Cyclamen persicum* 'Purple Flamed' line 3738-14 was obtained through the COST 822 WG2 collaboration from Dr. H.-G- Schwenkel, of Erfurt, Germany. These cultures are grown in the dark at 25 °C on solid medium. We followed their protocol for obtaining suspension cultures (Winkelmann *et al*., 1998) and inoculated in 2 l fully automated bioreactors (Hvoslef-Eide *et al*., 2005). The 'Purple Flamed' also had a line that had never shown any embryogenic capacity, termed non-embryogenic. We have run several experiments with the embryogenic cell line 3738-14 and have had the non-embryogenic as control (Munster & Hvoslef-Eide, 1996; Hvoslef-Eide & Munster, 1997, 1998a, 1998b; Hvoslef-Eide *et al*., 1998, Hvoslef-Eide & Melby, 1999). The conditions we have been varying are; light quality, temperature and dissolved oxygen. In most cases we have taken samples for protein analysis for comparison between embryogenic and non-embryogenic conditions, as well as the non-embryogenic line as control. We have used both one-dimentional SDS-PAGE electrophoresis (BIORAD) and two-dimentional Multiphor II system to compare the different treatments and cell lines.

Results

We see a lot of differences in protein patterns for the various treatments and especially between the embryogenic and non-embryogenic cell lines. One-dimentional SDS-PAGE electrohporesis give some differences, but the two-dimentional Multiphor II typically has a much greater resolution and more differences were spotted. We will report on the finding from the 2-D gel scans comparing the various environmental treatments given in embryogenic and non-embryogenic cultures.

Discussion

Both in *Cyclamen persicum* Mill. and birch (*Betula pendula* Roth.), we see large differences between embryogenic and non-embryogenic cultures with regards to proteins present in the cultures. Using a non-embryogenic cell line as control offers the possibility to eliminate the effect of removing growth regulators like auxin from the medium. These two model plants both show far more proteins disappearing from the cultures

than the very few that actually do appear as a result of growth regulator removal. This is also in accordance with several findings in carrot cultures (Gregor *et al*., 1974; Zung & Okimoto, 1981, 1983; Kiyosue *et al*., 1991). Those proteins that seem to disappear from the cultures may not actually cease to be present in the embryogenic cell lines, but may become cross-linked into the cell wall in such a way as to render themselves unextractable (Bradley *et al*., 1992). If they genuinely cease to exist, it can be postulated that they are either turned over at a fast rate and so cease to be synthesed or that they are normally stable and are actively degraded at this transition point. It is not possible from our studies what the role of these proteins might be, structural, cell signalling/recognition or enzymes in the cell wall modification. Proteins like these may have an active role in the cell to cell communication and interactions. The fact that they become cross-linked or disappear may alter their wall texture in such a way that the cells more readily adhere to each other and are thus able to organise and form bipolar structures like embryos.

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MOLECULAR BASIS OF SOMACLONAL VARIATION IN RYE

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Introduction

Although somaclonal variation has been widely described in many species, the molecular mechanisms involved in their appearance are still not fully known. Rye is one of the species in which the somaclonal variation rate is quite high. We have tried to ascertain the origin of those changes and we performed RAPD (Linacero *et al*., 2000), using as a criterion to detect the variation the possible modifications of the pattern of amplified bands. This study reveals the existence of hot spots of mutation: the same sequence varied in several plants obtained from different cell lines . The study of these sequences was carried out in order to deduce, if possible, which type of variation occurred. We will refer to the studies we have carried out lately in which several types of modifications were detected using different technical approaches.

Material and Methods

Rye (*Secale cereale* L.) regenerated plants were obtained from cell lines obtained from immature inflorescences (cv.Ailes) and immature embryos (cv.Ailes and Merced). Some of the plants obtained from a callus looked normal and reached maturity, but others were albino and as this is a lethal condition these plantlets were frozen to be studied subsequently. RAPDs were performed using primers from OPERON Technologies and several hypervariable bands were detected. Different oligonucleotides were designed in order to perform SCAR and to amplify internal regions of the variable bands. IPCR techniques and/or TAIL-PCR were used to amplify the borders of these bands. In one case a real-time PCR study was carried out to confirm the amplification of the F13c sequence. In vivo growing plants of the same cultivars were also used in some of these studies.

Results

The sequence of several of the studied amplicons showed some similarities with mobile elements, however in most of the cases no relation existed, or could be proved, between the detected modification and the element. However, the analysis of three of these amplicons indicate the activation of two mobile elements. We propose that one of them, the RYS1 element, could be a foldback transposon (Alves *et al*., in press) and the other, RYRE-1, is a BARE-like retrotransposon. In both cases preferential integration points in the rye genome existed because the new insertions seemed to be located, in all the studied cases, in the same genome positions. The amplicon F13c, obtained from regenerated plants with the OpF13 primer, showed a higher intensity in all of the albino plants than in the green plants regenerated from the same cell lines. When the sequence F13c was used as probe in Southern hybridizations to confirm the presence/absence of the F13c band in the RAPD pattern, a faint signal appeared in the green plants which contrasted with the strong one present in the albinos. IPCR techniques and TAIL-PCR were used to amplify the border of this band . The analysed sequences indicate that the band was amplified from a fragment of a gypsy retrotransposon integrated into the mitochondrial genome of rye. The presence of a very conspicuous band in the amplification pattern of RAPD in the albino plants was due to the amplification of their mitochondrial

genome as a real-time PCR study indicated. This amplification was due to the albino nature of the plants and was not related with the mutation/s promoting the albinism because, at least in some of the cases, the mutate locus/ci seems to be nuclear. The study of other variable amplicons indicated that deletions or insertions could have been involved in the changes. In most cases SCAR, specific PCR, IPCR and TAIL-PCR were performed.

Different plants from the *in vivo* growing cultivars showed polymorfism for the presence or absence of the variable amplicons.

Discussion

The study of the mechanisms involved in somaclonal variation requires the application of combined molecular techniques, mainly in species like rye, with a complex and highly variable genome. According to our experimental approach, although sometimes the study of the sequence from the variable amplicons indicated the nature of the change, in most cases the sequences flanking these bands had to be studied to know the kinds of mutations which originated the modification. Different mutations were involved in the variations of the RAPD pattern that we found in rye. However, as our study of the albino plants reveals, we must be critical about the interpretation of the presence/absence of some of the variable amplicons.

The polymorfisms that we found in some particular loci, in both *in vivo* and *in vitro* populations, could indicate that the mechanisms by which plants respond to stress in nature, are probably the same as those inducing the variation found *in vitro*, and that the modifications induced by somaclonal variation could be already present in normal populations

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PLANT CYTOMICS: A NEW WINDOW FOR PLANT BIOLOGISTS

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Abstract

The concept of cytomics as "*the multiparameter cytometric analysis of the cellular heterogeneity of **cytomes** (cellular systems/organs/body), resulting in the maximization of information on the **molecular cell phenotypes** from cell **genotypes** and **exposure***[1]" brought together three main fields (genomics, proteomics and cytomics) in approaching biological questions, in interdisciplinary and complementary perspectives.

This elegant definition of cytomics opened a new look to the multidisciplinary study of any living structure, where, the edge of any researcher will be to combine the ultimate information given by the genomic and proteomic approaches with a more holistic - and perhaps more real one - , that is, a multiparametric approach of the phenotype of that individual…

Although well developed in animal sciences, plant cytomics is still incipient. This communication aims to introduce the most important advances in plant cytomics (using flow cytometry, FCM) to beginners and explore some of its broad potentialities as well as good practices for achieving reliable results. The first example of the application of FCM will focus on nuclear DNA content and ploidy level of plant species (the most common application). These types of studies were already preformed in micropropagated plant species where FCM was used to assay the ploidy stability and cell cycle of some plant species (e.g. eucalyptus, cork oak, elm, olive). Also, FCM has potential for taxonomic studies as this technique can easily estimate the genome size and the ploidy level of different species/ecotypes. A last example is the use of this technique to assess genotoxicity in ecotoxicological studies.

We hope that the given examples may raise the interest on FCM and that researchers feel useful the creation of a network of European laboratories working on FCM and interested researchers.

Acknowledgements

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ASSESSMENT OF GENETIC VARIABILITY AND DIFFERENTIAL IMPRINTING OF ZYGOTIC AND APOMICTIC PROGENY IN Citrus VIA AFLP MARKERS

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Introduction

In the past, characterisation of Greek Citrus propagation materials has been based on phenotypic, terpene and isozyme characters (Protopapadakis and Papanicolaou, 1998; 1999). Further, distinction of apomictic from zygotic Citrus seedlings was not feasible until trees had passed juvenile stage. The objectives of the present study were twofold. The first objective was to assess genetic relatedness and to verify origin of Citrus hybrids via molecular markers while the second was to determine epigenetic differences between zygotic (sexual) and apomictic (nucellar) seedlings.

Materials and Methods

We have employed standardised Amplified Fragment Length Polymorphic (AFLP) molecular markers (Vos *et al*., 1995) in order to answer most of the horticultural and breeding questions of the present study (first objective). Plant material originated from various controlled crosses. For our second objective, we have attempted to assess imprinting status of sexual and apomictic (nucellar) *Citrus* progeny. An alternate AFLP methodology was employed for that purpose (Cervera *et al*., 2002). This second AFLP methodology involved, as restriction enzymes, isoschizomers that differ in their sensitivity in the methylation status of their recognition sequences. Restriction enzyme combinations were EcoRI/HpaII and EcoRI/MspI. Enzymes HpaII and MspI are isoschizomers but are expected to restrict differential DNA fragments. Subsequently produced amplification patterns would reflect methylation differences. For this objective sour orange (*Citrus aurantium*) was employed as pollen donor while citrumelo #1452 (*Poncirus trifoliata* X *Citrus sinensis*) and a local rootstock (belonging to the *Citrus limon* group) served as maternal plants. The descendants of these crosses were grouped *ex-ante* into zygotic (sexual; true hybrids) and nucellar (apomictic) seedlings based on their phenotypic (horticultural) characteristics as well as on their molecular fingerprints (standard AFLP).

Results and discussion

The phenogram produced following standard AFLP analysis of the citrus propagation material agreed well with classification produced before following horticultural and isozyme characters. The efficiency of a methylation sensitive AFLP methodology towards detecting methylation pattern changes between citrus progeny was also assessed.

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COMPARISON OF MICROSATELLITE MARKERS AND RETROTRANSPOSON BASED MARKERS (SSAPs) FOR THE DETECTION OF GENETIC VARIATION IN OAK SOMATIC EMBRYOS

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Introduction

Somatic embryogenesis as a vegetative propagation method requires monitoring methods to assess genetic uniformity. Cytological and molecular methods (RAPDs, AFLPs, SSRs) have been used to monitor genetic stability in somatic embryos (SEs) and produced plantlets of the genus *Quercus ssp*. (Pinto *et al.*, 2004; Sanchez *et al*., 2003; Hornero *et al*., 2001; Thakur *et al*., 1999; Endemann *et al*., 2002; Wilhelm *et al*., 2005). Retrotransposons are mobile genetic elements and ubiquitous in plant genomes. Stress activation of plant retrotransposons (e.g. via tissue culture) has been identified as a factor in somaclonal variation (Hirochika *et al*., 1996). Several long terminal repeat sequences of Ty1-copia retrotransposon elements have been identified in oak as markers for population studies (Berenyi *et al.*, in prep.), and they were used in sequence specific amplified polymorphism (SSAP) analysis. SSAP is a multiplex amplified fragment length polymorphism (AFLP) like technique, where one of the primers is based on specific retrotranspons sequences. We evaluated the applicability of the oak SSAP marker system for assessing genetic variability in SEs and derived plantlets of pedunculate oak (*Q. robur* L.) In addition we compared the results obtained from the SSAPs and SSRs marker systems. Comparisons were made among and within five embryogenic culture lines.

Material and methods

The plant material, DNA isolation, microsatellite analysis was performed after the procedures described in Wilhelm *et al.* (2005). The S-SAP markers and primers for oak were isolated after the method described in Berenyi *et al*. (2002).

S-SAP analysis:

Genomic DNA were digested with EcoRI restriction endonuclease and ligated with EcoRI adaptor sequences in one reaction at 37 °C four Hours or over night. The rest adaptor sequences were removed with PCR fragment purification Kit (Qiagen). PCR amplification was performed with the digested/ligated genomic fragments with adaptor specific (Eco1) and transposon specific (Qtr56G and Qtr 55T) primers. The transposon specific primers are labelled with fluorescent FAM6 dye. The PCR amplification was performed with HotStar DNA polymerase (Qiagen) in 25µl reaction volume without oil. Amplifications were performed on PTC 100 thermocylers (MJ Research, Waltham, Mass.) using the following amplification profile: one cycle of 15 min at 95 °C, 8 cycles of 30 s at 95 °C, 30 s at 55 °C, 90 s at 72 °C (-1 °C/cycle), one 30 s cycle at 95 °C, 35 cycles 30 s at 48 °C, 90 s at 72 °C and one-10 min cycle at 72 °C. 0.3µl PCR product was mixed with 15µl HiDi formamide and 0.25µl ROX500 marker than separated with ABI 3100 sequencer.

Analysis

Analysis of the amplified fragments were made by the Genotyper computer program. Phylogenetic tree analysis was made by the TreeCon computer program.

Results and discussion

The genetic variability of five embryogenic cell lines was assessed using SSRs and SSAPs marker systems. With the SSRs markers, DNA variation was detected among SEs within all embryogenic lines, whereas no genetic instability was found among the regenerated plants. However the two cell lines displaying the most mutated SSRs loci lost their regeneration potential.
The same oak DNA samples were also analysed via the SSAP marker technique. In total 180 insertions were found with primer Qtr56G and 203 insertions were detected with primer Qtr55T. However the distribution of single insertions per sample is of particular interest. Therefore primer Qrt56G was found to be more polymorphic compared to primer Qtr55T. Cluster analysis reveals that the SSAP marker system is a very sensitive method, differentiating each single embryo and plantlet. However most of the embryogenic cell lines and the regenerated plantlets are clustering into distinct phylogenetic groups. In addition, also several putative insertions have been detected. In contrast to the SSR markers, where no variation was found in the regenerated plants, the insertions were also detected both in somatic embryogenic tissue as well as leaf tissue derived from the regenerated SE plants.
In conclusion the SSAP is a very sensitive method for the detection of “genetic noise” in oak. In addition this method seems to be attractive for screening large sample numbers and good enough to predict new insertions, but not solid enough to prove them. To verify new insertions, sequencing of these particular regions would be necessary.

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MOLECULAR CHARACTERIZATION OF TOMATO MUTANTS (*Lycopersicon esculentum* L.)

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Introduction

The power of AFLP and ISSR is based upon the molecular genetic variations that exist between closely related genotypes. These variations are exploited by both techniques, so that "fingerprints" of mutant genotypes can be generated showing changes occurred in DNA sequences. The present study was therefore designed to assess DNA polymorphism between mutant tomato lines and a wild type line, which reveals genetic changes, obtained after applying distinct irradiations of explants *in vitro* with Co^{60} gamma-rays.

Material and methods

Included in this study were: wild type tomato CommodoreGCR758 (WT - fertile with indeterminate habit); M_3 to M_5 generations of mutant with male sterility (M_{ms}); irradiated fertilizing line (M_f); mutant with determinate habit (M_{dh}).

Mutants were developed using distinct irradiation by 20 and 30Gy of γ-rays ^{60}Co of callus from explants, micropropagated and grown in soil for studying and seed formation (descriptor list for *Lycopersicon* (IPBGR, 1996) .

DNA was isolated from 100 mg *in vitro* sustained leaf material from explants following the protocols of Tai *et al*. (1990) and Pich *et al*. (1993).

ISSR-PCR (Inter Simple Sequence Repeats Polymerase Chain Reaction) method was performed, using for DNA amplification primers: 5'-$(CA)_6$(G/A)(C/T)-3'; 5'-$(CA)_6$(G/A)G-3'; 5'-$(AGC)_4$(C/T)-3'. The amplified products were separated on 2% Agarose, high gel strength (NuSieve).

AFLP (Amplified Fragment Length Polymorphism) method was conducted according to the protocol of Kashkush *et al*. (2001). The reaction was performed on a genomic DNA using the enzymes *Eco*RI, *Mse*I, adaptor pairs and pre-selective primers and selective primer pairs *Eco*RI/*Mse*I: ACT/CAT, ACA/CAT, AGG/CTT, ACT/CAG, ACC/CAC. *Eco*RI-based primers were end labeled with γ-ATP-P^{32}.

Results

ISSR-PCR method was performed on all genotypes included in this study and polymorphism was shown in both studied mutants with one of the applied primers 5'-$(CA)_6$(G/A)(C/T)-3' only. M_{ms} differs from the WT by two bands among 11 well reproducible bands. The pattern of the sterile line (M_{ms}) and the fertilizing one (M_f) were identical and differed from the non-irradiated WT line. Polymorphism between mutant with determinate habit and wild type with indeterminate one was also revealed by one polymorphic band.

The selective primer combinations of AFLP, which proved the mutation changes in the male sterile mutant plants and differentiated them from the WT were: ACT/CAT, which revealed 4 polymorphic bands, ACA/CAT - 2 polymorphic bands, AGG/CTT - 4 polymorphic bands, ACT/CAG 1 polymorphic band. The primer combinations, which differentiate the mutant with determinate habit from the WT, were: ACT/CAT - 15 polymorphic bands, ACA/CAT - 3-4 bands, AGG/CTT – 6 bands, ACT/CAG – 6 bands.

Discussion

The induced mutation factors usually affect more regions all over the genome. Only a little part from the mutations is expressed in a change of a character, which can be detected as different phenotypic trait. The identity of both patterns of M_{ms} and M_f illustrated the established ISSR polymorphism, which was not related to the male sterility. A new fragment amplified in the M_{dh} assessed the change occurred on DNA level after irradiation. Most mutant individual plants possessed identical ISSR and AFLP patterns among them and differed from the WT with the same bands. However in single plants appertaining to the same mutant, variability was detected. The studied generations were M_3, M_4, M_5 and partially the polymorphism shown on the gels was due to the heterogeneity. To develop M_8 and subsequent advanced generation mutants useful for breeding, more backcrosses between M and WT and following selection for the target character would be achieved.

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DIRECT SOMATIC EMBRYOGENESIS IN PEA (*Pisum sativum* L.): THE STUDY OF GENETIC STABILITY BASED ON FLOW-CYTOMETRY, PROTEIN AND DNA MARKERS

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Introduction

In our previous studies we demonstrated that indirect (callus-mediated) somatic embryogenesis in pea (*Pisum sativum* L.) may generate relatively great phenotypic / genotypic variation in primary regenerants and their seed progenies (Stejskal & Griga, 1992; Griga & Stejskal, 1993; Griga & Létal, 1995; Griga *et al*., 1995; Griga, 2000, 2002; Wiesner *et al*., 2001). However, there is no information in the literature on the genetic status of pea regenerants produced via direct somatic embryogenesis (Tétu *et al*., 1990; Loiseau *et al*., 1995; Griga, 1998). Here we report the results of the evaluation of genetic stability / variability of direct somatic embryos and somatic embryo-derived plants based on the analyses of DNA content (ploidy level) as well as on the use of protein (total proteins, isozymes) and DNA markers.

Material and Methods

Plant material: Dry-seed pea line HM-6 and canning pea cv. Oskar (both with good embryogenic competence – Griga, 1998) were used in the experiments.

Flow cytometry: Nuclear DNA content was analysed with the use of Partec PAS (Münster GmbH, Germany) according to producer's manual. Control I represented 2-week-old plants grown from the seeds in perlite saturated with MS salts (16 h photoperiod) - shoot apices, leaves, stem segments and root tips were analysed. Control II represented 3-5 day-old seedlings germinating in dark (ordinary plant material for isolation of apical meristems for direct somatic embryogenesis) - shoot apical meristems and root tips were analysed. As tested samples, globular, heart, torpedo, and cotyledonary somatic embryos, and callus occurred on embryogenic explants were harvested. Finally, plants regenerated from the ordinary germinated somatic embryos (R0, R1) and plants regenerated from the somatic embryos after desiccation treatment (R0, R1) were evaluated - shoot and root apices, stem segments and leaves were analysed similarly as the control plants.

Total proteins and isozyme analyses: Total proteins and selected enzymes (ADH, EST, AAP, ACP) in individual somatic embryo stages were analysed by SDS-PAGE (Stejskal & Griga, 1995) and by NATIVE-PAGE (Pošvec & Griga, 2000), respectively, and compared to protein patterns of seed samples of particular pea cultivars.

DNA (RAPD) analyses: The globular, heart, torpedo and cotyledonary (regular and irregular) somatic embryos and *in vitro* regenerated pea plants, were evaluated using RAPD analyses. Altogether, eight primers (OPW01, OPW02, OPW08, P9, P10, P14, UBC556, UBC741) were used for analyses (Samec *et al*., 1998).

Results and Discussion

Flow cytometry: The results confirm literature data on certain cytological heterogeneity (ploidy changes; aneusomaty) of tissues of both intact pea plants as well as *in vitro* cultures and derived regenerants (Natali & Cavallini, 1987; Ochatt *et al*., 2000). Regardless the majority of cell population of various parts in the same plant was found in various phases of the cell cycle (e.g. shoot apical meristem in the G1, root apical meri-

stem in G2 phase), the results just show that control plant material (CI, CII) and plants regenerated *in vitro* (R0-, R1-regenerated plants after / without desiccation, somatic embryos and calli) had either standard (2C=2n=14, 4C=4n=28) or slightly altered DNA content (mostly diploid tissues exhibited also haploid 1C=1n=7, octoploid 8C=8n=56 or aneuploid cells: n<7, n<28) - Fig.1. However, detected aneusomaty (= presence of cells of various ploidy or euploid and aneuploid cells within certain tissue) was not connected with visually detectable morphological / physiological changes in analyzed plants.

Total proteins and isozyme analyses: Somatic embryos contained identical spectra of total proteins as compared to mature sexual seed - mainly typical storage proteins were expressed. They included 7S and 11S class globulins - vicilin (major subunits M_r ~ 47,000 - 50,000; minor subunits M_r ~ 25,000; 30,000; 34,000), convicilin (M_r ~ 70,000 - 75,000), acidic legumin α (M_r ~ 40, 000), basic legumin β (M_r ~ 20,000). Another proteins abundantly expressed, which may also behave as storage proteins, were lectin β (M_r < 20,000) and lipoxygenase (M_r ~ 97,000). Quantitative differences in total protein expression were detected between somatic embryos and seed (developmental expression of storage proteins from globular to cotyledonary stage). Desiccation treatments (rapid and slow desiccation in dark/light) did not affect the total protein spectrum but only intensity of protein accumulation or degradation (embryo germination). No new protein / polypeptide specific for somatic embryogenesis and not found in the seed was recorded. Isozymes of ADH, EST, AAP, ACP analysed in heart, torpedo and cotyledonary somatic embryos displayed stable spectra, only quantitative differences were recorded.

DNA analyses: RAPD analyses have shown negligible variation in pea somatic embryos and embryo-derived plants; mostly presence of quantitative differences between relevant DNA fragments was observed (Fig. 2). No phenotype alterations were detected in regenerants considered as "stable" by means of RAPD analyses.

Conclusion

Detected aneusomaty or minor changes in RAPD profiles probably do not have dramatic influence on development and viability of pea somatic embryos (direct somatic embryogenesis without callus intervention) and on embryo-derived plants. We did not observed any phenotype changes within R0 and R1 plants analysed in this study. In contrast to callus-mediated pea somatic embryogenesis which may be a source of significant instability (Griga, 2002), direct somatic embryogenesis seems to be a regeneration system less affected by somaclonal variation phenomenon and thus suitable for genetic transformation. The first results with the use of direct somatic embryogenesis protocol for biolistic transformation of pea are available in our laboratory (Švábová *et al*., 2003; Švábová – unpublished results).

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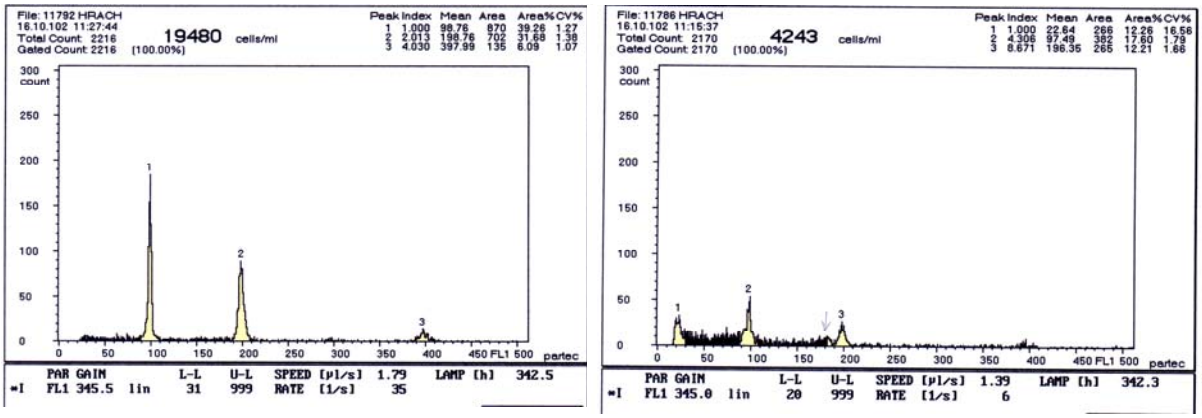

Figure 1 Examples of DNA content in cell populations of cotyledonary stage somatic embryos of pea cv. Oskar. Both graphs represent single somatic embryos. Peak numbers correspond to a DNA level 2C for peak 1, 4C for peak 2 and 8C for peak 3 (left graph), and 1C for peak 1, 2C for peak 2 and 4C for peak 3 (right graph)

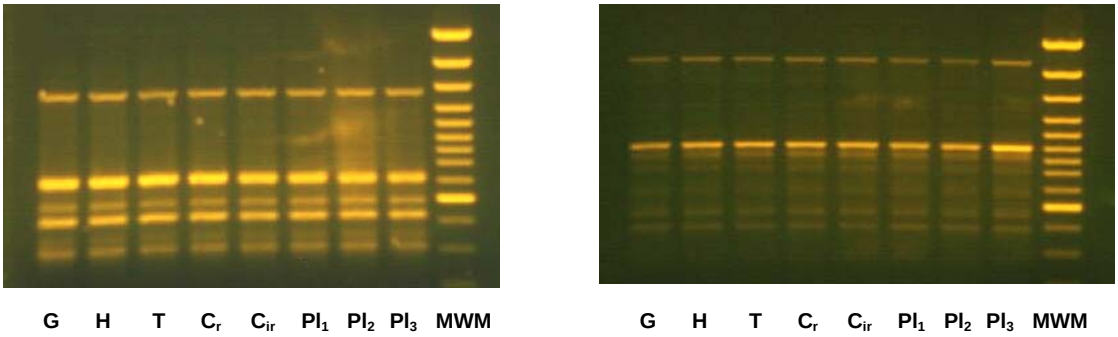

Figure 2 Examples of RAPD profiles generated by primer UBC741 (left) and P9 (right) in pea (line HM-6) somatic embryos (G - globular, H - heart, T - torpedo, C_r - cotyledonary regular, C_{ir} - cotyledonary irregular) and embryo-derived R0 plants (Pl_1 - Pl_3)

THE PCMV RESEARCH WORK ON BIOTECHNOLOGICAL APPROACHES AT THE URLEG, INRA DIJON

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Plant biotechnology has shown its large potential as a breeding tool in the past years, through the generation of genetic novelties by an enlargement of the variability between genotypes within a species. The characterisation of any novel clone obtained is, in this respect, a prerequisite for the efficient exploitation of in vitro biotechnology in breeding for an enhanced quality, for an increased resistance to biotic or abiotic stresses, or for an improved productivity in general and for all species of interest, but also for model species. Classical methods of hybridisation and selection have been used successfully over the centuries for the genetic improvement of plant species and have yielded a large array of cultivars responding to various production systems and the global food and feed demand. Conventional methods, though, are time-consuming and require the presence of the appropriate genes to succeed. There are nowadays several biotechnological approaches that have become very useful tools to accelerate the selection process, to increase the genetic variability, and event o improve the product quality, thereby speeding up breeding. This flow of methodologies also facilitates the detection of the genetic mechanisms underlying the phenotypic expression on which conventional breeding is based. The main challenge to any geneticist is to detect the maximum association possible between phenotypic variations and the genes responsible for them. On the other hand, the demanding selection processes, coupled with the permanent genetic erosion in plant species, be it by nature or due to the man's conquest of new productivity niches make the preservation of genetic resources and the generation of new ones of paramount economical, commercial and scientifically strategic importance for any country or culture. In this context, biotechnology tools represent a further step towards an ecologically sustainable agriculture.
Over the last years, the Cell Physiologie, Morphogenesis and Validation team (PCMV) at the URLEG in INRA Dijon has developed and exploited a rather large range of biotechnological strategies and applied both to the breeding of several economically important species and also to the understanding of some of the fundamental mechanisms underlying the in vitro responses observed and the novel genotypes obtained. Such research work has concerned protein legume species including *Pisum sativum* (protein pea), *Lathyrus sativus* (grass pea), *Vigna subterranea* (Bambara groundnut), *Medicago truncatula* (a model species of forage interest), *Arabidopsis thaliana*, and several horticultural, condiment and aromatic species such as the brown mustard (*Brassica juncea*), asparagus (*Asparagus officinalis*), onion (*Allium cepa*), garlic (*Allium sativum*) and tarragon (*Artemisia dracunculus*), as depicted in Figure 1.

The results obtained in the different programmes, described in an abridged manner, include [(1)]:

- In protein legumes: the development of reliable strategies for the regeneration of whole fertile plants from protoplasts, cell suspensions and protoplasts of a wide range of genotypes of the species studied coupled with a detailed characterisation of the regenerants produced through their phenotype and by flow cytometry, isoen-

zymes, molecular methods as compared to the mother plant genotype. A separate series of experiments with these species permitted the production of hybrids between pea and some of its wide relatives. Also with these species, and later on extended to others, an original strategy for the acceleration of generation cycles that encompassed the induction of flowering and fruiting on rootless shoots was developed and used for SSD. Somatic hybridisation studies were also performed between pea and grass pea, and several early indicators of the embryogenic competence of cells in culture (the cell wall thickness, the osmolarity of the medium and cells, cell size and volume, …) were identified. Also interest with this group of species were studies that revealed the genetic basis of hyperhydricity of regenerants. Finally recent experiments permitted the production of genetically transformed tissues and plants, the latter being used for the understanding of gene function in *Medicago truncatula*.

- With brown mustard, androgenesis from isolated microspores was applied for the production of a population of double haploid lines which were thereafter exploited as the genetic material for the establishment of the first gene map in *Brassica juncea*, whereby the genetic determinism of glucosinolate content, seed coat colour, fatty acid synthesis and several of the yield components was from partially to completely explained.
- In asparagus, the main goal of the ongoing research is to produce double haploids which are supermale, to improve the productivity and quality of turions. For this, both anther and microspore culture have been applied and, among significant results obtained, special mention is due to the promoting effect of microspore electroporation on their embryogenic competence.
- With onion, haplo-diploidisation this time via gynogenesis through regeneration from unfertilised flowers has served to improve the dry matter content of bulbs by increasing the level of high degree of polylmerisation fructans. On the other hand, work with garlic in the past years was aimed at understanding and exploiting the beneficial health (anti-cancer) properties of alliin present in the bulbs.
- Finally, work with tarragon, a hitherto little studied species in vitro, was aimed at improving the aromatic properties of this species and included a wide range of biotechnological approaches, from propagation to regeneration from callus, cell suspensions and protoplasts and followed by the characterisation of regenerants produced.

The availability of reliable regeneration strategies for the production of fertile plants permits to envisage now a better use of biotechnology for the breeding of many species now, be it through the application of in vitro selection procedures, somatic hybridisation strategies, haplo-diploidisation approaches or gene transfer technology. More generally, if coupled with the extensive characterisation of the regenerants produced, these results open up the way for the rapid and efficient utilisation of such regenerants as genitors for conventional breeding of *per se*. Several of our results underline, in addition, the utility of other, less conventional tools such as flow cytometry as an early screening strategy for the avoidance of hyperhydricity in cultured tissues but also for the optimisation of the plant regeneration process, since this was shown to depend on a true-to-type DNA content in the cultured tissues. Likewise, the possibility to reduce the duration of generation cycles is of the utmost importance to gain time for breeding of any species. On the other hand, doubled haploids can be used not only for immediate breeding purposes (as for asparagus and onion here), but also for a detailed study of the genetic mecha-

nisms underlying the various phenotypes observed, and mainly for gene mapping (as exemplified in our work with brown mustard).
The summation of results obtained with these various species stresses the mutual benefits of working with different species when a common intellectual approach is applied. More importantly, it stresses that biotechnology-based approaches are now of age, and can be successfully and efficiently applied for the generation of genetic novelties in a wide range of species.

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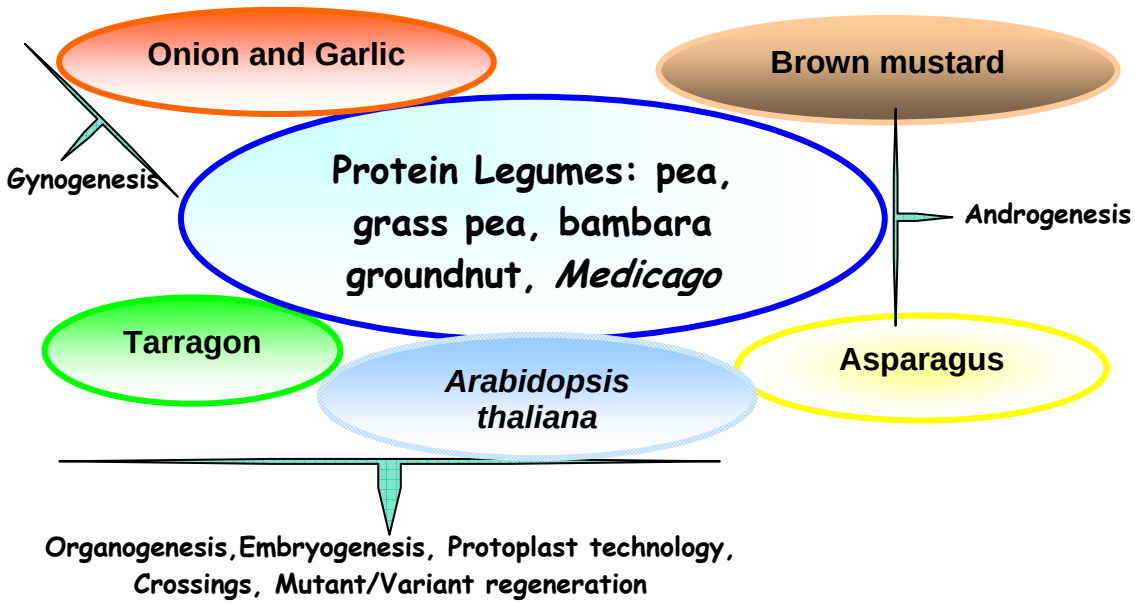
Figure 1 The biotechnology-based research programmes underway at PCMV team, URLEG, INRA Dijon



GENERATING A POPULATION OF TNT1 PRIMARY TRANSFORMANTS IN THE MODEL LEGUME *Medicago truncatula* FOR GENETIC ANALYSES

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Introduction

The annual forage lucerne species *Medicago truncatula* was proposed as a suitable model legume[2,4] and has been widely adopted for genetic and genomic analysis since. It can be regenerated by organogenesis[5] or somatic embryogenesis[1,3,4,6], transformed with *Agrobacterium tumefaciens* [1,3,5,6], nodulated by the symbiotic bacterium *Rhizobium meliloti*[2] and the true-to-typeness of regenerants examined [4]. This work has been aimed at i) comparing some of the regeneration methods in the literature[1,6] using J5 and 2HA, ii) generating a population of regenerants of BC4.1.tr from one in vitro plant with six leaves of *M. truncatula* Jemalong Tnt1 (BC4.1.tr), iii) producing primary transformants of genotype 2HA using AGL1 as bacterial strain, and iv) examining by flow cytometry the true-to-typeness of all regenerated materials (transgenic or not).
Comparison of regeneration methods by Trinh *et al.*[6] and Chabaud *et al.* [1]
The large numbers of J5 and 2HA plants produced and time required with each method showed that

➔ Chabaud's protocol1 was more efficient and less genotype-dependent

➔ Trinh's strategy[6] was well adapted to R108 but gave few regenerants with J5 and 2HA

Shortening the method from Chabaud[1] by deleting the EDM passage improved results. All plants produced skipping the passage on EDM were true-to-type. A similar trend was observed for J5.

➔ At least 50 % of embryos converted into plants, irrespective of the method used

➔ Embryo mortality and regeneration of enfeebled, hyperhydric plants were systematically correlated with endoreduplicated or non-homohistont tissues.

Production of regenerants from BC4.1.tr

On 22/07/04 we received one *in vitro* plant with six leaves of *M. truncatula* Jemalong *Tnt1* (BC4.1.tr). This was cut into 4 cuttings transferred onto MS0 medium (hormone-free MS) which gave 4 BC4.1.tr plants three weeks later. On 12/08/04, new cuttings were prepared from such plants and were subcultured onto SHb10[1, 6] medium to give 11 plants.
Leaf explants were regularly prepared and cultured on CIM medium for callus initiation, while the remaining material BC4.1.tr plants was multiplied on SHb10 medium for further experiments. After three weeks, green calluses were transferred to EIM medium for the induction of embryos. Embryos were regularly isolated in PDM medium for developpement of embryos into plants. These plants were cut into cuttings and grown on PDM. One cutting of each plant (number) was transfered to the greenhouse for seed production and molecular and cytometry analyses from leaves and another one was kept *in vitro* as a backup.
To date, 1897 explants have been cultured, 1062 calluses were obtained, 1910 embryos have been isolated from 306 individual regeneration events, transferred onto PDM medium and 644 plantlets of 187 numbers have been produced from different calluses. At present, 139 of such plants, representing each one regeneration event, are growing in the greenhouse. Analyses undertaken over the last few months have included DNA extraction, PCR and Southern blotting and also flow cytometry assessments.

Production of new primary transformants of 2HA

By the end of October 2004 we received 16 pods of *M. truncatula* 2HA, of which we kept 3, and recovered 100 seeds from the remaining 13 pods. Of these, 50 seed were scarified, disinfected and germinated on medium SHb10[1,6]. Several transformation experiments, using the bacterial strain AGL1 and including several hundred folioles from germinated seedlings of regenerants produced in our lab each were performed. Thus, with leaves from plants regenerated

in our lab, the best results were obtained, with about 40% of explants producing calluses on selection medium (78 of 200 and 67 from 150 explants). With genotype R108 and C58pMP90, carrying pCambia plasmid which confers resistance to hygromycin (*hphI*) and GUS intron gene under the control of 35S promoter, a final % of primary transformant plant production of about 8% of the initially transformed explants.

Assessment of primary transformants and regenerants by flow cytometry

We analysed both regenerated materials and primary transformants by flow cytometry, compared to tissues taken from the mother plant genotype 2HA. For a normal material, nuclei appear as populations with two peaks of epifluorescence corresponding, the first one, to G1 phase nuclei and, a second one, to G2/M nuclei. For normal tissues, MI should be equal to 2.000 and the frequency of division always lower than the MI values. Deviations in either or both of these parameters indicate an abnormal DNA synthesis during mitoses and are usually reflected in the analysed profile by a modification in the means of emitted fluorescence between the first and second peaks. Analysing the position of the G1 peak alone, already gives a clear indication of the trueness-to-type or not of the analysed tissues and, for some materials, we therefore decided to represent graphically this parameter alone. For a true-to-type material, the peaks should have a mean comparable to that of the mother plant (>15% in excess or in defect indicate an abnormal DNA content per nucleus), and not more than two peaks per profile. An additional analysis consists of looking at the mitotic index (MI = 4 X 4CDNA/2CDNA + 4CDNA) and the division frequency.

Of 238 regenerants of 2HA produced in our lab characterized by flow cytometry, 22% of those obtained with Trinh *et al*. method[6] were either endoreduplicated, mixoploid or tetraploid, while 8% of those produced with Chabaud *et al*. protocol[1] differed from the mother plants (mostly endoreduplicated, a few mixoploid). We therefore analysed also similar plants produced in other labs. One such series of experiments, concerned 22 primary transformants and 8 regenerants (one leaf per genotype) received from M. Chabaud's team in Toulouse (FR). Interestingly, of the 30 genotypes assessed, five only proved not to be true-to-type, in line with previous results from our laboratory[4] for regenerants obtained with this protocol.

Finally, we also analysed all regenerants of BC4.1.Tr obtained in our lab with our modified method described above, and found that very few of them deviated from the mother plant genotype, maybe due to the reduction of the number of culture passages we used.

Acknowledgements

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PRODUCTION OF TRANSGENIC GRAPE ROOTSTOCK 'RICHTER 110' HARBOURING THE *ROL*B GENE FROM *Agrobacterium rhizogenes*

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Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) affects European grape (*Vitis vinifera* L.) cultivars by destroying the roots. After its accidental introduction from North America to Europe in 1860, grafting of *V. vinifera* scions onto phylloxera-tolerant North American *Vitis species* hybrids serving as rootstocks has been developed as general practice of grapevine propagation. In addition to protecting from phylloxera, the available assortment of selected rootstock cultivars mediates adaptability to a wide range of soil and climatic conditions. Goals in grape rootstock genetic improvement comprise high tolerance / resistance to phylloxera as well as other soil-borne pests and diseases, high graft compatibility and rooting ability, nutrient effiency, draught tolerance and resistance to adverse soil conditions. Breeding of new rootstock cultivars through crossing is complicated by the high degree of heterozygosity in grapes, accounting for unpredictable recombination patterns and dissolution of valuable trait combinations. Genetic transformation could accelerate rootstock breeding, as it permits the addition of single traits, largely without affecting the genetic background. In the present paper we report first results of transferring the *rol*B gene of *Agrobacterium rhizogenes* to the grape rootstock *Vitis berlandieri* × *V. rupestris* 'Richter 110'. Insertion of *rol*B has been useful in promoting rooting in other woody fruit crops, *e.g.* apple (Welander *et al*., 1998) and pear (Zhu *et al*., 2003). In grape rootstocks, higher numbers of roots per graft and stronger root system could increase reliabilty and efficiency of propagation and also might improve adaptation to poor soil conditions.

Material and Methods

Embryogenic callus from somatic anther tissues was obtained by a two-step procedure basically according to Bouquet *et al.* (1982) and Mauro *et al*. (1986). Anthers excised from disinfected flower buds that had been stored during 2-5 days at 4 °C were incubated at 28 °C in the dark in liquid N69 (Nitsch, 1969) primary medium (IM) supplemented with 0.25 $mg.l^{-1}$ BA and $1mg.l^{-1}$ 2,4-D. After 4 weeks, anthers with callus were transferred onto agar ($7g.l^{-1}$)-solidified N69 secondary medium (EM) containing 0.5 $mg.l^{-1}$ BA, 0.1 $mg.l^{-1}$ NAA and 250 $mg.l^{-1}$ casein hydrolysate (CH), and were incubated in the dark at 24 °C. Embryogenic callus used for transformation was subcultured on EM modified according to Perl *et al*. (1996) by adding 4 $g.l^{-1}$ polyvinylpolypyrrolidone (PVPP) and 100 $mg.l^{-1}$ dithiothreitol (DTT). Transformation was mediated by *Agrobacterium tumefaciens* LBA4404 harbouring plasmid pHrB containing the *rol*B gene under control of its own promoter and the *hpt* gene conferring hygromycin resistance. Inoculation and co-cultivation with agrobacteria was performed with minimum handling according to Oláh *et al*. (2003). Ten to twenty µl each of undiluted overnight bacterial suspension were applied on top of embryogenic calli about 5-10mm in diameter which were then left for co-cultivation without prior washing and transfer to fresh medium. After 2 days, the calli were transferred to selection medium (= modified EM + 200 $mg.l^{-1}$ carbenicillin + 300 $mg.l^{-1}$ cefotaxim + 10 mgl^{-1} hygromycin). For embryo germiation and plantlet development somatic embryos were transferred to KM (= N69 + 0.2 $mg.l^{-1}$

BA + 150 mg.l^{-1} CH) and variations thereof, and were incubated at 24 °C with 14 h light per day. DNA of putative transgenics was examined by PCR for presence of transgene sequences, using *rol*B- and *hpt*-specific primers.

Results

Control calli which were not co-cultivated with agrobacteria and were placed on selection medium first stopped growing, then completely turned dark brown and finally died after a further transfer to fresh selection medium. In contrast, sectors of vigourously growing tissue with newly formed somatic embryos were produced from points of calli that had been co-cultivated with agrobacteria and transferred to selection medium. Well growing callus sectors were isolated and grown during up to 7 passages on selection medium without loss of vigour. Conversion of embryos proved difficult; most plantlets were malformed and stopped growing. Addition of activated charcoal (2 g.l^{-1}) to the medium improved the yield of normal plantlets. Until now, putative transgenic plantlets that are well developed could be recovered from >20 different original calli. Plantlets of 6 different origins have been transferred to soil and successfully acclimatized to greenhouse conditions. DNA analysis of plantlets of 6 different putative transgenic lines by PCR revealed the presence of *hpt* sequences in all of them, while *rol*B sequences were found in only four.

Discussion

Despite many reports on successful transgenic plant production in grape, routine transformation remains difficult. Mainly, this is due to highly genotype-dependent regeneration ability, often with low embryogenesis induction and/or low embryo conversion rates, the latter being true in 'Richter 110'. Further, conditions of *Agrobacterium* co-cultivation need to be carefully equilibrated, the avoidance of oxidative stress being one important point (Perl *et al*., 1996). The present results suggest that minimum handling (Oláh *et al*., 2003) may be equally important. Also, choice of bacterial strain, selectable marker and selection conditions need careful consideration for each grape genotype. In previous experiments we had used *npt*II as selectable marker gene, but never obtained transgenic plants of 'Richter 110', which may be attributed to relatively high kanamycin tolerance of this cultivar. According to our knowledge, this is the first report on whole plant transformation of grape with the *rol*B gene. Transgenic plants are currently multiplied *in vitro* and in the greenhouse for future investigation of the effects of *rol*B expression on rooting and growth performance.

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GENETIC DIVERSITY AND POLYMORPHISM IN CUCUMBER (*Cucumis sativus*) VARIETIES AS ASSESSED BY RAPD MARKERS AND MORPHOLOGICAL CHARACTERS

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Introduction

Although genetic analysis by RAPD molecular markers is a popular method for the assessment of genetic diversity, it's application to *Cucurbitaceae* species has been used only recently. The results of it's first application in cucumber indicated that the pattern of RAPDs is not affected by the age of the tissue, infection of plants from some diseases and the presence of fruits. RAPD markers have been used ever since in genome study, map construction and detection of polymorphism in varieties of different *Cucumis* species, and their advantages compared to RFLP markers have been reported (Staub *et al.*, 1996; Lopez-Seze *et al*., 2002; Staub *et al*., 2004). The purpose of the present work was the detection of genetic diversity among and within cucumber varieties from Greece and it's comparison with the variability of morphological characters. This is expected to contribute to the description and fingerprinting of landraces and to provide useful information for breeding purposes.

Materials and Method

RAPD analysis for the detection of genetic variability was applied for 8 traditional cucumber populations from different geographic regions of the country as follows: Knosos, Kalyvia, Crete 1, Crete 2, Crete 3, Kos 2, Ikaria and Samos. Young leaves or cotyledons were used for DNA isolation. Extraction of DNA was made by the CTAB method (Halbert and Bennetzen, 1991). All PCR cycles were made in a final volume of 25 μl with 1 oligonucleotide in each reaction. Final concentrations in the mixture of the reaction was as follows: 25 pmol of each oligonucleotide, 1.5 mM Taq polymerase, 50 μM KCl, 10 mM Tris-HCl, pH=9.0, 1.5 mM MgC l2, 0.1% Triton, 0.25 mM from each of the 4 nucleotides (dNTPs) and 50-100 ng of total DNA. PCR reaction took place with DNA denaturation in 94°C (5 minutes) followed by 45 cycles each with 1 min in 94 °C, 1 min in 36 °C and 1 min in 72 °C and at the end of the 45 cycles, 5 min in 72 °C. Product analysis was made by 1% agarose gel electrophoresis. DNA from phage λ was used as control of the following sizes: 23 kb, 7.7 kb, 62.2 kb, 4.2 kb, 3.4 kb, 2.7 kb, 1.9 kb, 1.5 kb, 0.9 kb, 0.4 kb and 0.1 kb. Repeatability was checked with reactions from at least 2 DNA isolations for each variety and each primer.

All reaction products were checked for presence/absence of RAPDs and their size was estimated in comparison with the control. Assessment of variability, genetic distance, and polymorphism was made by statistical analysis (Nei and Li, 1979) and the respective dendrograms were constructed by the UPGMA method.

Results

Detected RAPD markers are presented in Table 1. Different number of RAPD markers were detected in different varieties. The presence of polymorphism was also detected since several markers were not detected in individuals of each variety. The mean number per plant ranged from 16.67 to 37.10 and is smaller than the mean value of all individuals (42.6), thus indicating polymorphism within the varieties. Percentage of polymorphic loci for each variety was ranged between 36.76 and 73.53 and genetic variabi-

lity ranged between 0.1573 and 0.2947 (Table 2). Varieties Knosos and Kalyvia indicated the highest genetic variability. Genetic distance (as assessed by Nei mesure) indicated high genetic similarity between Knosos and Kalyvia, however some markers were detected only in the one or the other variety and could be used possibly for their distinction and fingerprinting. RAPD data analysed in groups resulted in the construction of a dendrogram according to genetic distance by Nei (Figure 1). The two main branches of the dendrogram include the varieties Knosos and Kalyvia in the first and all the other varieties in the second.

Discussion

RAPDs data in this work were repeatable and the analysis pattern was clear for each plant. Although the varieties studied come from different geographic regions of the country, their morphological differences were small. The results with the specific primers indicate high genetic similarity between Knosos and Kalyvia. Similar indications were found within the group of Kos 2, Ikaria, Samos and also the group of kriti 1, Kriti 2, Kriti 3. The genetic similarity of Knosos and Kalyvia is also prominent in the description of morphological characters. From the nine characters evaluated, differences were found in only 3 of them, probably as a result of segregation in the sample used. Similar comparison is also valid for the groups

Kos 2, Ikaria, Samos, and Kriti 1, Kriti 2, Kriti 3. Their close geographic origin could probably indicate their common genetic origin. Comparison of the genetic distances of the two main branches of the dendrogram suggests that the genetic distances of the 6 varieties in the first branch is larger than the distance between Knosos and the varieties Kriti 1, Kriti 2, Kriti 3, all of which originate from the same geographic region.

In conclusion, the RAPD markers used in the present work seems to be insufficient by themselves for genetic fingerprinting of the specific varieties, since the large number of polymorphisms within the varieties is making their identity obscure. This is in accordance with the results found by Bernet *et al*. (2003), when they tried to distinguish 36 cucumber varieties by DNA analysis. On the other hand individual plant DNA analysis within the varieties is underway and is expected to allow the comparison of genetic variability to phenotypic variability within each plant population.

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Table 1 Number of molecular markers and mean values per individual (+ :presence, - :absence)

Variety	Number of RAPDs		Mean value per individual	
	+	-	+	-
Kalyvia	57	11	37.10	30.90
Knosos	56	12	36.08	31.92
Kriti 1	43	25	27.75	40.25
Kriti 2	39	29	25.75	42.25
Kriti 3	51	17	32.75	35.25
Kos 2	34	34	22.00	46.00
Ikaria	28	40	19.67	48.33
Samos	33	35	19.75	48.25
Mean	42.6	25.4	27.61	40.39

Table 2 Number and percentage of polymorphic loci and values of genetic diversity by Nei

Variety	Polymorphic loci		Genetic diversity by Nei	
	number	%	Mean value	Standard deviation
Kalyvia	49	72.06	0.2947	0.2127
Knosos	50	73.53	0.2856	02154
Kriti 1	33	48.53	0.1886	0.2049
Kriti 2	34	50.00	0.2014	0.2087
Kriti 3	41	60.29	0.2480	0.2077
Kos 2	30	44.12	0.1646	0.1966
Ikaria	25	36.76	0.1573	0.2146
Samos	*30*	*44.12*	*0.1612*	0.1923

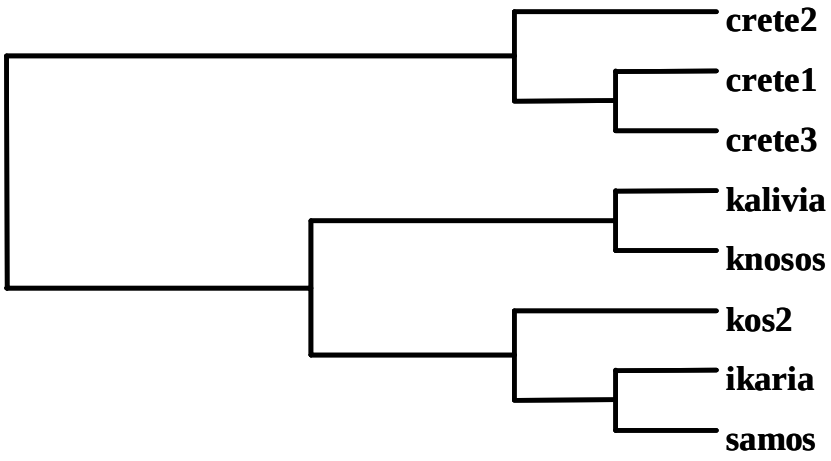

Figure 1 Dendrogram based on the genetic distances by Nei

ANALYSIS OF PHYSIOLOGICAL CHANGES DURING *IN VITRO* CULTURE OF WHEAT TISSUES USING AN EST MICROARRAY

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Different *in vitro* culture methods or the use of diverse plant propagules (roots, cells, embryos) may significantly modify growth and regeneration characteristics. We hypothesized that these changes can be studied through monitoring the expression of thousands of genes using an EST microarray.

We applied a newly developed wheat EST microarray to study gene expression within callus and cell suspension cultures from a winter wheat variety. The callus and suspension cultures were prepared from two different explants, immature inflorescences and embryos.

It has been observed that plants regenerated from immature inflorescences do not need to be vernalized for initiation of heading as compared to plants regenerated from embryos that require cold treatment in order to flower.

We prepared (1) cell suspension and (2) callus cultures from both immature inflorescences and wheat embryos. Gene expression within inflorescences and within embryos was then compared within each type of *in vitro* culture method.

For the cell suspension culture, (1), we found 16 ESTs, representing 7 different wheat genes, whose expression appeared to be altered (down-regulated; $P<0.0001$) within cultures of immature inflorescence tissue relative to embryonic tissue. Four of the 16 clones represented an identical sequence with homology to a rice gene encoding an unknown protein, and another group of six identical clones had high homology to a barley 26 S ribosomal RNA gene.

For the callus culture method (2), only one EST representing a putative ABC transporter gene appeared to be differentially expressed (up-regulated; $P<0.001$) within cultures of immature inflorescence tissue. When the threshold of significance was reduced to $P<0.005$, four additional genes would appear expressed at higher levels. In the callus cultures no significantly down-regulated genes could be detected.

This preliminary study demonstrates that EST microarrays are useful tools for studying the molecular bases of physiological processes resulting from different *in vitro* culture methods.

EFFECT OF HEAVY METALS ON AMMONIA ASSIMILATING ENZYMES IN CUCUMBER (*Cucumis sativus*) *IN VITRO*

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Introduction

Due to various anthropogenic activities (mining, smelting, dispersal of sewage sludge, intensive use of phosphate fertilizers) heavy metals concentrations are increasing rapidly in the environment. Heavy metal excess induce several phytotoxic symptoms in higher plants such as inhibition of seed germination, growth retardation, loss of photosynthetic pigments, lipid peroxidation *etc*. (Hall, 2002). Although ammonia assimilation is an important metabolic process in plants, few studies have been carried out with respect to heavy metals impact. In the present study we have examined the response to Zn and Pb of enzymes involved in the assimilation of ammonia (glutamine synthetase [GS], glutamate synthase [GOGAT] and glutamate dehydrogenase [GDH]) in cucumber plantlets grown *in vitro*.

Materials and methods

Cucumber (*Cucumis sativus* L. cv. Knossos) seeds were germinated *in vitro* in a MS basal medium (except NH_4NO_3) supplemented with 0, 50, 100 and 500 μM of $ZnSO_4$ or Pb-EDTA. Also, cuttings from the aforementioned seedlings were subcultured in hormone-free MS medium, modified to contain 20 mM KNO_3 as the only nitrogen source and supplemented with 0, 50, 100 and 500 μM of $ZnSO_4$ or Pb-EDTA. The plants were grown at 25±1^{o}C and 16/8-h photoperiod under cool white fluorescent light (2800-3000 lux) and were harvested three weeks later, sectioned in roots, shoots and leaves and stored at - 80 ^{o}C until further use. Protein extraction, enzyme activities, SDS-PAGE and western blotting were carried out as already described (Loulakakis *et al*., 2002).

Results and Discussion

The presence of Zn or Pb at any concentration in the medium negatively affected seed germination and growth parameters such as height and fresh weight. The percentage of seed germination decreased to 26% and 20% at the higher concentrations tested (500 μM) for Zn and Pb respectively. Similarly, fresh weight of seedlings decreased by 30% and 34% after three weeks of culture in the medium supplemented with the 500 μM of Zn and Pb respectively. The effect of the two metals on fresh weight was more pronounced when cuttings were used as starting material (37% and 43%). Such toxicity symptoms are well established by the literature (Hall, 2002). The presence of excessive amounts of heavy metals may cause a range of interactions at the cellular/molecular level leading finally to the inhibition of seed germination and growth. Toxicity may result from the binding of metals to sulphydryl, amino or imino groups in proteins or from the displacing of an essential element resulting in deficiency effects. Furthermore, high heavy metal concentrations may induce the generation of free radicals and reactive oxygen species, resulting in oxidative stress (Hall, 2002).

The concentrations of metals were negatively correlated with enzyme activities and immunoreactive protein of GS and GOGAT. A similar response has also been demonstrated for Cd, in maize (Boussama *et al*., 1999) and bean (Gouia *et al*., 2000). On the

contrary, the 500 µM of zinc increased GDH activity by 54% and 65% in leaves and shoots whereas for the same concentration of lead the increase was 66% and 73%, respectively. The above changes in GDH activity were accompanied by analogous changes in GDH immunoreactive protein. The induction of GDH activity is a predominant feature under several stress conditions such as salinity, water deficiency and leaf senescence. It seems that the excess of heavy metals invokes a stress response. As in Cd-treated plants, where elevated endogenous ammonium levels are observed (Boussama *et al*., 1999; Gouia *et al*., 2003), Zn and Pb may induce ammonium accumulation in cucumber, which in turn increases the expression of GDH. On the other hand, the increase in GDH protein and activity by zinc and lead may be necessary to replenish glutamate pool needed for the synthesis of heavy metal binding peptides such as metallothioneins and phytochelatins.

Further work is needed, in order to elucidate the correlation of the aforementioned results with the endogenous levels of heavy metals, using a simpler *in vitro* system, like calluses or cell suspension cultures, where possible interactions with root uptake and xylem transport of the metal ions are lacking.

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VALUE-ADDED STRAWBERRY PROPAGULES THROUGH HOLISTIC BIO-CONTROL

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Introduction

Adding value to microplants is a way to improve the competitiveness of micropropagation versus conventional vegetative propagation. Added-value may be achieved by improving the stress resistance of microplants by inoculation with beneficial microorganisms (Vestberg *et al*., 2002). Strawberry (*Fragaria x ananassa*) is an appropriate model for such investigations as the crop is affected by a number of serious diseases including *Verticillium* wilt and powdery mildew. High dependence of the crop on chemicals for disease control and the ban on the soil fumigant, methyl bromide, has led research on alternative approaches. PGPR (plant growth promoting rhizobacteria) and AMF (arbuscular mycorrhizal fungi) inoculants, singly and in combination, have been evaluated for their potential to control strawberry pathogens (Cordier *et al*., 2000). Here, VAMINOC (a commercial AMF inoculant), *Bacillus subtilis* FZB24–WG (a PGPR inoculant) and a fungal elicitor analogue N,O-carboxytmethyl chitosan (NOCC) were investigated singly and in combination for their potential to control *Verticillium* wilt and powdery mildew (*Sphaerotheca macularis*) in strawberry.

Materials and Methods

Strawberry microplants were transferred to the glasshouse where they were inoculated, at establishment, with PGPR and/or AMF for the control of *Verticillium* wilt and inoculated with PGPR and/or AMF and/or sprayed with NOCC for the control of powdery mildew. Plants were acclimatized and grown on in the glasshouse. After acclimatization the plants were challenged with either *Verticillium wilt* or powdery mildew, as appropriate. The effects of the treatments on disease development and fruit yield were recorded.

Results and Discussion

In the *Verticillium* experiment, inoculation with AMF, PGPR and AMF+PGPR increased yields compared with the uninoculated control. Biocontrol agents were shown to sustain crop yield when challenged with *Verticillium* and a significantly higher marketable fruit yield was observed in the AMF and AMF+PGPR inoculations compared with the *Verticillium*-challenged controls (Fig. 1). There was no significant difference however, between the treatments and the unchallenged control. In the case of powdery mildew, *Bacillus*, NOCC and combined treatments reduced disease progression when compared to the control treatment (Fig. 2). Plants treated with both biocontrol agents and the elicitor had the highest level of infection. The effect of the treatments on fruit yield appeared to relate to the level of protection conferred on the plants (increased protection leading to increased yield) with the exception of the NOCC treatment where reduced disease did not result in increased yield.

The results are discussed in the context of developing holistic, reduced pesticide input and possibly organic/ecological strategies for crop production from microplants.

Acknowledgements

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Figure 1 The effect of AMF and PGPR on *Verticillium dahliae* and marketable fruit yield in strawberry microplants. Treatments sharing a common letter are not significantly different (P<0.05), using the Kruskal Wallis test

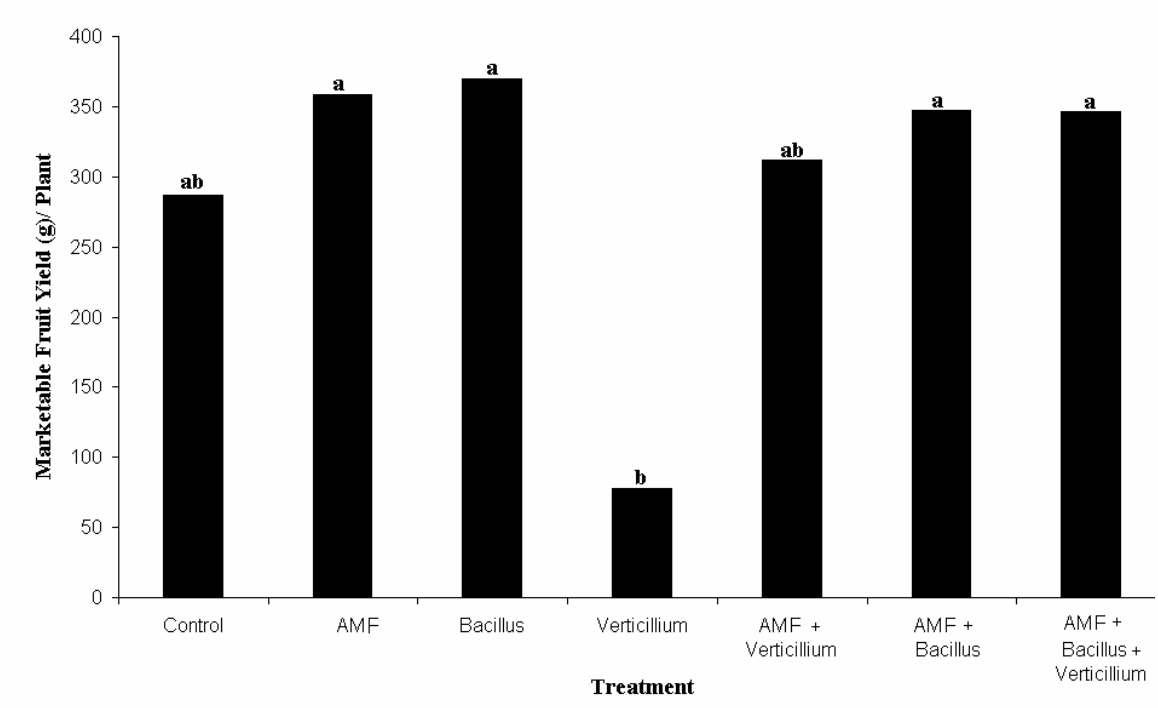
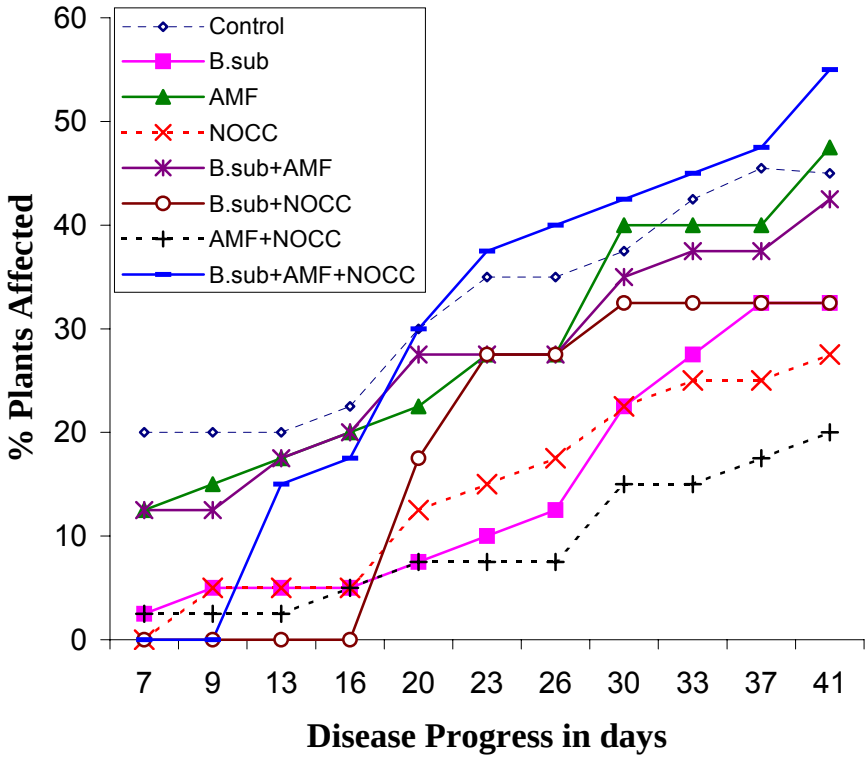


Figure 2 Disease progress curves for powdery mildew in control and treated strawberry plants



CRYOPRESERVATION AND ABIOTIC STRESS TOLERANCE IN POTATO: A PROTEOMIC APPROACH

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The improved commercial potato varieties are derived from only one species and thus possess a limited genetic variation, when compared to the total gene pool of potato. Therefore it is necessary to store safely all the potato varieties.
Cryopreservation involves the storage of biological material in liquid nitrogen (-196°C). At this temperature all the chemical and physical processes are arrested, allowing a safe storage over an unlimited period of time. Standardized cryopreservation protocols are not yet available for potato.
Using 2D-gelelectrophoresis, protein composition is being analyzed of samples (i) differing in pre-treatment, (ii) at different stages of the cryopreservation protocol and (iii) of different genotypes. To develop improved cryopreservation protocols, identified proteins will be linked to the recovery percentage of cryopreserved shoot tips and – in turn – to tolerance towards cryopreservation. Since cold and drought stress will be used as pre-treatment and since cryopreservation and dehydration resistance are closely linked, the results will also lead to a better understanding of the stress mechanisms of potato in the field.

REGENERATION AND TRANSFORMATION OF *Rhododendron* WITH THE *ROLB* GENE

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Introduction

Rhododendrons are important ornamental plants in the world. Propagation by cuttings is one of the methods used for rhododendron production. As the genus *Rhododendron* is very large and the genotypic variation is great among different species, the rooting ability of cuttings varies considerably among species and even between hybrids with the same parents. It is thus necessary to improve the rooting ability of difficult to root species and cultivars. The aim of this study was to work out regeneration protocols for five rhododendron cultivars and to transform the cultivars with the *rolB* gene which has been proven to significantly increase the rooting ability of some woody plant species (van der Salm 1998; Welander *et al*., 1998; Zhu *et al*., 2001, 2003).

Material and Method

In vitro shoot cultures of the cultivars 'Elviira', 'Haaga', 'Pekka', 'Pohjala's Daughter' and 'St Michel', kindly provided by Dr. Marjatta Uosukainen, MTT, Laukaa Reasearch and Elite Plant Station, Finland, were used in this study. Young unfolded leaves from 3 weeks old cultures were used as explants. The regeneration medium consisted of basal WPM, 1, 5 or 10 µM IBA in combination with 20, 40 or 60 µM 2iP or 1, 5 or 10 µM TDZ. Each combination or treatment consisted of 30 explants. The results were recorded after 9 weeks in the 2iP tests and 10 weeks in the TDZ tests. Based on the regeneration results, transformation of the cultivar 'Pohjala's Daughter' was carried out using the *Agrobacterium tumefaciens* strain C58C1 (pGV3850) carrying the transformation vector pCMB-B. The vector, kindly provided by Dr. C. Maurel, contained the *rolB*, *nptII* and *gus* genes.

Results and discussion

For the 2iP tests, the combination of 2iP:IBA (40:5 µM) resulted in 100% regeneration for 'Haaga', 'Pekka' and 'Pohjala's Daughter', and 91.7% regeneration for 'St Michel'. In general, the cultivars 'Haaga', 'Pekka' and 'Pohjala's Daughter' had high regeneration ability regardless of growth regulator combination, while 'Elviira' showed a poor regeneration rate at all combinations. For the TDZ tests, almost all combinations tested gave 100% regeneration for the cultivars 'Haaga' and 'St Michel' and better results for 'Elviira' compared with the 2iP tests. Figure 1 shows the visual differences of regeneration rate and colouration among the four cultivars tested with 2iP:IBA (40:5 µM). The cultivar that showed the least hyperhydricity, red colouration or other signs of stress was 'Pohjala's Daughter'. Thus this cultivar was chosen for transformation. Ten putative transgenic clones were verified by PCR and all the clones contained the *rolB* (Fig 2) and *nptII* (data not shown) genes. However, none of them contained the *gus* gene, indicating the possible deletion of the *gus* gene which is located near the left border on the T-DNA. Further verification of the transgenic clones by Southern blot and *in vitro* rooting tests are underway.

Acknowledgements

We thank Dr. Marjatta Uosukainen for kindly providing initial *in vitro* cultures.

Figure 1 Leaf explants of ‘Haaga’ (a), ‘Pekka’ (b), ‘Pohjala’s Daughter’ (c) and ‘St Michel’ (d) grown on medium containing 2iP:IBA (40:5 µM)

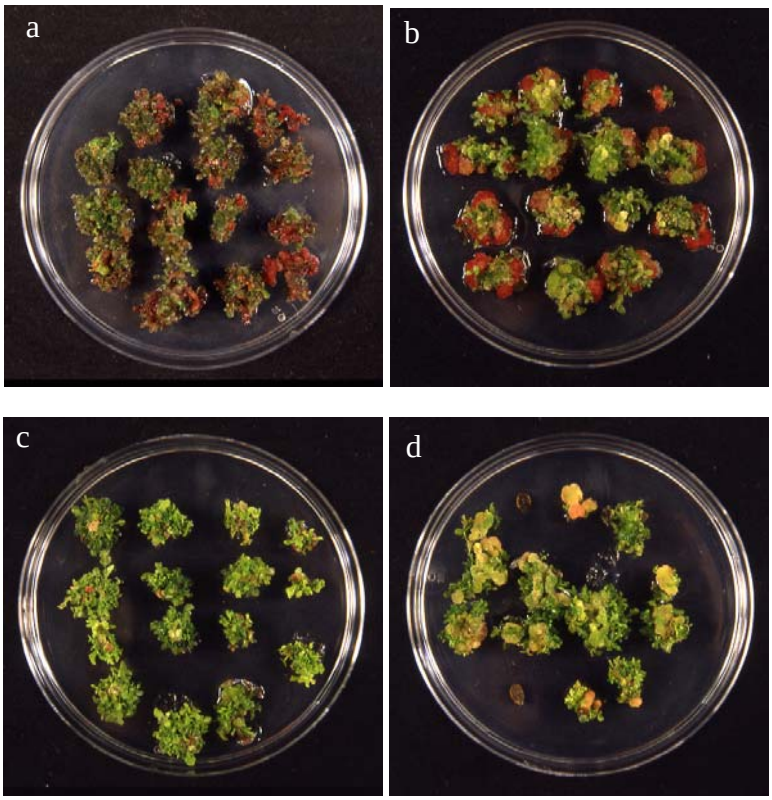
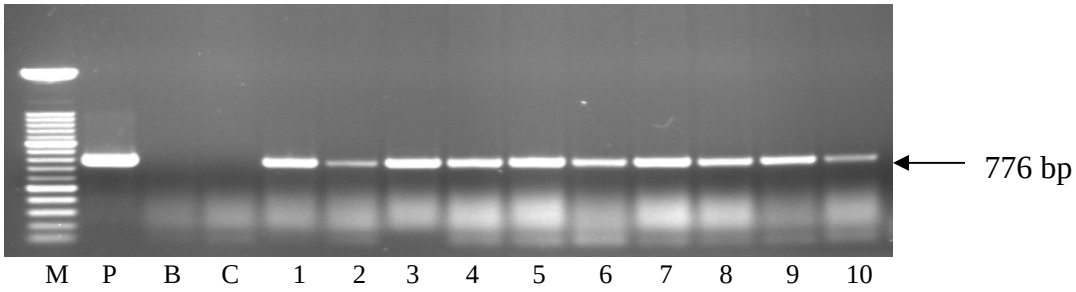


Figure 2 DNA fragments of the *rolB* gene (776 bp) amplified with PCR from the transgenic clones and untransformed control. M = markers, P = plasmid DNA, B = blank, C = untransformed control, 1-10 = transformed clones



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GRADIENT SDS-PAGE ANALYSIS OF SOLUBLE AND INSOLUBLE PROTEINS OF SOMATIC AND ZYGOTIC EMBRYOS OF ALGERIAN FIR (*Abies numidica* De Lann.)

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Introduction

Comparative study on zygotic and somatic embryogenesis in conifers has shown that except of morphological similarity there exists a high degree of biochemical homology between zygotic and somatic embryos of conifers, especially with respect to their storage proteins (Hakman *et al*., 1990). The greater biochemical similarity of somatic embryos to their zygotic counterparts is believed to improve the conversion of somatic embryos to plants (Klimaszewska *et al.*, 2004). Because of their accumulation during embryo development the storage proteins were reported to be excellent markers for comparison of zygotic and somatic embryo programs (Flinn *et al*., 1993).

In present paper we have followed the depletion dynamics of both soluble and insoluble proteins during cultivation and germination of somatic and zygotic embryos of Algerian fir aiming in assessment of the nutritive role of these proteins.

Material and Methods

As a material for gradient SDS-PAGE analysis of soluble and insoluble proteins, the cotyledonary somatic embryos, emblings and zygotic embryos of Algerian fir (*Abies numidica* De Lann.) were used. Induction and proliferation of embryogenic tissue as well as desiccation and germination of somatic embryos was described in our previous work (Vooková and Kormuťák, 2002). Three types of maturation treatments were used to achieve maturation of somatic embryos:

- Embryogenic tissue proliferated on SH medium (Schenk and Hildebrandt, 1972) with 1 $mg.l^{-1}$ BAP was transferred to SH maturation medium. This medium contained SH original macro-, micro-elements, FeEDTA and vitamins.
- Embryogenic tissue proliferated on SH medium with 1 $mg.l^{-1}$ BAP was transferred to MS maturation medium (Murashige and Skoog, 1962). This medium contained ½ strength MS macro- and micro-elements, FeEDTA and modified vitamins (1 $mg.l^{-1}$ nicotic acid, 1 $mg.l^{-1}$ thiamine HCl, 1 $mg.l^{-1}$ pyridoxin HCl, 2 $mg.l^{-1}$ glycine, 100 $mg.l^{-1}$ myo-inositol).
- Embryogenic tissue was transferred from SH proliferation medium with 1 $mg.l^{-1}$ BAP to the same SH medium lacking growth regulator. Cultures were maintained on this medium for two weeks. As a maturation medium was used MS medium.

All maturation media contained 40 $g.l^{-1}$ maltose, 100 $g.l^{-1}$ polyethylene glycol 4000 and 10 $mg.l^{-1}$ abscisic acid.

Dormant zygotic embryos (following their 24 h imbibition in water) along with stratified (3 months in moisture sand, 4 ºC) and germinating embryos (early stage) were analyzed.

Both somatic and zygotic embryos were subjected to gradient SDS-PAGE analysis using 5-20% polyacrylamide gels. Soluble and insoluble proteins were extracted according to Jensen and Lixue (1991).

Results and discussion

About 16 abundant fractions of soluble proteins were detected in somatic and zygotic embryos of algerian fir with molecular size ranging between 130 and 14 kDa (Fig. 1). Of these, most abundant were the proteins of 100, 80, 55, 42, 25, 20, 16, and 14 kDa size. No differences in soluble protein profiles were observed between non-desiccated and desiccated somatic embryos on MS medium (Fig. 1 E, F) as well as on SH medium lacking growth regulator (Fig. 1 H, I). The only exceptions were in this respect the desiccated embryos on SH medium with growth regulator lacking the proteins within a range of 42-30 kDa (Fig. 1 C). Zygotic embryos were similar but not identical with somatic embryos. In particular it concerned the 42 kDa protein which was much more abundant in zygotic embryos (Fig. 1 K) than in somatic ones. The same was true also of the 28, 24 and 14 kDa proteins. On the contrary, the proteins of 30, 25 and 10 kDa size characteristic for zygotic embryos have not been detected in somatic embryos at all. The most characteristic feature of the soluble proteins as a whole is their nutritive function as evidenced by their complete absence in regenerated emblings (Fig. 1 D, G, J) as well as by their progressive depletion during early stage of zygotic embryos germination (Fig. 1 L, M).

Contrary to soluble proteins, the insoluble proteins of somatic and zygotic embryos were less numerous consisting of 13 abundant fractions only (Fig. 2). Among these, the proteins of 57, 55, 40, 30, 25, 22, 18, and 14 kDa were most distinct. Both non-desiccated and desiccated somatic embryos shared identical protein profiles on SH media (Fig. 2 B, C, E, F, H, I). However, their respective regenerants differed profoundly possessing 55 kDa proteins with conspicuously increased abundancy and the novel proteins of 38, 36 and 25 kDa size (Fig. 2 D, G, J). Zygotic embryos seem to possess the same insoluble proteins as their somatic counterparts (Fig. 2 K). A nutritive role of individual proteins was assessed according to their depletion dynamics upon germination. Based on this criterion, the storage reserve function was ascribed to the 40, 30 and 22 kDa proteins of Algerian fir embryos what is a little deviating figure from *Picea glauca* embryos where the corresponding proteins were reported to have 42, 34.5-35 and 22.5-23 kDa size (Misra and Green, 1990). Still other explanation of this discrepancy is offerred by Jensen and Lixue (1991). According to the authors *Abies* species differ in storage proteins from *Cedrus*, *Larix*, *Picea*, *Pinus* and *Pseudotsuga*, mainly because of the lack of 55 kDa αβ-dimer legumine-like proteins in their seeds. This aspect of seed biochemistry needs to be verified further.

Acknowledgement

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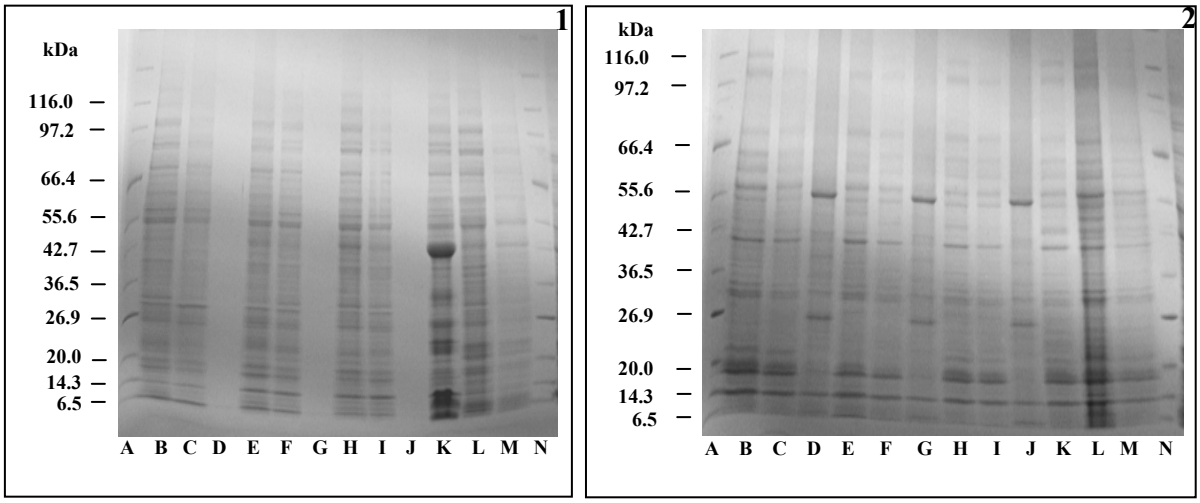

Figs.1-2 Gradient SDS-PAGE profiles of soluble (1) and insoluble (2) proteins of somatic (SE) and zygotic embryos (ZE) of Algerian fir

B - SE on SH medium before desiccation
C - SE on SH medium after desiccation
D - corresponding emblings
E - SE on MS medium before desiccation
F - SE on MS medium after desiccation
G - corresponding emblings
H - SE on SH medium before desiccation, cultivation without PGR
I - SE on SH medium after desiccation, cultivation without PGR
J - corresponding emblings
K - dormant zygotic embryos
L - stratified zygotic embryos
M - germinating zygotic embryos
A, **N** - molecular size markers

STUDY OF SOMATIC AND ZYGOTIC EMBRYOS OF ALGERIAN FIR (*Abies numidica* De Lann. Carrière) BY AQUASEM

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Specimens for the usual scanning electron microscope (SEM) must be clean, dry, electrically conductive, and must tolerate vacuum. "Environmental" scanning electron microscope (ESEM) is the only solution for problematic specimens. The essence of ESEM consists in the possibility of observing specimens with a certain volume of water and of observing specimens at certain pressure in the specimen chamber.
In SEM, the detection system is used for detection of signal electrons emitted by the specimen as a result of interaction of primary electrons (PEs) with the specimen, primarily for secondary (SEs) and backscattered (BSEs) electrons (Autrata 1995). Detection of backscattered electrons in SEM is a method of studying material composition (Madea and Autrata, 1995). AQUASEM (TESCAN) was used for studying non-fixed pine megagametophytes under the following conditions: pressure in the specimen chamber 400 Pa, BSE detector and 15 kV. Storage organelles, protein bodies, and especially their globoids and spherosomes were observed as particles with a high level of brightness (Hřib *et al*., 2001).
The aim of this study is to show non-fixed somatic and zygotic embryos of Algerian fir by AQUASEM using a BSE detector.

Material and Methods

Seeds of Algerian fir (*Abies numidica* De Lann. Carrière) were obtained from Mlyňany Arboretum, Slovakia. The coat was removed from the dry seeds under a preparation microscope and zygotic embryos were prepared for making longitudinal sections and their observation in a scanning electron microscope.
Somatic embryogenesis. Embryonal suspensor mass (ESM) of Algerian fir (*A. numidica*) was initiated from immature zygotic embryos on SH medium (Schenk and Hildebrandt, 1972) with 1 $mg.l^{-1}$ benzylaminopurine and 10 $g.l^{-1}$ sucrose. Maturation of somatic embryos was achieved on modified MS medium (Murashige and Skoog, 1962) supplemented with 40 $g.l^{-1}$ maltose, 100 $g.l^{-1}$ PEG-4000 and 10 $mg.l^{-1}$ abscisic acid. Cotyledonary somatic embryos were subjected to partial desiccation in the dark at 21-23°C for a period of three weeks (Vooková and Kormuťák, 2002). AQUASEM scanning electron microscope (TESCAN, Brno) was used to observe longitudinal sections of zygotic and desiccated somatic embryos of Algerian fir (*A. numidica*). The AQUASEM was used under the following conditions: pressure in the specimen chamber 40-60 Pa, temperature -20 °C, BSE detector, voltage 20 kV.

Results and Discussion

Previous experiments, during which an examined specimen was supposed to be placed in an "environmental" chamber isolated from the main tube by one or more differential pumping apparatuses, were complicated by lack of suitable electron detectors working in the chamber atmosphere. Boston Company ElectroScan developed a secondary electron detector which works in gaseous environment. The detector uses cascade intensification not only to intensify the signal of secondary electrons, but also to create positively charged ions which are attracted by negative charge on the isolated surface of the specimen and they suppress (neutralize) this charge effectively.
Our specimens were examined with the BSE detector set to low vacuum, and frozen at -

20°C. In situ observation could thus be performed on non-fixed zygotic and somatic embryos of Algerian fir (*A. numidica*). It was discovered that occurrence of storage organelles with a higher level of brightness is lower in the somatic embryo (Fig. 1), while in the zygotic embryo occurrence of these organelles is higher (Fig. 2).
AQUASEM scanning electron microscope can be utilizable for studying reserve organelles also in specimens with higher water content, as indicated by this *in situ* experiment.

Acknowledgments
I thank Dr. B. Vooková from Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra for providing of somatic embryos and Ing. L. Ilkovics from Institute of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno and Ing. P. Bojda from DIPEZ Ltd. for technical assistance.

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Figure 1 Longitudinal section trough a desiccated somatic embryo of Algerian fir (*A. numidica*)
(A – apex, C – cotyledons; arrows point to reserve organelles). Magnification 565.4 x

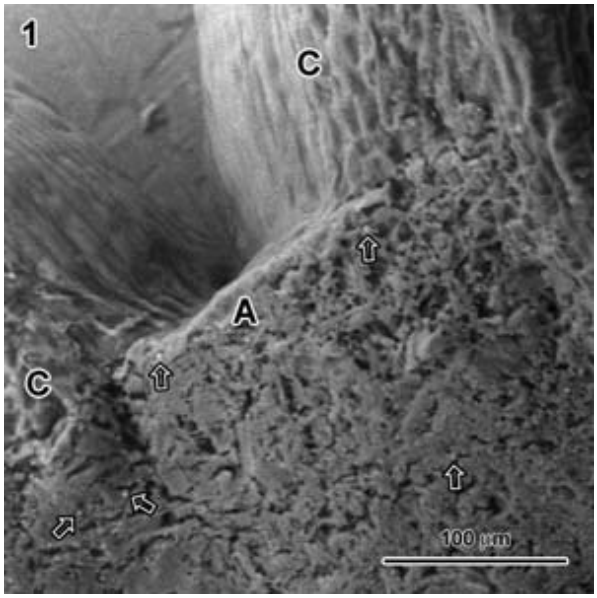
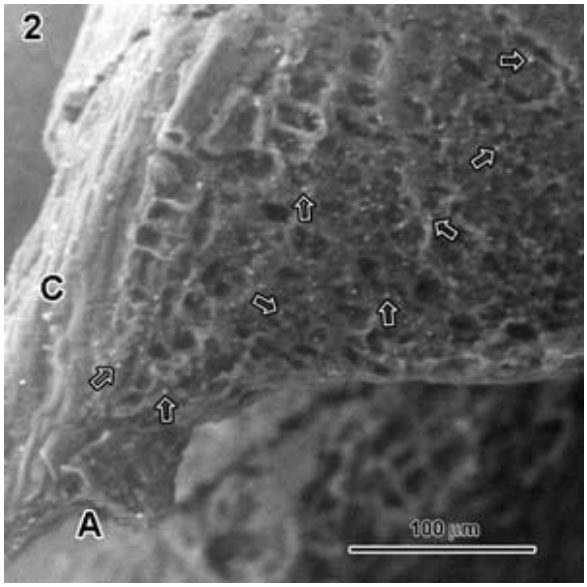


Figure 2 Longitudinal section trough a zygotic embryo of Algerian fir (*A. numidica*)
(A – apex, C – cotyledons; arrows point to reserve organelles). Magnification 576.4 x



MONITORING OF GLUCANASE AND CHITINASE ACTIVITY IN SOME CROP PLANTS STRESSED WITH Pb^{2+}

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Introduction

Most crop plants are sensitive to higher concentrations of heavy metals in soils. Such contamination can cause serious damages in plants including poor growth, defects in respiration and photosynthesis, inactivation of enzymes. On the other hand, plants possess a broad scale of different mechanisms that are connected with processes of detoxification (Mithöfer *et al.*, 2004). For tolerance of plants to heavy metals are directly responsible protein products of few genes only (1-3) (Macnair *et al*., 2000). In addition to these, activities of more than 30 different proteins are also induced during the plant defence. Next to heat shock proteins, phytochelatins, metalothionins, prolin rich proteins etc., glucanases and chitinases are likely to be involved (Nasser *et al*., 1990). Plant glucanases and chitinases are glucanhydrolases belonging into the group of so-called pathogenesis related (PR) proteins. They have been mostly detected during plant defence against different plant pathogens such as viruses, bacteria and fungi. Activity of these enzymes with respect to heavy metal stress has been, however, much less studied (Przymusiński and Gwóźdź, 1999). Here we present the monitoring of several crop plants for changed glucanase and chitinase activity when exposed to high concentration of lead. Effect of Pb^{2+} primary root length was scored. Plate clearing assay using specific enzyme substrates was used to compare activity of glucanases and chitinases in roots of stressed and control plants.

Material and Methods

Seeds of peas (*Pisum sativum* L., *var.* Olivín), beans (*Vicia faba* L. *var.* Aštar, *Vicia faba* L., *var.* Piešťanský), barley (*Hordeum vulgare* L., *var.* Garant), maize (*Zea mays* L., *var.* Quintal) and soybean (*Glycine max* L., *var.* Korada) were pregerminated on water-wet filter paper on large Petri dishes for two days. Subsequently they were replaced on filter paper with 300 $mg.l^{-1}$ Pb^{2+}. Daily length increments of primary roots were measured for four days.

Total protein extracts were isolated from roots of each control (non-stressed) and lead-stressed plant. Petri dish plates containing 0.01% glycol chitin or 0.01% laminarin, 0.8% agarose in 0.1 $mol.l^{-1}$ sodium acetate buffer (pH 5.2) were prepared, and filter paper discs (Ø 5 mm) were positioned around the dish. From each sample, 10 μg was applied on discs. After incubation for 60 min. at 37 ºC the discs were removed. In case of laminarin as specific substrate, the staining was performed with 500 $mg.l^{-1}$ of Congo Red. Degraded substrate was observed as a clear zone on the red background. In case of glycol chitin, plates were stained with 0.01% Fluorescent Brightner 28 in 0.5 $mol.l^{-1}$ Tris-HCl (pH=8.9). Degraded substrate was visible after illumination by UV as dark zones on the contrasting background.

Results and Discussion

Toxicity of lead on different crop plants was studied. Seeds of peas, barley, maize, soybean and two cultivars of beans were germinated with- or without the presence of 300 $mg.l^{-1}$Pb ions. The toxic effect of lead was obvious on the morphology and root lengths. The most damaged were the roots of barley that was reflected in fivetimes shorter primary roots comparing to roots of non-stressed control seeds. Peas, maize and soybean exerted less sensitivity to the metal ions (respectively). In contrast, lengths of bean roots were not affected by the presence of given Pb concentration. Differences in plant sensitivity to heavy metals were observed by several authors (Raskin and Ensley, 1999).
The effect of Pb^{2+} on occurrence and activity of stress proteins in examined crop plants was studied in plate clearing assays. Seeds of peas, barley, maize, soybean and two cultivars of bean were pregerminated in water and subsequently exposed to 300 $mg.l^{-1}$ Pb^{2+}. A set of seeds continued root development under Pb-free condition. Total proteins were extracted from root material after two days of exposure to Pb^{2+}. From these, equal amounts were assayed for glucanase and chitinase activity on Petri plates containing media with appropriate enzyme-specific substrates. Our preliminary experiments showed that enzymes from all root extracts (including control) were able to digest glycol chitin reflecting to active chitinases, as well as laminarin suggesting to glucanases. For example in pea there were no differences in chitinase activity in extracts from both control and stressed roots. These activities probably correspond to constitutive enzymes that are involved in processes different from those during stress (e.g. morphogenesis) and activity of which is regulated developmentally and tissue- or organ-specifically (Kasprzewska, 2003). On the other hand, differences in glucanase activity were observed between stressed and control pea, barley and bean roots. Deviancies in chitinase activity appeared in case of bean and maize. Quantitative measurements of these activities in examined crop roots will confirm in what extent the presence of lead causes increase or decrease of glucanases and chitinases. Interestingly, the root sensitivity to Pb did not always coincide with altered enzyme activity. For example, bean roots revealed almost no sensitivity to lead based on morphological measurements but exerted significant alterations in glucanase activity. Results of this work will contribute to evaluation of Pb impact on morphology and activity of some stress proteins in selected crops for better understanding the plant defence mechanisms in heavy metal- contaminated environment.

Acknowledgments
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THE ROLE OF HYDROGEN PEROXIDE AND ANTIOXIDANT ENZYMES DURING FORMATION OF EMBRYO LIKE STRUCTURES IN FLAX

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Introduction

While the stress responses of reactive oxygen species (ROS) and antioxidants and their function in plants have been relatively well studied, limited information about their role in develomental processes in plants exists up to now. We showed previously, that hydrogen peroxide substantially affect the shoot, root and embryo-like structures regeneration from flax hypocotyl segments (Takáč, Preťová, 2004). This study was elucidated to reveal the function of antioxidant enzymes – superoxide dismutase (SOD), catalase and peroxidases during flax embryo-like structures (ELS) formation. The endogenous variability in hydrogen peroxide concentration was studied as well.

Material and Methods

Flax hypocotyl explants (cv. Super with low organogenic capacity) were cultivated 10 days on MS media supplemented with NAA (1 $mg.l^{-1}$) following 14 days on MS media with NAA+BAP (0.5+0.5 $mg.l^{-1}$). Whole explants were harvested 2, 5, 7, 10 and 14 days after transferring calli to NAA/BAP media. Activities of superoxide dismutase, catalase and guaiacol peroxidase (POX) and hydrogen peroxide concentration were examined spectrophotometrically.

Results and Discussion

Results showed that activities of studied enzymes changed throughout the all cultivation of explants on NAA/BAP media. The activities of SOD and peroxidases reached their maximum after 5 days, when ELS began to appear on the surface of explants. After this increase strong decline in their activity was observed. On the other hand, activity of catalase was moderately enhanced showing maximum value after 10 days of cultivation. Exogenous addition of H_2O_2 to media significantly influenced activities of SOD, catalase and peroxidases in explants. We found, that activities of studied enzymes were the highest in the case of media with 70 μM hydrogen peroxide. Higher H_2O_2 concentrations inhibited their activities. Previous experiments (Takáč and Preťová, 2004) showed that the 70 μM concentration of H_2O_2 in media induced the highest regeneration of ELS from explants.

We could summarize, that the activity of SOD and peroxidases correlated with flax ELS formation. In addition the response of antioxidant enzymes activities to growing H_2O_2 concentrations in media showed similar pattern as the response of ELS number. These results emphasize the important role of ROS and antioxidant enzymes during flax ELS formation.

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OXIDATIVE STRESS DURING *IN VITRO* MORPHOGENESIS IN FLAX

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Introduction

Reactive oxygen species, ROS (superoxide $O_2^{\cdot-}$ radicals, hydrogen peroxide H_2O_2 and hydroxyl radicals ·OH) can accumulate in response to stress. They can have a detrimental effect on the metabolism, growth and development through their ability to initiate reaction cascades, which result in the production of toxic chemicals such as lipid peroxides and their aldehydic breakdown products leading to cell dysfunction and death (Alscher *et al*., 1997). In parallel, ROS may also have a positive role in plant growth and development. The balance between essential and damaging oxidative reactions is influenced by the physiological and developmental status of tissues and exogenous factors such as stress, disease or wounding and the application of plant growth regulators (Benson, 2000). A non-destructive assay for hydroxyl radicals (·OH) utilising DMSO as a radical trap is applied to determine ·OH formation during flax tissue culture and morphogenesis.

Material and Methods

Flax (*Linum usitatissimum* L.) genotypes: Atalante, Jitka, Super, Flanders and Szegedi-30 were used. Hypocotyl segments were cut from the central part of the hypocotyl of 7-day old flax seedlings, placed on the surface of MS media with 2% sucrose and 0.8% agar and supplemented with plant growth regulators in different concentrations (Table 1) and screened for organogenesis (shoot and root induction) and embryo-like structure production.

Assay for hydroxyl radicals (·OH), utilising DMSO as a radical trap, was used to determine ·OH formation during tissue culture and morphogenesis. DMSO can be used as a hydroxyl free radical scavenger with the subsequent release of methane, which is quantified by GC headspace sampling (Benson and Withers, 1987). Headspace samples were measured according Obert *et al*. (2005).

Results

Flax hypocotyl segments demonstrated responses after cultivation on media supplemented with various plant growth regulators (Table 1). Hypocotyl segments of all flax genotypes cultivated on medium containing BAP showed shoot formation. Greater variability was observed for root formation on medium supplemented with NAA, ranging from 7 roots/hypocotyl segment in case of Szegedi 30 up to 17 roots per hypocotyl segments in Flanders. After an initial 10 days of cultivation on NAA containing medium hypocotyl segments for ELS formation were transferred to medium containing mixture of NAA and BAP. After a further 2 weeks the hypocotyl segments were examined for ELS. Embryo like structure formation was higher in oil cultivars and lower in the case of fibre flax cultivars Jitka or Super.

Hydroxyl radical formation was detected in all genotypes during the morphogenic responses (Fig. 1). Highest levels were detected during callus formation. Low levels were

detected in hormone free medium and during shoot formation. A negative correlation was observed between hydroxyl radical formation and the number of induced roots. Higher levels of methane were detected in genotypes, which had the lowest number of roots. In genotypes with higher ELS induction, higher levels of methane were detected during the early stages of cultivation.

Disscussion

Organogenesis and somatic embryogenesis require the re-initiation of cell division and moderation of cell differentiation by the application of appropriate plant growth regulators (Benson, 2000). The morphogenetic responses achieved in this study corresponded with those expected and published previously (daCunha and Ferreira, 1996; Dedičová *et al*., 2000; Tejavathi *et al*., 2000). Different flax genotypes varied in their responses to the different combination of growth regulators, but genotype differences were not significant. Differences in response of oil and fibre cultivars were observed in root initiation and ELS formation. In both cases, oilseed cultivars Atalante and Flanders showed a higher response than fibre cultivars Super and Jitka.

The monitoring of hydroxyl radical activity within plant tissue cultures may give an indication as to the potential for free radical mediated stress in *in vitro* cultivated tissues. Different developmental processes and signalling pathways involved in the different morphogenic response of flax hypocotyl segments, may therefore be correlated with changes in oxidative status. The number of ELS produced, correlated positively with hydroxyl radical formation particularly in genotypes with higher levels of ELS induction. Concomitantly higher levels of hydroxyl radical activity were thus detected during the early stages of cultivation. This indicates that hydroxyl radical and peroxidation reactions are involved in the early stages of ELS development.

Highest levels of methane were detected in the case, when only callus was formed. The association of enhanced free radical activity with dedifferentiation and the application of synthetic auxins in flax concurs with previous finding for other in vitro plant cultures (Benson, 2000; Benson and Roubelakis-Angelakis, 1992). In contrast, lower levels of methane detected in flax tissue cultivated on hormone free media and media for shoot induction) indicates lower level of free radical mediated stress.

Differences in methane production during the occurrence of different phases of the morphogenetic processes, indicates that hydroxyl radicals, or active oxygen species that are involved in the formation of hydroxyl radical, may have role in development and during the induction of shoots or roots in hypocotyl segments, callogenesis and ELS formation.

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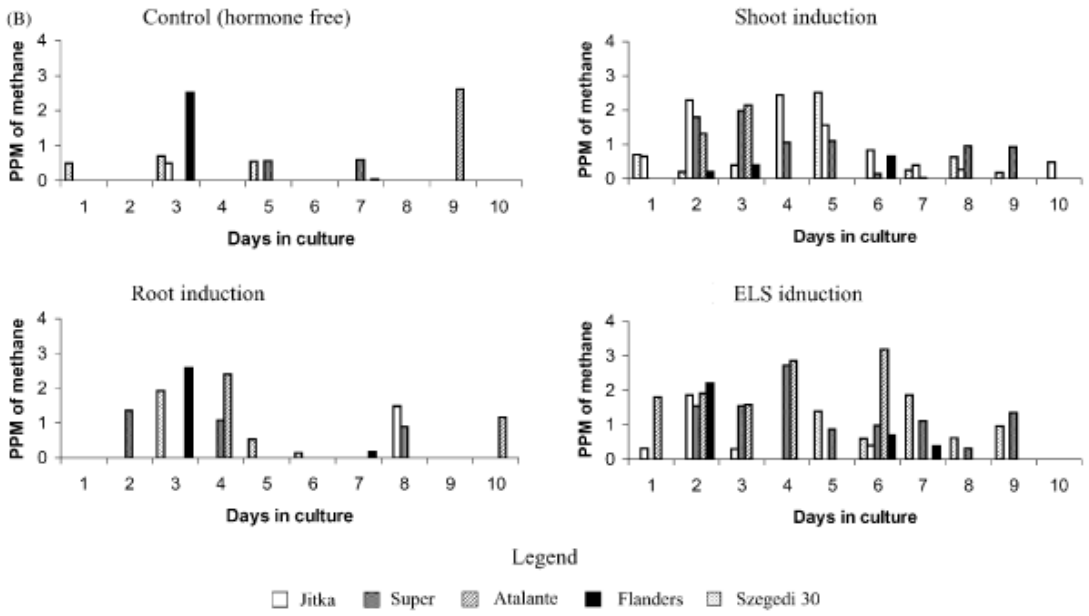
Table 1 Morphogenic response of different flax genotypes after 3 weeks of cultivation on media containing different plant growth regulators

Plant growth regulators	Hormone free	1 mg.l^{-1} BAP	1 mg.l^{-1} NAA	1 mg.l^{-1} NAA after 10 days transfer to 0.5 mg. l^{-1} BAP+ 0.5 mg.l^{-1} NAA
Morphogenic response	Control	Shoot	Root	ELS
Atalante	0.3±0.3	7.6±1.0	13.5±1.6	13.5±2.0
Flanders	2.5±1.2	6.0±1.9	17.2±1.5	11.5±2.0
Szegedi 30	2.1±0.6	6.3±1.5	7.0±1.1	15.2±2.2
Jitka	1.4±0.6	4.8±0.8	12.0±1.1	4.5±1.5
Super	1.8±1.3	6.8±1.2	8.5±1.6	9.3±1.2

Figure 1 Methane production by hypocotyl segments of different flax genotypes on different media



STUDY OF *DBAT* AND *DBTNBT* GENE EXPRESSION IN *Taxus baccata* L. *IN VITRO* CULTIVATED YOUNG SEEDLINGS

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Taxanes belong to the most important recently developed cancer chemotherapeutic agents paclitaxel (TAXOL®) and docetaxel (TAXOTERE®) are effective in the treatment of many types of cancer, including carcinomas of the ovary, lung, head and neck, bladder and esophagus (Jordan *et al*., 2002).

The most difficult problems encountered in clinical development of TAXOL® is supply limitation and its high cost. The low yield of paclitaxel from natural source, yew (*Taxus*) species and the lack of a commercially viable total synthesis have rendered the semisynthetic coupling of the side chain to baccatin III as the principal means of producing the drug to meet increasing demand (Walker *et al.*, 2002).

Baccatin III is presently isolated from the needles of *Taxus baccata* L. For the foreseeable future, the supply of paclitaxel and its synthetically useful precursors must continue to rely on biological methods of production, either in *Taxus* species, or potentially by cell cultures derived from these plants (Schoedorf *et al*., 2001).

Improving the biological production yields of the drug depends on detailed understanding of the biosynthetic pathway, enzymes catalyzing the sequence of reactions, genes encoding these enzymes and their expression (Jennewein *et al*. 2001). Recently, 9 cDNAs encoding pathways enzymes have been isolated from a *Taxus* cDNA library.

The present study is focused on the molecular-biological study of expression of genes coding for the key enzymes in paclitaxel biosynthetic pathway, particularly the *dbat* gene coding for 10-deacetyl baccatin III-10-O-acetyltransferase (DBAT) and *dbtnbt* gene coding for 3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase (DBTNBT). DBAT catalyzes formation of the baccatin III, one of the last intermediate known in the paclitaxel biosynthetic pathway. DBTNBT catalyzes the final reaction in this biosynthesis (Walker, Croteau, 2000; Croteau *et al*., 2002).

The aim of this work is to evaluate the *dbat* and *dbtnbt* gene expression during ontogenesis of *in vitro* cultivated *T. baccata* young seedlings and in different tissues of these plants. Recently, real-time reverse transcription PCR (real-time RT-PCR) method presents the most sensitive, reliable and high-throughput technique to study gene expression.

Material used for the study was *T. baccata* seedlings cultivated *in vitro* on RM medium supplemented with 1 $g.l^{-1}$ casein hydrolysate, 1 $g.l^{-1}$ yeast extract and 5 $g.l^{-1}$ activated charcoal. Several ontogenetic stages (from mature embryos to 2-months-old differentiated seedlings), in which the expression of *dbat* and *dbtnbt* was confirmed by reverse transcription PCR (RT PCR), were chosen for the expression analysis. Real-time RT PCR was used to compare the expression level of these genes during ontogenesis as well as in different plant parts such as needles, stems and roots of 2-months-old seedlings.

Understanding the patterns of expression of the crucial genes encoding for key enzymes in the paclitaxel biosynthetic pathway and their regulation can be used for effective production of paclitaxel using *in vitro* technologies.

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EXPRESSION OF THE *HYP-1* GENE IN RELATION TO THE TOTAL HYPERICIN CONTENT IN *Hypericum perforatum* L.

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Hypericum perforatum L. is a broad source of secondary metabolites with significant pharmaceutical effects. Hypericin and its derivates attract great attention due to their anti-viral and anti-cancer effects (Bombardelli and Morazzoni, 1995). It has been found that these compounds are localized in dark glands present primarily on the margins of leaves and flower petals (Briskin and Gawienowski, 2001). The amount of hypericin in plants of *H. perforatum* varies. The variability is affected by environmental as well as by genetic factors, as the influence of individual genotype or ploidy level is (Bütter *et al*., 1998; Košuth *et al.*, 2003). The amount of hypericins varies also in course of ontogenesis of the plant (Repčák and Martonfi, 1997), what can be related to enlarging or reducing of the plant organs where the hypericins preferentially accumulate or by activation/repression of the biosynthesis during ontogenesis. There is only a little knowledge about the biosynthesis of hypericin, other that it lays on the polyketide pathway, presumably through emodin, and with protohypericin as the penultimate precursor. The only one known since the year 2003, cloned and characterized gene/enzyme involved in this biosynthetic pathway, is the gene encoding for HYP-1 protein, which catalyzes direct and complex conversion of emodin to hypericin (Bais *et al.*, 2003). The cDNA nucleotide sequence information of this gene enabled us to study and compare the production of hypericin in relation with expression of this gene.

The aim of this study was to investigate the expression of *hyp-1* gene in *H. perforatum* plants differing in production of hypericins and to compare the *hyp-1* expression with the amount of the hypericins produced by these plants.

The expression of *hyp-1* was determined in *H. perforatum* plants with defined chromosome number. Individual plants differing in the hypericin content were clonally multiplied to the amount necessary for concurrent extraction of hypericin and total RNA. For the quantification of steady-state mRNA level of *hyp-1* gene in the samples, the fluorescence-based real-time reverse transcription PCR (RT-PCR) with double-stranded DNA-specific binding dye SYBR Green was employed. The assay is easy to perform, capable of high throughput and combines high sensitivity with reliable specificity. The differences in the expression pattern of *hyp-1* gene between the plants detected by quantitative RT-PCR will be presented and discussed.

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GENETIC (IN)STABILITY OF *Hypericum perforatum* L.: REASONS AND CONSEQUENCES

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Hypericum perforatum L. (Saint John´s wort) is a medicinal plant considered as an important natural source of secondary metabolites with a wide range of pharmacological effects. The most important biologically active constituents in the plant are the naphtodianthrones, hypericin and pseudohypericin and phloroglucinol derivatives, hyperforin and adhyperforin. These constituents show antidepressive, anticancer, antiviral and antibiotic activities (Anker *et al.,* 1995; Diwu 1995; Schempp *et al.,* 1999). The content of these metabolites which may be influenced by genetic, physiological, metabolic and environmental conditions is very variable (reviewed by Kirakosyan *et al.,* 2004). Therefore, a search for genotypes with high and relatively stable content of these compounds and conditions for their preservation without genetic and metabolic alterations is of great interest.

In natural populations, *Hypericum perforatum* L., a facultative apomict, is very variable species. The variation is especially due to physiological (peculiarities in the mode of reproduction and use of several reproduction pathways) and environmental factors. Different ways of seed formation, even on one plant, result in production of plants with different ploidy levels. This has an impact on traits that exhibit ploidy-dependent behavior. Environmental factors over the seasons contribute to the variation of secondary metabolites as well.

The problem of genetic variation can thus be studied with genetically well characterized plants under defined nutrient and physical conditions. For *Hypericum perforatum* L., an effective regeneration system *in vitro* has been developed by Čellárová *et al.* (1992). This system is based on the use of young seedlings or shoot tip meristems that provide multiple shoot formation under the effect of BAP. While the meristems differentiate directly into shoots with subsequent rooting, the seedlings respond to culture conditions by dedifferentiation followed by shoot formation indirectly through callus stage. The former result in almost identical plants, but the latter represent a source of induced somaclonal variation. Significant variation in somaclones and several generations of their progenies has been detected at genetic (DNA polymorphism, chromosome number alterations), physiological (reproduction pathways), morphological (leaf shape, density of hypericin accumulating glands) and biochemical (content of hypericins and acylphloroglucinols) levels (Košuth *et al*., 2003; Koperdáková *et al*., 2004). The genetic stability can only be assured in tetraploid obligate apomicts that are very rare. Most of diploids reproduce sexually.

Reproduction of *Hypericum perforatum* L. plants with desirable properties by seeds or by *in vitro* multiplication does not assure maintenance of their quality. The quality can be restored when the plant material is subjected to cryopreservation. Cryostorage in liquid nitrogen has relevant role in conservation of plant cells and tissues characterized by high production of important secondary metabolites. As our results show, cryopreservation using the slow freezing method did not affect genetic stability of *Hypericum perforatum* L. plants regenerated from cryopreserved meristems. No alterations were detected in chromosome number and mitotic activity. By applying the analysis of va-

riability of the VNTR sequences in our samples before/after cryopreservation, only minor alterations in the amplified profiles between cryopreserved and non-cryopreserved plants were found. The content of hypericin was not significantly changed in plants differentiated from cryopreserved *H. perforatum* L. meristems (Urbanová *et al*., 2005, submitted for publication).

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Taxus baccata CELL CULTURES – EXPERIMENTAL SYSTEM FOR STUDY OF EXPRESSION OF PACLITAXEL BIOSYNTHETIC GENES

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Plant cell cultures represent a useful production system for many pharmaceutically valuable secondary metabolites. In comparison with the whole plants they possess several advantages – growth in controlled conditions, stability of yield and quality of product, no contaminations with agrochemicals or pests, simplified extractions of metabolites as well as economic viability – plant cells are inexpensive to grow and maintain.

Although worldwide many high-yielding cell cultures of different plant species with unique characteristics have been established, only two plant secondary metabolites – shikonin and paclitaxel have recently been produced on a commercial scale (Hellwig *et al*., 2004).

Paclitaxel (TAXOL®) was approved for the treatment of refractory ovarian cancer by *Bristol Myers Squibb* in 1992. The unusual mechanism of action, promising spectrum of antineoplastic activity but also poor water solubility and acute hypersensitivity reactions initiated further investigations of second-generation analogues. Semisynthetic docetaxel (TAXOTERE®; *Rhône-Poulenc Rorer* – now *Aventis*) and PROTAXOL were developed to overcome these difficulties (Zhao *et al*., 2004). Recently TAXOL® and TAXOTERE® are well-established drugs for the treatment of breast, ovarian, non-small cell lung cancers and Kaposi's sarcoma (Oberlies *et* Kroll, 2004). Since TAXOL® is used in the treatment of ovarian cancer, the survival rate has more than doubled (Crown *et al*., 2000).

Recently, rapid progress has been made in explanation of crucial steps in biosynthetic way of taxanes. Biosynthesis of paclitaxel involves 19 enzymatic steps from the universal diterpenoid progenitor geranylgeranyl diphosphate (Jennewein *et al.*, 2004). Several enzymes catalyzing subsequent pathway steps were identified; the sequence of 9 corresponding genes was established (Walker *et al*., 2002).

Our attention was aimed at the study of the expression of two genes - *dbat* and *dbtnbt* - involved in the biosynthetic pathway of paclitaxel. A full-length cDNA clone for 10-deacetylbaccatin-III-10β-*O*-acetyltransferase (*dbat* gene) which catalyses the formation of the last diterpene intermediate, baccatin III, has been isolated from *T. cuspidata* in 2000 (Walker *et* Croteau, 2000). The isolation and analysis of the cDNA encoding a 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase (*dbtnbt* gene), catalysing the last step of paclitaxel biosynthetic pathway, was performed in *T. canadiensis* in 2002 (Walker *et al.*, 2002).

The gene expression was studied in callus cultures initiated from one-month-old *in vitro* grown *T. baccata* L. seedlings. The callus cultures were initiated and maintained on Gamborg's B_5 medium using a 1.5 % phenolic-binding compound, soluble polyvinylpyrrolidone (PVP), 3 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 $mg.l^{-1}$ kinetin. The cultures were cultivated in darkness at 22 ± 2 °C. The expression of *dbat* and *dbtnbt* genes in callus cultures was confirmed by reverse transcription PCR (RT-PCR). The level of expression was evaluated periodically during one subculture interval by real-time RT-PCR. It is the most sensitive and the most flexible quantification

method (Wang *et* Brown, 1999) and can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyze RNA structure. The expression data obtained from analyses of different growth phases of *Taxus baccata* cell cultures are compared with the accumulation of 10-deacetylbaccatin III and paclitaxel during subculture.

Because the supply of taxanes from their natural sources – the bark of *T. brevifolia* Nutt. is limited, modern biotechnology may provide an alternative way to improve taxane production. The knowledge of biosynthetic pathway, the responsible enzymes and coding genes as well as well established *in vitro* experimental system of plant cell cultures would help to provide the taxane production more reliable and effective and to save the natural resources.

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VARIABILITY OF HYPERICIN CONTENT IN FOUR GENERATIONS OF SEED PROGENY OF *Hypericum perforatum* L. SOMACLONES

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Introduction

Hypericum perforatum L. is a perennial plant with broad spectrum of secondary metabolites of significant pharmacological effects. Phloroglucinol hyperforin is a major antidepressant component in the extract of the plant (Zanoli, 2004). Naphthodiathrone hypericin and its derivatives (assigned as hypericins) are the active antiviral components of the extracts (Park *et al*., 1998). Hypericin is a potential antitumor photosensitiser in photodynamic cancer treatment (Agostinis *et al*., 2002).

The aim of this work was to study total hypericin content in four lines of diploid and tetraploid plants derived from a single somaclone and to compare total hypericin content in apomictic progeny of tetraploid plants.

Material and Methods

Plant material: *In vitro* regenerated somaclones (R0) of *H. perforatum* L., cv. Topas originated from seedlings (Čellárová *et al*., 1992). Diploid and tetraploid plants were obtained from seeds germinated on hormone-free medium followed by acclimation to *ex vitro* conditions and field cultivation consecutively during the years 1992-2002 (R1-R4 generations) or simultaneously (R'1-R'4 generations) under the same cultivation conditions during the years 2003-2004.

Hypericin assay: Total hypericin content in methanol extracts of flowering parts of two-year old plants was quantified by spectrophotometer Spectronic 20 Genesys at 592 nm wavelength in 3 replicates.

Chromosome number determination: Chromosome numbers were determined by counting mitotic chromosomes in root tips pre-treated with 0.002% (w/v) 8-hydroxyquinoline at 4 °C for 4 h, fixed for 16 h in a mixture of 3:1 ethanol/glacial acetic acid, hydrolysed for 6 min in 1 N HCl at 60 °C and subsequently stained with 5% (w/v) aceto-orcein for 5 min. Squash preparations were prepared by cellophane technique followed by Giemsa staining for 30 min.

Statistical analyses: Differences in the content of hypericins were evaluated by Kruskal-Wallis analysis and analysis of variance, respectively followed by multiple range analysis. Association between the total hypericin content in mother plants and total hypericin content in their progenies was determined as Spearman rank correlations. The results of analyses were evaluated at 0.05 significance level using Statgraphics statistical software.

Assessment of DNA polymorphism: Total genomic DNA was isolated using the method of Haberer *et al*. (1996). High molecular weight DNA (30 to 40 ng) was used for 20 µl PCR reaction containing: 1x concentrated Taq polymerase reaction buffer with 1.5 mM $MgCl_2$, 0.75 U DyNAzyme^{TM} II DNA polymerase, 0.2 mM dNTP, and 1 mM primer (HVR-, HVR+, YNZ22, FVIIexB-C, FVIIex8, HBV5 or OGRB01). PCR fragments were separated in 1.6% agarose gel dyed with ethidium bromide (0.5 $\mu g.ml^{-1}$). The molecular weight of the bands was evaluated using TotalLab 1D analysis 2.0 software.

Results and Discussion

Four plant lines consisting of four successive generations of seed progenies derived from a single somaclone were studied. Plant line 7 contained only diploid plants, while lines 64 and 93 consisted of tetraploids only. The line number 104 was derived from diploid regenerant which progeny was partly diploid and partly tetraploid. Flowcytometric seed screen (FCSS) revealed that diploid plants were obligate sexuals, while tetraploid plants were facultative apomicts (Koperdáková *et al.*, 2004). Hypericin content differed significantly among different plant lines. As supposed, there were no differences in hypericin content between the plants of R'1-R'4 generations. Comparison of hypericin content in diploid and tetraploid plants revealed that di-

ploids had higher hypericin content than tetraploids when growing during successive years. Spearman rank correlations revealed correlation between hypericin content of the plant and that of its mother plant in both groups of plants. However, if only the plants of one line were considered, statistically significant correlation has been found only in R0-R4 plants of lines 7 and 93.
Broad DNA variability obtained with seven non-specific VNTR primers was detected in plants of line 7. This is in agreement with the sexual mode of reproduction of diploid plants. Based on the FCSS of mother plants of the lines 64 and 93, their tetraploid progeny could have originated by sexual or apomictic seed formation. VNTR analysis showed prevalence of apomictic seed formation in lines number 64 and 93. DNA variability occurred only in progeny of one mother plant, which implies sexual origin of the progeny. Three pairs and four triads of plants with identical profile were revealed, indicating that these plants represent clonal – apomictically derived progeny. Although these plants were cultivated under the same conditions, they showed variability in total hypericin content.
It is necessary to determine contribution of the genetic components of variability to overall variability. A little is known on biosynthesis of hypericin. It is synthesised by polyketide pathway through emodin (Torsell, 1997). Except phenolic oxidative coupling protein HYP-1, which converts hypericin from emodin, neither specific enzymes and intermediates involved in hypericin biosynthesis in *H. perforatum* have been identified or purified; nor there is any information concerning the regulation and molecular biology of the pathways involved (Bais *et al*., 2003). Moreover, the rate-limiting step for precursor regulation at the enzymatic level is still unknown (Kirakosyan *et al*., 2004). Knowing all genes of the whole biosynthetic pathway and its regulation in temporal and spatial manner would help to elucidate the production of hypericin in plants and would help to directly manipulate/influence the production of hypericin.

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GENETIC STABILITY OF HOP CALLUS DERIVED PLANTS AND *AGROBACTERIUM* - MEDIATED TRANSFORMATION

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Introduction

Plant regeneration protocols have been lately developed for hops, but the genetic stability of the recovered plants has not been tested yet. Callus-derived plants may undergo genetic and epigenetic changes along the *in vitro* culture stages, which is an undesirable phenomenon when the main objective is *in vitro* micropropagation or transformation of selected material. Therefore, it is important to assess the frequency of variation and try to infer the mechanism and factors affecting it.

Materials and Methods

Humulus lupulus L. *cv*. Chinook plants from clonal propagation were obtained from a commercial plantation (León, Spain), and an *in vitro* proliferative line was started. Plant regeneration from callus derived from *in vitro* shoot segments was carried out according to Gurriarán *et al.* (1999).

Analysis of variation.

Field and *in vitro* plants were compared to regenerated plants collected along the 1st, 2nd and 3rd subculture of the organogenic calli. AFPL and MSAP analyses were performed according to Vos *et al.* (1995) and Xiong *et al.* (1999). AFLP was carried out using sixteen combinations of *Eco*RI primers with an *Mse*I primer. Three primer combinations of both isosquizomers (*Msp*I/*Hpa*II) were tested to detect the methylation level of cytosines in the restriction sites. The presence/absence of bands was scored by visual inspection, and only those showing a clear signal were taken into account. The relationships among samples were estimated by UPGMA cluster analysis.

Agrobacterium-mediated transformation procedure.

Stem segments of *in vitro* plants were employed as starting material. The transformation experiments were performed using the *Agrobacterium tumefaciens* strains AGL1, EHA105 and LBA4404, that harboured the pBINUbiGUSint plasmid (Humara *et al*., 1999). This plasmid carries the neomycin phosphotransferase II (*nptII*) and the β-glucuronidase (*uidA*) genes.

In vitro shoot segments were incubated for 20 min in a 0.05 ($O.D._{600nm}$) bacterial suspension and cocultured for two days in solid medium in the dark. Next they were transferred to regeneration medium plus antibiotics (100 $mg.l^{-1}$ kanamycine and 500 $mg.l^{-1}$ cefotaxime) and subcultured at 15-day intervals in a 16/8 photoperiod.

Putatively transgenic calli were subjected to molecular (PCR for *nptII*) and histochemical (GUS staining) analyses.

Results and Discussion

In the AFLP analysis, 876 loci were detected. All the banding patterns of the regenerated progenies were identical to the field and *in vitro* mother plants. MSAP analysis (Fig. 1) showed that plants shared a 70% of the total detected loci, a 45% of the identified variation was produced by singletons contrasting with the small amount of the total of variation that was present in all the callus-derived plants (13.37%) suggesting that the process potentially induces random methylation changes in many sites along the hop genome. Control, 1st and 2nd subculture pools clustered in the same main branch of the

phenogram while the 3rd subculture pools were placed in other bifurcations (Fig. 2). 117 out of the 298 detected loci (39%) were polymorphic when compared to the control pools, being the most common change the demethylation of the tagged sites (84%). Polymorphic fragments in the regenerated pools were isolated from polyacrylamide gels, re-amplified and sequenced but no significant homologies with known genes or sequences were found.

A 63.3% of kanamycin-resistant calli were obtained with LBA4404 strain. A 20.0% and a 6.7% were obtained with AGL1 and EHA105 carrying the pBINUBiGUSint plasmid, respectively. The kanamycin-resistant calli were subjected to PCR and histochemical analyses, and their transgenic nature was confirmed.

Epigenetic changes have been detected with the MSAP analysis, and could be due to the length of the *in* vitro culture. However, the AFLP results have shown that no major genetic rearrangements occurred in the regenerated plants. A protocol for hop genetic transformation has been developed and, supported by the AFLP results, we can conclude that true-to-type transformed plants could be produced by indirect organogenesis.

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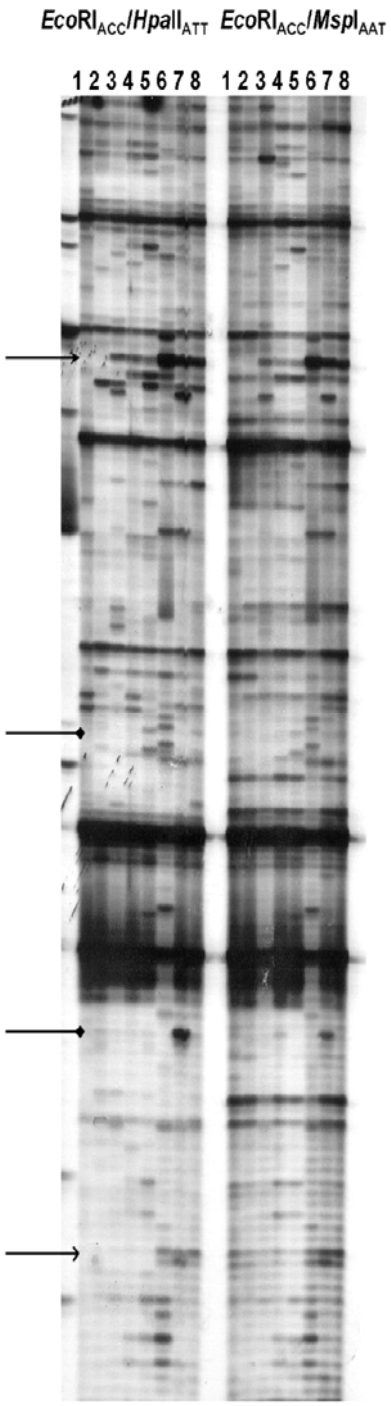

Figure 1 MSAP patterns. Lane 1-2 *in vitro* material; lane 3-8 plants regenerated from callus, 1st subculture, lane 3; 2nd subculture lanes 4-5; and 3rd subculture, lanes 6-8. Differences in the presence of a fragment between the controls and the callus-derived pools are signalised with a full arrow, differences among some of the pools showed with an empty arrow, and singletons with a diamond arrow. All the differences signalised correspond to demethylation processes

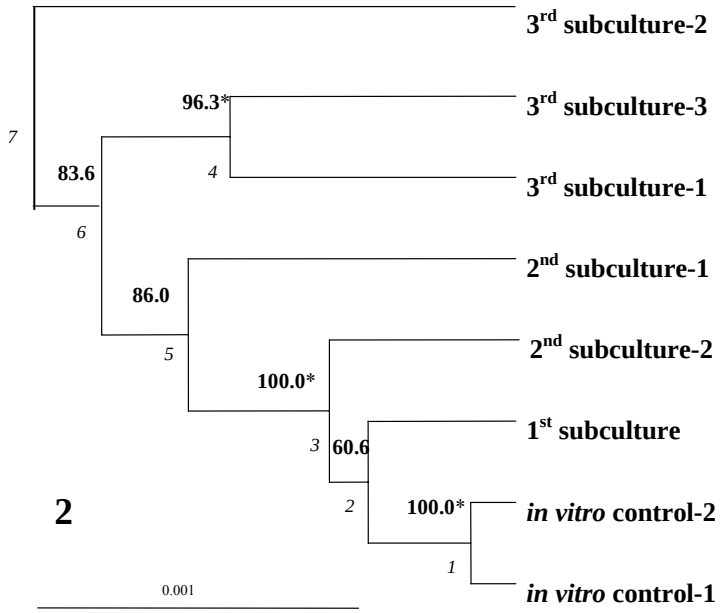

Figure 2 UPGMA tree. In cursive, the node numbers; in bold, the bootstrap value. The branches grouped together in a cluster with a bootstrap confidence value higher than 95% are signalized with an asterisk. The third subculture pools are clustered apart of the others pools

UK *Narcissus* CROP IMPROVEMENT RESEARCH

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Introduction

The UK *Narcissus* industry is the largest in the world with some 4300 ha under cultivation and a farm gate value of around £31M per year. A large proportion of the bulbs and flowers produced are exported throughout Europe and the USA. Currently, the industry relies on only a small number of commercial cvs and there is a need to extend and improve the range of cvs in production.

There are two significant problems faced by the UK *Narcissus* industry. Firstly the slow clonal propagation rate of *Narcissus*: it takes about 25-30 years to produce sufficient bulbs for commercial release of a new variety. This greatly impedes the development of improved varieties. Secondly there is a requirement for resistance to basal/neck rot (caused by *Fusarium oxysporum* f. sp. *narcissi*) which is currently the worst UK *Narcissus* disease. Half of the *Narcissus* grown in the UK comprises cvs (primarily 'Golden Harvest' and 'Carlton') that are highly susceptible to basal/neck rot so currently there is heavy use of fungicides for control. It was recently estimated that some 20% of UK *Narcissus* production could have unacceptably high levels of bulb rot, making it unsaleable and amounting to losses of £6M.per year.

Improvements in both of these areas would be of great benefit to the industry.

Current and proposed research:

Clonal propagation: At Warwick HRI we are working to optimise a 'RITA' bioreactor protocol for micropropagation of *Narcissus*. Bioreactors are a rapid, automated plant production system which could accelerate development of new *Narcissus* cultivars. We are comparing nodular callus vs. shoot cluster explants which have been obtained from *Narcissus pesudonarcissus* cvs Golden Harvest and St. Keverne to determine the best starting material for use in the bioreactors and which will acclimatize best into the glasshouse.

It is widely recognised that variation is sometimes induced during tissue culture and that this is undesirable where clonal propagation is the aim. Amplified Fragment Length Polymorphism (AFLP) is extremely useful for detection of variation between closely related genotypes and this technology is ideally suited for detection of genetic variation during tissue culture. Tissue culture induced genetic variation is rare in monocots, however, we need to verify that no such aberrations occur during tissue culture of *Narcissus.* We have now developed AFLP methodology to confirm clonal fidelity of bioreactor produced *Narcissus* plants.

Basal rot resistance: AFLP technology can also be used to identify molecular markers for quantitative traits such as resistance to basal rot. However, there are difficulties in attempting to find molecular markers in a tetraploid species, especially when there is also a high level of heterozygosity as common in commercial *Narcissus* cvs. To address this we will produce a defined diploid population from two parental diploid *Narcissus* species, one basal/neck rot resistant and the other basal/neck rot susceptible. Because we are working at the diploid level we anticipate that it will be more feasible to utilise AFLP's to identify molecular markers for basal/neck rot resistance in this population.

Significant natural genetic variation is known to occur in the wider gene pool of *Narcissus* species for many traits including basal rot resistance. However, exploitation of natural diversity is difficult as infertile triploids result from interspecific crosses between tetraploid commercial *Narcissus* cvs and diploid *Narcissus* species.

Chromosome doubling techniques will be developed for *Narcissus* so that we can produce tetraploid forms of diploid wild species with useful traits such as basal rot resistance. These tetraploids can then be used as parents in breeding programs to produce improved commercial tetraploid cultivars. Chromosome doubling through shoot cultures has been successful in many species including the geophyte *Alstroemeria*. Regenerative callus might be good material to use initially in the doubling work as the calli are believed to regenerate at least in part by somatic embryogenesis originating from single cells. They are therefore likely to produce totally tetraploid plants without the possibility of chimeras consisting of tetraploid and diploid cells. Regenerated plants will be tested by flow cytometry to confirm their ploidy level.

Discussion

Both nodular callus and shoot cluster explants in the bioreactors are showing promise and with full evaluation and optimisation we believe we can achieve rates of at least 1000 clones per starting bulb in 1.5 years in the near future. The rate of bulbil production in the bioreactors is likely to be influenced by immersion time, frequency, inoculum density and medium volume. Our objective is to optimise *Narcissus* production in bioreactors to produce large numbers of clonal regenerants.

The AFLP work carried out on *Narcissus* cvs Golden Harvest and St. Keverne has shown differences between them so enabling the technique to be used for marker selection. Repeats of the same sample from cv Golden Harvest shows an AFLP experimental error of 5% Using this we have been able to confirm clonal fidelity between bulbs of cv Golden Harvest and tissue culture derived material from it.

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MICROPROJECTILE MEDIATED DNA TRANSFER IN *Pinus nigra* ARN.: EMBRYOGENIC TISSUE REGENERATION AND STABLE GUS–EXPRESSION

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Introduction

Gene transfer via particle bombardment or *Agrobacterium* mediated, occurred in several conifer species. Embryogenic tissues have been included in many transformation experiments due to their high regeneration ability. Transgenic somatic plantlets have been produced in conifer species belonging to genera *Picea*, *Pinus*, *Abies*, *Larix* (Minocha and Minocha, 1999). In *Pinus nigra* two embryogenic cell lines have been selected for genetic transformation by biolistic method. In the present study we describe the transformation procedure and tissue regeneration after bombardment.

Material and Methods

Plant material: the embryogenic tissues used in transformation experiments have been derived from immature zygotic embryos of *Pinus nigra* Arn. Two cell lines (E103, E104) have been selected. Both of them produced mature somatic embryos and subsequently plantlets.

Preparation of tissues for bombardment: 4g embryogenic tissue was resuspended in 40 ml liquid DCR medium containing 2mg.l^{-1} of 2, 4 D and 0.5mg.l^{-1} of BA. Two ml of suspension was pipetted on filter paper disc and the liquid was discarded by filtration. The filter papers with tissues were transferred to mentioned DCR medium containing manitol (45 mg.l^{-1}) and placed to culture room in dark for 24 hours. After 24 hours the tissues were bombarded.

Plasmids and microprojectile bombardment: the plasmid pCW 122 (Walter *et al.*, 1994) containing the reporter *uidA* gene encoding β-glucuronidase (GUS) controlled by double Ca MV 35S promoter and the *nptII* gene is controlled by single Ca MV 35S promoter. For bombardment the biolistic particle delivery system (PDS 1000, BioRad, Hercules, California) has been used. The gold particle size was 0.6 μM.

For each cell lines four bombardments with 28 Petri plates (9 cm) have been carried out. Non-bombarded tissues were prepared by the same way as the bombarded, and served as control (14 Petri plates four times). Immediately after bombardment the Petri plates with tissues were placed to culture room (dark, 24 ˚C). After three days, the tissues were transferred to selection medium (the mentioned DCR containing geneticin 20 mg.l^{-1}). Five Petri plates from each replicate were taken for the histochemical assay to evaluate transient expression. The tissues (bombarded and non-bombarded) were cultured in dark and subcultivated regularly to fresh selection medium. The cultures were kept on selection medium until maturation.

***Histochemical and fluorimetric analysis of GUS expression*:** Histochemical assays were conducted essentially as described by Jefferson. Fluorimetric assays (Mlynarova *et al*., 1994) were performed using a Fluoroskan II microplate reader (Titertek, Finland). Concentration of proteins in extracts was determined according to Bradford. GUS activities were expressed as nanomoles of methylumbelliferone (MU) per minute per milligram of soluble protein.

***Southern hybridization*:** Genomic DNA (15 μg) was digested overnight with restriction enzyme *Hind*III, separated on 1% (w/v) agarose gel and blotted onto a Hybond N^+ membrane (Amersham) using alkali transfer buffer and hybridised at 65 ^oC with *nptII* gene fragment as a probe. The probe was radioactively labelled (Amersham Megaprime DNA labelling Kit). Hybridisation signals were visualised by autoradiography using a BAS2000 PhosphorImager (Fuji).

Results and discussion

After particle bombardment tissue regeneration occurred in cell line E104. Totally 5 putative sublines were achieved out of 112 bombarded Petri plates. In cell line E103 no tissue regeneration occurred. Similarly, no growth was observed for control (non bombarded) tissues. Tissue regeneration was relatively slow. The first recovery was observed 8-10 weeks after bombardment. Following this period the recovered tissues grew rapidly and showed features characteristic for embryogenic tissue of *Pinus nigra*. Southern hybridization further confirmed integration of the *nptII* gene into genomic DNA of the transgenic sublines E 104. No hybridization signals were detected in non-transformed control tissues whereas signals were observed in transgenic tissues.

Histochemical assay four months after bombardment showed extensive GUS positive activity concentrated mainly in meristematic cells of the embryonal part. GUS expression was stable over a period of time and one year after bombardment GUS loci were observed in sub-cultured transformed embryogenic tissues maintained on selection medium. GUS-like staining was never observed in non-transformed tissues. The expression of the GUS gene in embryogenic tissues of stably transformed sub-lines of line E 104 was also investigated using a fluorimetric approach. Transclones displayed differences in expression of GUS activity. Variability in the transgene expression between individual transgenic plants or cell lines carrying the same transgene(s) in conifers was also observed by others (Walter *et al*., 1998; Klimaszewska *et al*., 2003).

The maturation of transformed embryogenic tissues was very poor and no transgenic plantlets were obtained.

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