SECTION 5 - *IN VITRO* CULTURES

Uptake of ^{14}C-sucrose and ^{14}C-NAA by tissue cultures of *Nicotiana tabacum* and *Brassica oleracea* in regenerating and non-regenerating cultures

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Biol. Plant. 34 (Suppl.): 533, 1992

Uptake of ^{14}C-sucrose and ^{14}C-NAA applied into MS medium supplemented with relevant growth substances in a period of four weeks, one week prior to transfer and on transferring to hormone free media was investigated using callus forming explants of tobacco (*Nicotiana tabacum* L.) and cabbage (*Brassica oleracea* L. var. *capitata*) hypocotyl explants. Different uptake of both labelled substances was observed prior to visible organogenesis, *i.e.* prior to transferring onto hormone free media. This suggests that this process is connected with an increase of sucrose and auxin (NAA) uptake. A more detailed study on the uptake of age of cabbage hypocotyl explant on the uptake of labelled sucrose revealed that the uptake of ^{14}C-sucrose increases up to the age of 12 d of the donor plant. During this period the difference in uptake was obvious between explants of different capacity of organogenesis. The results indicate that the process of organ initiation affects the whole metabolism of culture resulting in an increase in both substances uder study. More intensive uptake of ^{14}C-NAA compared to ^{14}C-sucrose suggests that the process is selective as early as in the period prior to visible organogenesis.

Production of naphthoquinones in callus culture of *Drosophyllum lusitanicum* Link.

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Biol. Plant. 34 (Suppl.): 533, 1992

In the present study the relation between growth rate and production of secondary metabolites (naphthoquinones) was investigated. The content of dry mass was the highest on the 7th d after inoculation on MS medium supplemented with 2,4-D and NAA and on medium with IBA and NAA. The dry mass content was 6.39 % and 5.05 %, respectively. From 1,4-naphthoquinone derivates only the plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) was found in high amount (1.65 % of dry mass) after 42 d of incubation.

Subcellular localization of sanguinarine in cells of callus culture of *Papaver somniferum* L. after treatment with elicitor

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Biol. Plant. 34 (Suppl.): 534, 1992

Many efforts have been devoted to improve the productivity of specific natural compounds, especially various secondary metabolites of medicine plants. Elicitation is one of the new techniques, which help us to enhance the accumulation of secondary natural products in the plant tissue cultures. This paper presents data showing the effect of fungal elicitor prepared from *Botrytis cinerea* on sanguinarine alkaloid formation and accumulation in cells of the callus culture of *Papaver somniferum* L. Fungal elicitor was added 5 weeks after inoculation. The callus turned brown after 24 - 48 h of incubation under the influence of the elicitor. Ultrastructural changes have been observed in association with the accumulation and secretion of sanguinarine in elicitor-treated cells. Alkaloid content in elicited cells was shown as an electrondense material (osmiophilic aggregations), which occurred on the tonoplast and in freely floating bodies in the vacuole. This cell compartment represented the most conspicuous storage place of secondary compounds including alkaloids. There was positive correlation between the PAL (phenylalanine ammonia-lyase) enzyme activity, number of electrondense bodies and content of sanguinarine after elicitation. The 30-times increasing of sanguinarine content was observed in elicitor-treated cultures. Our results showed a possible way of primary biosynthesis of dense material in the endoplasmic reticulum. The dilated elements of the endoplasmic reticulum contain electrondense material in the area of the enchylema. Membranes were free of ribosomes on these sites.

Morphological and anatomical study of *Hypericum perforatum* L. intact plants and R_0 regenerants

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Biol. Plant. 34 (Suppl.): 534-535, 1992

In comparison with intact plants, there are several anatomical and morphological alterations in plants regenerated *in vitro* due to altered physiological functions. *In vitro* regenerated plants of *Hypericum perforatum* which were placed on RM medium supplemented with BAP in a concentration of 2.22 μM, showed differences in the anatomical structure of leaves. The cuticle was thin, and palisade parenchyma consisted of monolayer of cells and occurred only on the upper side. In comparison with the native roots, the anatomical structure of *in vitro* regenerated roots did not show any difference. On the other hand, morphological differences have been detected. *Hypericum perforatum* is of great interest especially due to the content of tannins, dianthrones, essential oil and other secondary metabolites. Dianthrones and essential oil are accumulated in the special internal secretory structures. There are two types of glands scattered throughout the lamina that contain essential oil, and blackish clusters of cells containing a wax impregnated with dianthrones arrayed mostly around the leaf margin. Both types of secretory structures have the same relationship to minor veins and vein endings. These glands differ from each other in more than just colour. The translucent cavities extend from abaxial to adaxial epidermis and the large central space is surrounded by a uniseriate epithelium of flattened cells. The blackish glands consist of a core of

large cells surrounded by an uni- to bi-seriate sheath of flattened cells and do not quite span the entire height of the mesophyll being separated from adaxial epidermis by a layer of flattened palisade parenchyma cells.

Microspore culture of potato

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Biol. Plant. 34 (Suppl.): 535, 1992

For studies of androgenesis in potato, the dihaploid clone LH 43/85 was used. The androgenic response was induced using the starvation method, for the first time described by Kyo and Harada (1986). Sterile flower buds were kept at a temperature of 4 ± 2 °C for 7 d in starvation medium. Microspores isolated from these buds having one nucleous were cultivated in Murashige and Skoog medium supplemented with 0.3 M mannitol, 2,4-D (1.4 mg l^{-1}) and kinetin (0.4 mg l^{-1}). After 8 d of cultivation we could observe the first divisions, and first multicultural structures started to form. The androgenic embryos passed their globular and heart stages. After 30 - 34 d of cultivation the embryos reached their torpedo stage.

Reference:
Kyo, M., Harada, H.: Planta 168: 427, 1986.

Structural analysis of organogenesis in potato stem explant culture

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Biol. Plant. 34 (Suppl.): 535, 1992

Histological investigation of the structural changes in potato stem internode segments enabled us to classify and to localize the individual steps of regenerative processes, in particular of *de novo* bud formation, in this primary culture. Stem internode segments of cv. Desirée were cultivated on the agar medium MS containing 6-BAP (2 mg l^{-1}), IAA (0.1 mg l^{-1}) and adenine (40 mg l^{-1}). The cross sections (10 μm thick) of the segments embeded in paraffin were stained by Alcian blue and Kern Echt Rot. The method enabling to evaluate the gradual differentiation of the callus tissue as well as starch incidence has been worked out. Special attention was payed to the *de novo* bud formation which was observed at about the 20th d of culture. These buds were organized in two possible ways: *1.* they differentiated either from meristematic layers localized on the surface of the wound callus parenchyma or on the surface of the globular structures, morphologically distinguishable as "nodules". *2.* they differentiated from endogeneous meristemoids, groups of small meristematic cells, localized under the callus surface. In this case, the arrangement of the cells into tunica-like layer represents the first step of the shoot apical meristem differentiation. This tunica layer formed frequently on the surface of voluminous neighbouring callus parenchyma cell. Further development of the bud primordia was followed as well: initiation and growth of leaf primordia, procambial strands differentiation and stem internode growth. The reversion of the shoot meristems into callus tissue in case of malformed or developmentally inhibited buds was described.

Multiple shoot formation in *Hypericum perforatum* L. and variability of R_0 regenerants

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Biol. Plant. 34 (Suppl.): 536, 1992

Culture requirements promoting morphogenesis and organogenesis in *Hypericum perforatum* L. resulting in a very effective way of clonal propagation were studied. Seedlings of *Hypericum perforatum* were placed on RM medium supplemented with BAP within the concentration range between 0.44 and 4.40 μM. Green shoot primordia appeared within 10 d along the whole seedlings. By 4 weeks, all explants had produced multiple shoots with minimum callus formation. Numbers of shoots per explant were influenced by concentration of BAP. Increasing the level of BAP enhanced the number of regenerated shoots per explant. In average, 885 regenerated shoots per one seedling were obtained under the influence of 4.40 μM of BAP. Regenerated shoots were easily rooted on RM medium without plant growth regulators and later transferred to perlite following the cultivation in hotbed. Several characters such as fresh and dry mass of herbage, height of plants, number of branches per plant and number of dark glands containing dianthrones per leaf area were evaluated in R_0 regenerants and compared with those in the control plants. A significant decrease had been detected in fresh and dry mass of regenerants. On the other hand no significant differences between the control and *in vitro* regenerated plants occurred in the number of branches per plant and number of glands containing dianthrones per leaf area. We have detected great variability within a particular genotype as well as within individual groups of regenerants obtained on different concentrations of BAP. Significant differences occurred also within the control group. Concentration of BAP did not influence the observed characters.

Fluorescence and immunofluorescence labelling of calmodulin during protoplast regeneration of potato

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Biol. Plant. 34 (Suppl.): 536, 1992

The Ca^{2+}-binding protein calmodulin is a biologically active and important compound. The aim of this study was to find out if such gradients also are present during protoplast regeneration. The number of protoplasts isolated ranged between 3 and 7×10^6 per gram of fresh mass. The first cell divisions could be observed after 3 - 5 d of cultivation. The 10-d old culture was supplemented with fresh culture medium and the plating efficiency at that time was 7 - 13 %. Freshly isolated potato protoplasts showed a bright signal which originated from the cytoplasm after staining with fluphenazine.HCl (1:2). In dividing cells a strong fluorescence near the plasma membrane was observed. The protoplasts had a relatively uniform cytoplasmic immunofluorescence staining. Cells from vascular-parenchymatic tissues had more anticalmodulin fluorescence in organels than meristematic cells. We conclude that a polarized subcellular calmodulin gradient was not observable during regeneration of potato leaf protoplasts.

The study of adventitious bud initiation on isolated zygotic larch embryos (*Larix decidua* Mill.)

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Biol. Plant. 34 (Suppl.): 537, 1992

Isolated larch zygotic embryos were cultivated on modified B5 medium with various concentrations of cytokinin benzylaminopurine (BAP). The greatest amount of adventitious buds per one embryo was observed at the concentration of 2 mg BAP l^{-1}. Paraffin sections stained with Heidenhein's haematoxylin were used for anatomical study of the adventitious bud initiation process. Metabolical changes caused by BAP led to the changes of the achromatic spindle orientation in the dividing epidermal cells which started their periclinal division on the second day of culture. The division of the cells formerly originated in the epidermis continued in the same way during the following days of culture and resulted in meristematic tissue forming several layers (4 - 10). This process was mainly locakized in the hypocotyl below cotyledons and in cotyledons. In this meristematic tissue some cells differentiated exhibiting vacuole enlargements during the following week of culture. Later these cells degenerated. On the other hand, some other cells continued in the mitotic division and formed adventitious buds. These buds then elongated and formed shoots when being cultivated without growth regulators. Rhizogenesis of adventitious shoots was not observed. Isolated larch embryos were paralelly cultured as a control on the same medium without growth regulators. These embryos developed into seedlings and their epidermis retained the character of one layer as a result of anticlinal division.

Origin of somatic embryos from single cells *in vitro* in Norway spruce (*Picea abies* (L.) Karst.)

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Biol. Plant. 34 (Suppl.): 537, 1992

The early somatic embryo of Norway spruce (ESM), as well as the zygotic one, is a longitudinal structure which consists of a more or less spherical group of isodiametric embryonal cells (pE), embryonal tubes (Et) and embryonal suspensor (Es) which both consist of longitudinal cells. The new ESM develop by cleavage of the original pE or the new ESM can originate from certain cells in the region of Et or Es. These cells, megakaryocytes, maintain the ability to divide. At first the nucleus of megakaryocyte divides forming two nuclei. One of them (embryonal nucleus - EN) moves towards one pole of the cells. A plasmatic membrane probably separates the EN from the rest of the megakaryocyte forming protoplast, resp. The EN starts to divide forming a group of free nuclei. After that the cell walls are formed among these nuclei and so a base of a new group of embryonal cells develops. The original megakaryocyte gradually collapses. The pE cells develop new Et. The new Es originate from that new developed Et. The new ESM is gradually liberated from the original ESM.

Somatic polyembryogenesis in Norway spruce - induction and characterization of different embryogenic clones

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Biol. Plant. 34 (Suppl.): 538, 1992

The embryogenic cultures were induced in the culture of not fully mature embryos of Norway spruce (*Picea abies* (L.) Karst.). Embryos were isolated from 2 cones which were taken in late August 1991 from one tree (the cones were green at that time). Some seeds were cold treated (4 °C) for one week before culture. The modified medium von Arnold (1987) with different concentrations 2,4-D, KIN and BAP was used for culture (dark, 25 ± 2 °C). 20 different clones were induced from different embryos in about 10 weeks. The first one was isolated 4 weeks after culture initiation. The obtained clones were subcultured in 10-d periods. During subcultures the clones were "cleaned" from callus. So only embryo suspensor masses (ESM), otherwise early somatic embryos, had been cultured. Some clones tended to develop callus-like globules. The induced clones were characterized according to: early somatic embryos shape, cultured colony shape, senescence during prolonged subculture and increase of fresh mass.

Reference:
Von Arnold, S.: J. Plant Physiol. 128: 233, 1987.

The use of tissue cultures in breeding of red clover (*Trifolium pratense* L.)

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Biol. Plant. 34 (Suppl.): 538-539, 1992

Tissue cultures have been used at our Plant Breeding Station for some years. The first attempt was micropropagation of red clover genotypes Kvarta and Radegast, which were resistant to damage by nematode *Ditylenchus dipsaci* in the field. Recently we try to produce viral resistant materials from the tetraploid variety Kvarta. We excised top meristems from tillers of mother plants from field nursery or meristems from aseptically germinated seedlings in combination with thermoterapy as initial explants. Callus was induced on PC-L2 agar medium with picloram or NAA and shoots were regenerated on the same medium with addition of NAA and BAP. Rooting was initiated on liquid medium with NAA as the only regulator. Regenerated plants were tested for resistence and transferred into the field nursery. The second aim in our laboratory is developing methods usable for production of interspecific hybrids in genus *Trifolium*. We tested 44 genotypes from 11 species of genus *Trifolium* for ability to induce callus, its cultivation and regeneration of plants. These were: *T. pratense* 2n (1 genotype) and 4n (12 gen.), *T. hybridum* (1 gen.), *T. repens* (4 gen.), *T. medium*(10 gen.), *T. ambiguum* (5 gen.), *T. meneghinianum* (1 gen.), *T. vesiculosum* (3 gen.), *T. pannonicum* (3 gen.), *T. incarnatum* (2 gen.), *T. resupinatum* (1 gen.), and *T. alexandrinum* (1 gen.). Explants were excised from aseptically germinated *Trifolium* plants and initial cultures were established from cotyledons, hypocotyles, roots, petioles and the first trifoliate leaves of seedlings. The best for callus induction and growth in most of the species and types of explants was PC-L2 agar medium with NAA and BAP. Satisfactory growth of callus was recorded in *T. medium, T. vesiculosum, T. pratense, T. pannonicum, T. incarnatum, T. alexandrinum* and *T. resupinatum*. Poor growth of callus was recorded in *T. meneghinianum, T. ambiguum* and *T. repens*. Callus failed to induce in *T. hybridum*. Callus with green centres was formed in *T. pratense, T. medium, T. vesiculosum, T. meneghinianum, T. pannonicum* and *T. ambiguum*, but plants were regenerated only from callus

cultures of *T. pratense* (2 genotypes), *T. pannonicum* (1 gen.) and *T. medium* (1 gen.). Callus was induced from petioles. We also tested crossing and growing of seeds on cut stems of *Trifolium pratense* 4n. Cut stems were cultivated in polyethylene bottles with Hoagland solution and 5 % saccharose. Crossing was made in greenhouse, flowers were pollinated with honey bees. Mean setting rate of seeds of 11.27 % was achieved in these experiments (in range from 0.6 % to 32 % of flowers in inflorescence) in comparison to 23 - 63 % in the field nursery. This method is to obtain results fully usable in hybridization (topcross, polycross, diallelic cross or wide hybridization) of *Trifolium* species.

Isoenzyme patterns and peroxidase activity during growth cycle of alfalfa (*Medicago sativa*) cell suspension culture

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Biol. Plant. 34 (Suppl.): 539, 1992

Isoenzyme patterns and peroxidase (EC 1.11.1.7) activity were investigated in the cells and in the growth medium of alfalfa cell suspension culture during the growth cycle. Peroxidase activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm. Peroxidase isoenzymes were separated by anodic and cathodic PAGE. The guaiacol stained gells were recorded at 470 nm using Beckman DV-6 scanner densitometer. Two peaks of the soluble peroxidase activity were observed in cells after their transfer into fresh medium. The first increase of peroxidase activity occurred in the early growth phase and depended on inoculation size. This phenomenon could likely be associated with dilution of culture after inoculation. The total peroxidase activity in cells increased continuously during the growth and reached the second maximum value at the late exponential phase. Peroxidase isoenzymes spectrum obtained with PAGE revealed one marked anodic and two cathodic bands in the first peak. On the contrary, dominant cathodic isoenzymes and only insignificant anodic band were found in the second maximum of the soluble peroxidase activity. The total peroxidase activity in the medium increased incessantly during exponential growth phase of alfalfa cell culture and reached the maximum value at the period of descelerating growth rate. The increase in the peroxidase activity of culture medium was at first caused by the release of cathodic isoenzymes and later, in the post-exponential growth phase, by anodic isoenzymes. The release of peroxidase into medium correlated with the growth of alfalfa cell suspension culture.

Effect of caffeic acid on Norway spruce [*Picea abies* (L.) Karst.] callus culture

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Biol. Plant. 34 (Suppl.): 539-540, 1992

Caffeic acid acts as an auxin protector and important metabolite on the metabolic pathway to lignin biosynthesis in the plant. This phenolic compound has often been studied in phytopathological aspects. Moreover, caffeic acid is an allelopatic compound. A long-term root callus culture of Norway spruce [*Picea abies* (L.) Karst.] derived from the primary root of the seedling was precultivated on a modified MS medium with 5 mg l^{-1} NAA and 0.1 mg l^{-1} BAP (Hřib and Rypáček 1981), and 1, 10 and 100 mg l^{-1} caffeic acid. The control was only the basic medium with NAA and BAP but free of caffeic acid. The precultivation on the medium with caffeic acid was

responsible for an increase of endogenous IAA level in calli. To estimate IAA the fluorimetric method was used. It was found that the mean at n = 3 was 12 ng IAA g^{-1} fresh mass of callus in the control, the highest mean at n = 3 being in calli precultivated on the medium with 100 mg l^{-1} caffeic acid, namely 16 ng IAA g^{-1} fresh mass of callus. The long-term root calli of Norway spruce precultivated on individual caffeic acid concentrations were tested for defense reaction in dual cultures with a tester, the fungus *Phaeolus schweinitzii* (Fr.) Pat. It was revealed that the calli precultivated on the medium with caffeic acid inhibited more intensive defense reactions as compared to control. This marked inhibition of the growth of tester's mycelium which was found in dual cultures using calli precultivated on the medium with caffeic acid, was not observed in cultivation of the fungus alone on the same medium with caffeic acid (1, 10 and 100 mg l^{-1}).

Reference:
Hřib, J., Rypáček, V.: Eur. J. Forest Pathol. 11: 270, 1981

Anatomical study of shoot development in long-term callus cultures of kiwifruit (*Actinidia deliciosa* var. *deliciosa*)

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Biol. Plant. 34 (Suppl.): 540, 1992

Long term organogenic callus cultures were established from axillary buds of female kiwifruit on solidified MS medium with Gamborg's vitamins, 30 g l^{-1} sucrose, 80 mg l^{-1} adenine, 170 mg l^{-1} NaH_2PO_4, 0.03 mg l^{-1} IBA and 2 mg l^{-1} BAP under illumination of 2 000 lx with photoperiod 16 h. Calli were cut from primary cultures and routinely subcultivated on the above mentioned medium for 2 years. Each callus produced 10 - 20 shoots. After 2 years samples for anatomical study were taken. They were fixed, dehydrated and embedded as for electronmicroscopical observations. Semithin sections were stained with methylene blue and basic fuchsine. Organogenic kiwifruit callus cultures were green and composed mainly of highly vacuolated interphase or unorganized dividing cells. After transfer to new medium many starch grains appeared in cells of some callus *loci*. Such cells often continued to divide but remained small. At this time starch grains disappeared and the relative amount of cytoplasm increased. In this way groups of small cytoplasmic cells with large nuclei and prominent nucleoli appeared in the calli. These cells were distinct from the original, highly vacuolated cells. Cytoplasmic cells were organized into a small layer at first and then typical dome-like structures were developed at the surface or in subsurface regions of calli. No vascular elements were present in or beneath these structures. Later a leaf primordium appeared in a lateral position and a leaf gradually differentiated. Subsequently vessels were formed in leaves and developing shoots.

Effect of humic substances on growth rate of the European orchid species *in vitro*

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Biol. Plant. 34 (Suppl.): 540-541, 1992

Humic substances can have both an indirect and a direct impact on the growth of orchid species *in vitro*. The former can take place mainly by buffering and complexation of ions in the medium, by affecting the hydrogen ion concentration, redox potential and by interaction with phenolic exudates. The last promotes plant growth by affecting water and water solutions reception, by enhancing rates

of respiration and photosynthesis, and by affecting other important physiological processes in plants. The purpose of this contribution was to investigate the complex effect of humic substances - Natrium humate (NaHu) and hymatomelanate (NaHy) in medium for asymbiotic *in vitro* germination of two wild European orchid species *in vitro*. Obtained results indicated that humic substances, especially NaHy at the concentration 10 - 50 mg l^{-1} significantly increased mass and size of protocorms, length of leaves and roots, number of rhizoids and percentage of surviving protocorms. Especially the last is important for the management of orchid species and decreasing of selective pressure *in vitro*.

Differentiation of multicellular glands in the course of development of regenerants in *Hypericum perforatum* L.

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Biol. Plant. 34 (Suppl.): 541, 1992

Multicellular glands containing dianthrones in *Hypericum perforatum* L. are distributed on the aerial parts of plant. They are usually not present on the cotyledons. During the course of ontogeny they start to differentiate on the first pair of foliage leaves and are located especially on the leaf margin. We have studied the course of development of dark glands containing dianthrones in plants regenerated *in vitro* in several stages of ontogeny. Regenerants were originated from seedlings of *Hypericum perforatum* L., cv. Topas, cultured on Linsmaier-Skoog's medium supplemented with 0.5 mg l^{-1} 6-benzylaminopurine. We have evaluated the number of glands on leaf blade and the leaf area during the course of development of regenerants until the values remained unchanged. Comparison between the *in vitro* regenerated plantlets and those from seeds in the stage of development of the first pair of leaves did not show significant difference in the mean number of glands per leaf. At this stage of development, the plantlets regenerated *in vitro* contained in average 4.85 glands per leaf. Regenerants with two pairs of leaves contained in average 7.4 glands per leaf. The number of glands per leaf blade has been completed in the stage of five pairs of leaves (in average 11 glands per leaf). The area of the leaf blade at this stage was approximately 7 mm^2 and during the further development had enlarged. The functional dependence of the number of differentiated dark glands on the leaf area was evaluated.

Some detection markers of heterofusion in interspecific and intraspecific somatic hybridization in potato

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Biol. Plant. 34 (Suppl.): 541-542, 1992

Somatic hybridization based on protoplast fusion represents a very promising way for creation of new potato genotypes. The wide use of that method is hindered by a lack of markers suitable for heterokaryons detection in protoplast population after fusion. As markers, differences in structures and plastid pigmentation, cytoplasm density, auxotrophy, resistance and the different ability to develop *in vitro* conditions and other morphological peculiatiries can be utilized. In interspecific somatic hybridization we used the fluoresceindiacetate staining of protoplasts derived from etiolated potato plants. For intraspecific fusion we have elaborated a detection system based on the natural

differences in protoplast pigmentation of both parental components. One component had anthocyanin intensively stained *calli* cells.

Synthesis of Norway spruce [*Picea abies* (L.) Karst.] seedlings with three species of ectomycorrhizal fungi *in vitro*

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Biol. Plant. 34 (Suppl.): 542, 1992

An ability of various strains of selected ectomycorrhizal fungal species to form mycorrhizal structures in roots of spruce seedlings, cultured under sterile conditions, was tested. Main intention of the procedure was to find the best way of quick development of mycorrhizis under aseptic conditions. The following fungal species were tested: two strains of *Laccaria laccata, Inocybe lacera* and *Lactarius deterrimus*. Growth rate of tested fungi on agar nutrient medium and on the newly developed artificial substrate was tested. Three growing techniques were used: growth in Erlenmeyer flasks with inorganic substrate, growth in Petri dishes with substrate inoculum and sandwich paper method. In the system with substrate inoculum some physical characteristics of newly developed inorganic substrates were tested. Some kinds of infusorial earth and clay burnt with sawdust and with perlite were used for experiments. After 7 months cultivation only 20 % of short roots created mycorrhizas and according to the anatomical investigation none or only one layer fungal sheat was formed. Between newly developed substrate and currently used peat-vermiculite no significant differences were ascertained but both techniques were more suitable compared with the paper sandwich technique. The presence of fungal inoculum caused shortening and beginning of branching of the roots.

Protein composition of non-embryogenic and embryogenic calli of silver fir (*Abies alba* Mill.) and its hybrid form *A. alba* × *A. nordmanniana*

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Biol. Plant. 34 (Suppl.): 542, 1992

Protein composition of both the embryogenic and non-embryogenic calli derived from zygotic embryos of silver fir (*Abies alba* Mill.) was analyzed in two lines of the species which have originated from a controlled cross-pollination of its individuals as well as in three lines of the interspecific hybrid *A. alba* × *A. nordmanniana* using SDS electrophoresis. It follows from the results that calli of both hybrids tested so far differ in number of their protein fractions. Individual lines of embryogenic calli were distinguished by different vitality and proliferation intensity. In addition, the non-embryogenic calli of both hybrids were used as a control. About 30 fractions of proteins were revealed in *A. alba* species. Its embryogenic lines differed mutually in their protein profiles, one being the identical with a non-embryogenic callus, the other containing the fast moving fraction (Rf 0.94) which had not been detected in any of the above calli. The protein pattern of *A. alba* × *A. nordmanniana* calli was reduced, consisting of only 24 fractions. Two lines of the embryogenic calli were identical in their protein composition with a non-embryogenic control, whereas the remaining line of callus with embryogenic activity lacked the fraction of relative mobility Rf 0.73.

The use of micropropagation in the protection of genofond of *Dianthus arenarius bohemicus*

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Biol. Plant. 34 (Suppl.): 543, 1992

Tissue cultures can also be used for the propagation of endangered and rare species of plants and therefore can contribute to the protection of phytogenofond. *D. arenarius bohemicus* is an endemic and endangered species which is included in the Red Book of Threatened and Rare Flora of Czechoslovakia. *D. arenarius bohemicus* can be found only in the protected reserve "Kleneč" in North Bohemia. Today the population of this species includes only about 30 clusters. The application of traditional methods of propagation of *D. arenarius bohemicus* has not been successful. A study was therefore undertaken to develop a technique for rapid clonal multiplication and to establish a germplasm bank of *D. arenarius bohemicus in vitro*. Actively growing shoots were excised from plants grown in the field. After surface sterilization in 10% commercial bleach, single node explants were placed into test tubes with a modified MS medium. The effect of different concentrations of BAP and IBA for shoot proliferation and rooting was tested. The cultures growing on the medium with a high concentration of BAP (1.0 - 2.25 mg l^{-1}) and with a low concentration (0.1 mg l^{-1}) or without IBA had a very high propagation coefficient (up to 120). The plants were small, reaching only about 30 % of the height of plants cultivated in the medium with a higher concentration of IBA. The roots did not usually form. On media with a higher concentration of IBA (0.1 - 1.0 mg l^{-1}) or without hormones the propagation coefficient was very low and one nodal segment usually produced only one long shoot with roots (rooting percentage from 55 - 83 %). The rooting of shoots in nonsterile conditions was also tested. After proliferation, single shoots were dipped in 0.05 % (m/v) IBA and transferred into perlite or perlite:sand (1:2) mixture wetted by distilled water. Rooting percentage was 73 % (the former) or 65 % (the latter). Rooted plantlets were transferred to the soil and after acclimation in the greenhouse to the field. Shoots rooted "*in vitro*" showed similar survival rate (85 %) after transfer to non sterile conditions, the same as shoots rooted in non sterile conditions. Tissue cultures of *D. arenarius bohemicus* have good genetic stability and they did not lose the morphogenetic capacity after one year of cultivation.

Somatic embryogenesis and plant regeneration in triticale

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Biol. Plant. 34 (Suppl.): 543-544, 1992

Somatic embryogenesis from immature zygotic embryos of triticale (*Triticale* Wittm.) was induced. Embryogenic ability and regeneration responsibility of four triticale genotypes (Bolero, Dagro, Grado, Lasko) were tested and highly responsive genotypes were found with a 75-100 % portion of embryogenic callus per cultured explant and high regeneration efficiency. Initial portion of embryogenic callus from the whole biomass of primary callus was found as the determining factor for a long-term retention of the morphogenic ability. This portion is influenced by genotype. Regeneration efficiency (ratio of the number of regenerated plants and the number of cultured zygotic embryos) was also found as genotype-dependent. Three successive phases of somatic embryogenesis in triticale were characterized: induction, development of somatic embryos and germination and regeneration of individual plants A basal MS-medium was used in three stages differing in their 2,4-D content. Improvement of somatic embryo conversion into complete plants was achieved by supplementation of cytokinins (0.1 mg BAP) into the medium. Conditions for

reliable transfer of regenerants to a nonsterile environment were found. In total, 415 green and 8 albino plants of four triticale genotypes were obtained. Morphogenic ability of the responsive genotypes was retained for at least one-year period.

Isolation and cultivation of embryogenic *calli* protoplasts of *Abies alba* × *Abies nordmaniana*

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Biol. Plant. 34 (Suppl.): 544, 1992

Somatic embryogenesis was initiated on hybrid immature zygotic embryos *Abies alba* × *Abies nordmaniana*. The stage of embryo development was the most important factor in initiation of embryogenic calli. Hybrid seeds of *A. alba* × *A. nordmaniana* were collected on the 13th of August. Embryogenic calli were initiated on SH solid medium with 1 mg l^{-1} BAP, 2 % saccharose and 50 mg l^{-1} inositol. Later all cultures were maintained on the same medium supplemented with 500 mg l^{-1} glutamine and 1000 mg l^{-1} casein hydrolysate. Cultures were initiated and maintained in the dark at 25 ± 2 °C. Embryogenic calli were subcultured every two weeks. Callus culture for protoplasts isolation was used after ten days of culture. Enzymatic solution contained 0.2 % BSA. 1.5 % cellulase, 0.3 % macerozyme, 0.1 % driselase, 0.3 % pectinase, 5 mM sucrose, 5 mM $CaCl_2.2H_2O$, 5 mM MES/KOH, pH 5.6. 2 g of *calli* were incubated in 15 ml enzymatic solution for 20 h. Protoplast suspension was purified three times. Pure protoplasts were immediately diluted at a density of 2×10^4 in Petri dishes containing 3 ml of semisolid basic MS medium, supplemented with 1 mg l^{-1} BAP. Formation of a new cell wall was observed four days after the protoplast isolation. First division in the cultivated protoplasts took place 10 d after isolation.

Osmotic potential of media and growth of plants *in vitro*

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Biol. Plant. 34 (Suppl.): 544, 1992

Sugars added to media used for cultivation of plants *in vitro* influence growth of cultures, dry matter accumulation and its partitioning. In order to distinguish between the two different effects of exogenous sugars, *i.e. 1*) source of carbon and energy and *2*) component of the media affecting osmotic potential, we investigated the effect of mannitol, which is considered by many authors as unmetabolizable. Excised embryos cultivated *in vitro* on media containing 0 - 9 % of mannitol were used for analysis. Increasing concentration of mannitol in the media brought about a decrease in root, shoot and total dry matter and an allocation of dry matter in favour of the roots. The dry matter per one cm of root and leaf increased remarkably above 4 % of mannitol. Endogenous levels of sucrose, glucose and fructose (per dry matter basis) were not influenced by different concentrations of mannitol in the medium. Mannitol was taken up by plants, transported to leaves and accumulated in tissue up to 0.25 and 0.4 mg mannitol per mg dry mass in shoot and root, respectively. It is concluded that osmotic relationship between *in vitro* grown plants and medium is considerably affected by increasing sugar concentration in the medium.

The effect of exogenous sugar supply, nitrogen deficiency and different light conditions on dry matter accumulation and partitioning in rape seedlings (*Brassica napus* L.) grown *in vitro*

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Biol. Plant. 34 (Suppl.): 545, 1992

Plant cultures grown *in vitro* are often supplied with exogenous sugars. We determined the interactive influence of sucrose supply and nitrogen deficiency on dry matter accumulation and shoot/root ratio in rape seedlings grown *in vitro* in light (550 μmol m^{-2} s^{-1}) and darkness. Total dry mass of seedlings increased with increasing sugar concentration and additional increase was exhibited under nitrogen deficiency in dark. However, in light the values determined under different nitrogen nutrition remained unchanged. Shoot/root ratio decreased with improving sugar supply and nitrogen shortage as expected but surprisingly was higher in dark as compared with light. The experimental data are discussed with regard to *1*) the contribution of exogenous sugar and sugar produced by photosynthesis to dry matter accumulation, *2*) the importance of the development of root system for exogenous sugar uptake and utilization and *3*) possible role of phytochrome system in dry matter partitioning.

Morphological and anatomical investigation of tissue culture of *Drosophyllum lusitanicum* Link.

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Biol. Plant. 34 (Suppl.): 545, 1992

Morphological and anatomical investigation was made on tissue culture *Drosophyllum lusitanicum* Link. (*Droseraceae*) during 42 d incubation under artificial illumination and in the dark. The differentiation of various types of cells was observed and by its quantification the periodicity was revealed (changing of meristematic and differentiation periods). By evaluation of these processes a differentiation at three different levels was found.

Experimental improvement of the regeneration ability of potato explants

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Biol. Plant. 34 (Suppl.): 545-546, 1992

Cultivar-specific differences in regeneration ability, *i.e.* possibility to induce *de novo* bud and shoot formation in the culture of primary explants were estimated in a set of 30 potato cultivars. The experimental system of stem internode segments (SIS) taken from *in vitro* cloned plantlets and cultivated on the inductive medium MS containing 6-BAP (2.1 mg l^{-1}), adenine (40 mg l^{-1}) and IAA (0.1 mg l^{-1}) has been used. The effect of various treatments on the regeneration capacity of genotypes with low or none frequency of bud formation under standard conditions has been tested: *1*) Replacement of the 6-BAP by zeatine induced regeneration in some of these genotypes. GA_3

(10 mg l^{-1}) exhibited no effect on the bud initiation itself, but in combination with cytokinin stimulated further development of even very small bud primordia. *2)* Growth retardants *Paclobutrazol* and *Retacel* (CCC), resp., were applied in the concentration inhibiting growth of stock plantlets up tu 50 %. SIS taken from these retarded plants exhibited both earlier and higher bud regeneration. *3)* Lower temperature (20 °C) selectively inhibited callus formation, being applied in the period of bud meristems differentiation, prevented their degradation into callus tissue. *4)* Wound callus formation on cut surfaces of nodal segments was stimulated by growth of axillary bud. After it subsequent excision (10th d), new buds regenerated from callus tissue - more easily than in SIS culture. *5)* Natural biorhythm of *in vitro* cloned stock plantlets has been preserved for a long time, in spite of the constant cultivation conditions. The regeneration ability has been few times higher in spring and summer seasons compared with the winter one (X-XII). However, the differences among the genotypes has been preserved all year round.

Morphogenic and regeneration potential of oaks (*Quercus* sp.) in culture *in vitro*

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Biol. Plant. 34 (Suppl.): 546, 1992

Improvement and optimization of *in vitro* techniques of oaks as a means of achieving the reproducibility of experiments and effective coefficient of propagation is important not only from economical point of view but also genetically, *i.e.* for the preservation and propagation of rare genotypes and hybrid froms of these trees. In our experiment we have tested both the morphogenic potential and regenerating ability of tissues in some species of the genus *Quercus* (*Q. robur* L., *Q. cerris* L., *Q. virgiliana* Ten, *Q. rubra* L.). The observed degree of morphogenic competence and responses of tissues was variable and depended on the type of primary explants and composition of cultivating media. The type, concentration and proportion of plant growth substances applied may to some degree initiate morphogenesis. The auxin 2,4-D in combination with BAP has in explants stimulated proliferation of a callus. Despite the fact that callus cultures were derived from the leaf segments, cambium of stem segments, of cotyledon segments and of anthers, respectively, we have not succeeded in inducing organogenesis in any of them. Direct organogenesis of isolated apical and axillary buds as well as mature zygotic embryos was positively affected by BAP alone or in combination with kinetin and/or with NAA and GA_3. This type of morphogenesis is the most effective as evidenced by the progressive multiplication of buds and obtaining the culture of multiple shoots. Low concentration of auxins (IBA and NAA - 0.1 mg l^{-1}) lead to rhizogenesis of isolated shoots and subsequent regeneration of plantlets. The activation of embryogenic potential of immature zygotic embryos and differentiation of somatic embryos were achieved on medium with BAP, kinetin and IBA as well as on medium with BAP and 2,4-D.

Production of peroxidase by callus cultures of horseradish (*Armoracia rusticana* L.)

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Biol. Plant. 34 (Suppl.): 546-547, 1992

The richest plant source of peroxidase is horseradish (*Armoracia rusticana* L.). Horseradish peroxidase (HRP) is used for analytical purposes and particularly in many different immunological tests in clinical biochemistry. The HRP cannot be replaced, because peroxidases from another plant

sources differ in the carbohydrate side-chains. There is a relative shortage of HRP, because the preparation of the enzyme from the roots is connected with many disadvantages, some of which can be overcome by cultivation *in vitro*. To verify this suggestion the effect of type and concentration of growth regulators and light regime of cultivation on growth and production of HRP by callus cultures was observed. The course of growth curves was particularly changed by cultivation in daylight. It connected with the transfer from heterotrophic to mixotrophic nutrition. The largest amount of biomass was obtained from cultures cultivated on medium MS containing NAA and BAP. These growth regulators caused partially differentiation. On the other hand, the level of peroxidase activity was three times lower than in calluses cultivated on MS-medium with 2,4-D and kinetin. It was brought about by the low peroxidase activity in young differentiating tissue. The enzyme activity from 1 g of inoculum was established to compare overall yield of different variants. The most attractive were the callus cultures cultivated in daylight on MS-medium containing 2,4-D and kinetin. The highest yield of enzyme activity was obtained after 28 d of callus cultivation.

Androgenic response in microspore cultures of *Zea mays* L.

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Biol. Plant. 34 (Suppl.): 547, 1992

The androgenic response - regeneration of microcalli and embryos - of cultured microspores has been examined. Various culture media and pretreatments were tested. Viability of cultured cells was traced using FDA and the development of cultured microspores was followed by DAPI and Hoechst 33288, the nucleic dyes. It was found that pre-incubation of dissected flowers floating in a liquid medium containing only macronutrients, mannitol and ascorbic acid at 8 °C for 14 d stimulates the division of the microspores. Only the microspores with one nucleus and vacuole gave highest rate of cell division. First divisions were observed 4 - 6 d after the onset of cultures. Multicellular pollen grains were found after 9 - 10 d of culture. At 12 d microcalli emerged from the burst pollen grains. We observed nine different pathways of the microspore responses in culture. Four led to multicellular structures, four to coenocyte (multinuclear) structures and one led to formation of large lobed nucleus inside the microspore, not followed by division. Multicellular structures developed to loose or compact calli. Compact calli formed embryo-like structures. Among the four growth regulators tested, we found PAA and TIBA to have an inductive effect on pollen mitosis in the 4cl maize line, but TIBA was the most effective in our experiment.

Induction of somatic embryogenesis in hybrid firs (*Abies* sp.)

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Biol. Plant. 34 (Suppl.): 547-548, 1992

Possibility of induction of embryogenic calli (EC) from immature zygotic embryos of interspecific hybrids *Abies alba* × *A. cephalonica* and *A. alba* × *A. numidica* were tested on three different media involving Schenk and Hildebrandt medium supplemented with 1 mg l^{-1} BAP (SH), Litvay *et al.* medium prepared in a half concentration with 2 mg l^{-1} of both BAP and NAA added (LM) and medium of Gupta and Durzan supplemented with 2 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} BAP (DCR). The

samples were collected during the period of maturation of zygotic embryos, *i.e.* from 10th of July till 30th of August. After 2-week's cultivation of explants the formation of EC from suspensor cells of zygotic embryos took place. Intensively growing, white and mucilaginous callus, contained numerous sometic embryos each of them consisting of elongated and vacuolized suspensor cells attached to the meristematic cells of embryonic apex. The formation of EC was profoundly influenced by the cultivating media as well as by the developmental stage of zygotic embryos. No EC has appeared on 1/2 LM medium whereas on the DCR medium only the samples of *A. alba* × *A. cephalonica* embryos taken on July 10th have given rise to the EC in 5.2 % proportion. A viable EC has developed only on the SH medium supplemented with 1 mg l^{-1} BAP. The highest potential for *in vitro* embryogeny has as a rule displayed the samples of zygotic embryos collected during July (38 - 44.6 %), while those obtained in the next month have a reduced ability to develop into EC (4.4 - 15.8 %). A further development of somatic embryos in EC was stimulated by ABA. Following its application, the differentiation of cotyledons and prolongation of hypocotyls was observed but no root formation had taken place.

Cryopreservation of microspore suspension of *Brassica napus* L. and *Brassica oleracea* L.

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Biol. Plant. 34 (Suppl.): 548, 1992

In order to increase the efficiency of the rapeseed haploidy system a simple procedure for long-term storage of initial material for microspore cultures has been developed. Isolation and cultivation of microspores was performed after slightly modified protocol published by Sato *et al.* (1988). For cryopreservation of microspores, resuspended in a medium (Lichter 1982) containing 13 % saccharose, no other cryoprotectans were needed. A multistep slo freezing protocol (Glacier, SY-LAB) with sample freezing point below -5 °C was used followed by thawing at room temperature. Successful cryopreservation without loss of viability and pollen embryogenic capacity followed by plant regeneration has been achieved in *Brassica napus* spring cultivar "Topas" and dihaploid DH line of winter cultivar "Darmor". After performing about 70 sample-cycles during the winter period 1991-1992, thawing and regenerating one fourth of this material, our attention is directed to further refinement of the cryopreservation method followed by an examination of embryogenic capacity in microspore cultures, rate of plant regeneration, ploidy levels of regenerants (frequency of spontaneous dihaploids) and somaclonal variability. The ability to conserve isolated microspores for a long-tem period allows further improvements in the recent systems of production of haploid rape and cauliflower plants through microspore system by allowing better distribution of activities in a haploid breeding program and reducing the need for costly indoor growth facilities. Of course, standardized methods and reliable technological system capable to raise healthy source plants as well as regenerants is essential.

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Selection of potato microtubers on higher level of nitrogen substances

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Biol. Plant. 34 (Suppl.): 549, 1992

Induction of axillary microtubers on stem segments of different potato cultivars is a suitable method for the study of tuberization abilities and also for deriving better potato clones. Arising of tubers depends both on photoperiod and growth regulators (Sladký and Bartošová 1990), and on level of nitrogen substances in the medium. The over-supply of nitrogen limits tuber formation, supports elongation of axillary shoots and gives rise to roots. Suitable over-supply of NO_3^-, NH_4^+, urea, aspartic and glutamic acids or casein hydrolysate increases the quantity of proteins and amino acids. The increasing of nitrogen ratio in the media of explants during 8 passages led to the selection of individuals which formed microtubers in the media with 5.7 mg KNO_3, 575 mg urea, 50 mg aspartic and glutamic acids or 2 g of casein hydrolysate. The variability of explants extended, the microtubers were longer and resembled stolons. They retained the ability to accumulate higher level of proteins and amino acids. The content of amino acids in edible cvs. Nicola, Bentje and Ostara oscillated from 150 to 220 μmol g^{-1}(fresh mass) in aspartic acid, in glutamic acid from 36 to 152 μmol g^{-1}(fresh mass) and proline from 39 to 159 μmol g^{-1}(fresh mass). Microtubers of industrial cv. Oreb contained from 90 to 270 μmol g^{-1}(fresh mass) aspartic acid, 80 to 130 μmol g^{-1}(fresh mass) glutamic acid and 5 to 12 μmol g^{-1}(fresh mass) proline. In agreement with the results obtained *via* meristem selection for chlorate resistance (Chwilkowska 1991) it is possible to suppose a selection in potato mutants with higher activity of nitrate reductase, urease, transaminase *etc.*

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Morphogenesis on spruce and fir resting vegetative buds

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Biol. Plant. 34 (Suppl.): 549, 1992

The adventitious buds forming ability of *Picea alba* and *Abies alba* × *A. cephalonica* resting vegetative buds was found to be at maximum immediately prior to their natural flushing. Flushing dormant buds *in vitro* on a cytokinin free medium increased organogenic capacity in spruce by 45 % and in fir by 18 %. Vegetative buds isolated with crown formed in spruce by 25 % and in fir by 15 % more adventitious buds than buds without it. Thidiazuron in concentration 0.1 μM was the only cytokinin effective at inducing adventitious buds in both species from late December to late March in two following years with one maximum prior to natural flushing. BAP in concentration 10 μM induced buds only in spruce. Pulse treatment with Thidiazuron (100 μM, 2 h) induced more adventitious buds when compared with ten day exposure on cytokinin media by 27 % in spruce and 10 % in fir. Transport of ^{14}C-activities in crowns, basal and apical parts of buds in spruce and fir was not found to be significant as well as the difference in activities in buds isolated with and without the crown.

CO_2 and ethylene effect on growth of regenerants *in vitro*

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Biol. Plant. 34 (Suppl.): 550, 1992

During routine cultivation of autotrophic plant regenerants *in vitro* (16/8 h day/night), photosynthetic activity is limited for a longer part of light period by CO_2 concentration in cultivation vessels. This results in retardation of biomass accumulation and plantlet growth. Another factor with a negative effect on plantlet growth is ethylene accumulation in cultivation vessels. In less tightly closed vessels concentrations of both CO_2 and ethylene may be regulated by their concentrations in ambient air. In small glass cultivation chamber different mixtures of K or Na carbonate and bicarbonate solutions (Warburg buffers) can be used for CO_2 concentration control; similarly $KMnO_4$ solution for ethylene absorption. In such an inexpensive equipment regenerants cultivated on medium without and with saccharose (*Allium sativum, Chenopodium rubrum, Dianthus caryophyllus, Nicotiana tabacum, Solanum tuberosum* and *Triticale*) were exposed in atmosphere both with and without ethylene, and with 600, 10 000 and 40 000 mg(CO_2) m^{-3}. A stimulating effect of higher ambient CO_2 concentration was in all cases more expressive on medium without saccharose. As concerns plant species, effect of higher CO_2 concentration is more conspicuous on regenerants with higher leaf area ratio. The inhibitory effect of ethylene accumulation on growth appears to be more evident in regenerant morphology than in reduction of dry mass.

Influence of temperature on vitrification of carnation apices *in vitro*

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Biol. Plant. 34 (Suppl.): 550, 1992

Vitrification is one of the problems encountered in bud cultivation of carnation. Vitrification of explants is characterized by a vitreous appearance, decrease in chlorophyll content and lignification of cell walls. These plantlets are not able to survive transfer to *in vivo* conditions. The growth rate of bud explants *in vitro* is very strongly influenced by temperature: Decreasing the temperature to 15 °C decreases the number of vitreous explants. The aim of our experiments was to characterize the positive influence of low temperature during cultivation and to determine the effect of concentration of agar, kinetin, NAA and ABA on vitrification. Lateral and terminal buds of carnation (*Dianthus caryophyllus* cv. Lena, Pallas) were sterilized in 30 % *Savo* for 20 min. MS medium was used with 3 % sucrose, vitamins and different concentrations of hormones. Incubation was under a photoperiod of 16 h, with temperatures of 15, 25, 30 °C or a gradient of an atmospheric temperature of 25 °C and a medium temperature of 15 °C. Decreasing the temperature to 15 °C during cultivation of bud explants of carnation resulted in a low number of vitreous explants. Similar results were obtained in both cultivars. The low temperature was more effective than the temperature gradient. High concentration of agar (0.8, 1.0 and 1.2 %), ABA (1 mg l^{-1}), kinetin (0.05, 0.5 and 0.1 mg l^{-1}) and NAA (1 mg l^{-1}) had no significant effect on the number of vitrified plants.

Growth retardants (Alar, Retacel, $AgNO_3$) for *in vitro* propagation of potato (*Solanum tuberosum*)

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Biol. Plant. 34 (Suppl.): 551, 1992

The use of plant growth retardants to regulate growth processes of potato plantlets from *in vitro* cultures was verified. Three substances were used for experiments: *Alar* and *Retacel* which interact with metabolism of gibberellins, $AgNO_3$ which affect the response to ethylene. Experimental material included plants propagated from the potato stem cuttings (cv. Kamýk, Lada, Blaník) grown for 5-6 weeks on media with retardants in the concentration 10^{-3} - 10^{-6} mol l^{-1}. Statistical significance through comparison of the 95 % median reliability intervals for treatments was determined. Minimal concentration of the tested substances significantly inhibiting stem growth was estimated with these results: *Alar* and $AgNO_3$ in concentration 10^{-5} mol l^{-1}, *Retacel* 10^{-4} mol l^{-1}. Only treatment with $AgNO_3$ brought about inhibition of leaf formation. *Alar* stimulated the rooting of stem cuttings, while effect of $AgNO_3$ resulted in marked inhibition. An overlasting effect of *Retacel* (in concentration higher than 10^{-3} mol l^{-1}), *Alar* and *Retacel* (in concentration higher than 10^{-4} mol l^{-1}) was observed even in the case of removal of the whole plants from media with retardants and passaging their stem nodal cuttings on retardant free media. Individual potato genotypes differ in their sensitivity to the retardants.

Hypocotyl protoplast culture of sunflower (*Helianthus annuus*)

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Biol. Plant. 34 (Suppl.): 551, 1992

Screening of six genotypes of sunflower was made to evaluate their regeneration potential. Hypocotyls of seedling plants of sunflower inbred line HNK-81 were used as a source of protoplasts. Optimal conditions of isolation and cultivation of protoplasts were established. Using the method of individual cultivation in microdrops the 95 % platting efficiency can be achieved. Cytokinins, especially zeatin, showed a significant influence on the formation of embryo-like structures. These embryoids failed to develop to mature embryos and after addition of auxins they turn to grow as undifferentiated calli, which can grow on the agar solidified media without growth regulators.

The distribution of peroxidase activity in early somatic embryos of Norway spruce

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Biol. Plant. 34 (Suppl.): 552-553, 1992

The method of vacuum infiltration was used to study peroxidases released by the cells of Norway spruce somatic embryos towards the cell walls and extracellular space. This fraction constituted

27.38 % of the overall activity of peroxidases in the culture. The percentage of other fraction was as follows: soluble cytoplasmic peroxidases 33.39 %, peroxidases ionically bound to the cell wall 14.69 %, peroxidases covalently bound to the cell wall 11.49 %. The overall activity decreased during the culture period but the percentage of the individual fractions remained the same. All fractions had distinct isozyme patterns of both acidic and basic peroxidases.

The effect of different types of culture vessel stoppers and the consequences for *in vitro* cultivation

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Biol. Plant. 34 (Suppl.): 552, 1992

The growth and development of a plantlet *in vitro* may be affected by the gaseous environment of the vessel. The gaseous environment will be largely determined by the extent of gas exchange between the vessel and the expternal atmosphere. Gas exchange may be affected both by the type of vessel and the type of stopper. This communication evaluates some physical properties of various kinds of stoppers (both foreign and domestic products) in terms of rate of evaporation from nutritional medium, CO_2 diffusion rate, irradiance of cultivation vessels and growth of nodal cuttings of carnation (cv. Mascotte Rosa). The rate of evaporation from liquid or agar media was determined by gravimetric analysis, the rate of CO_2 diffusion was determined from the change in pH of bicarbonate solution, irradiance was measured using a quantum photometer *LAMBDA LI-170*. The stoppers tested were the products of *Sigma Chem. Co.*, *Magenta Corp.* (USA), *Equilab* (Brno, Czechoslovakia) and stoppers hand-made in our laboratory. Metal lids without a diffusion hole on "baby" glass vessels and aluminium foil covers on Erlenmayer flasks were used as controls. The rate of evaporation from media in vessels at laboratory temperature increased in the following order: Al-foil covers on "baby" flasks - 3 times of control, polypropylene covers with a diffusion hole in an antibacterial membrane filter (*Equilab*) on "baby" flasks - 7 times of control, polypropylene cover for Magenta vessels (*Sigma*) - 10 times of control, metal lids with a diffusion hole and polyurethan tamping - 20 times of control, porous cellulose steristops on Erlenmayer flasks - 30 times of control. With the steristops, a rate of evaporation of 0.3 g water *per* day from an initial volume of 25 cm^3 water means that 30 % diffusion into the vessels followed the same sequence as for evaporation. The decrease in the water potential of agar media was found to be highest in vessels with porous cellulose steristops and cotton-wool plugs (50 % after 40 d). The polypropylene plastic covers are light transparent (90 - 95 % transmittance), while the metal lids reduced irradiance to 70% at plant height (10 cm below the lid). Dry mass of aerial parts of carnation plantlets did not differ with the various kinds of stoppers used, but the root mass was very much decreased in vessels with very high rates of evaporation. The autoclavable plastic polypropylene closure with membrane filter in diffusion hole from *Equilab* are comparable with foreign products and represent a new type of cover which can be recommended for use.

ABA in early stages of somatic embryogenesis of *Medicago sativa*

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Biol. Plant. 34 (Suppl.): 553, 1992

Changes in endogenous abscisic acid (ABA) concentration during somatic embryo formation and development in alfalfa (*Medicago sativa*) were analysed. Embryogenic calli were cultivated in suspension and the fractions mentioned below were separated by sieves with different mesh size. ABA concentration which is relatively high in the fraction of embryonic cells and cell clumps decreases markedly in the phase of formation of globular embryos and then ABA concentration subsequently increases in the more advanced embryo stages. Treatment of sieved cells with ABA (1 - 10 mmol m^{-3}) resulted in slight decrease of the total sum of embryos formed, with concomitant reduction in fresh mass and in slower development into more advanced embryo stages. The effect of exogenous ABA is more pronounced in cultures treated with ABA immediately after omitting 2,4-D from nutrient media than in cultures grown in medium without plant growth regulators during the last preceding subcultivation. It is suggested that ABA seems to influence embryo development but fails to prevent embryo formation if there is a time-break between the presence of 2,4-D and ABA treatment.

Anthocyanin synthesis in calli of four cultivars of grapevine (*Vitis vinifera* L.) under growth regulators influence

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Biol. Plant. 34 (Suppl.): 553, 1992

Anthocyanins synthesis was studied in four blue berry grapevine cultivars (Pinot noir, André, Saint Laurent, Blauer Portugieser). For explants, fragments of young sprays were used. They were placed on solidified MS medium containing Gamborg's vitamins, 26 g l^{-1} saccharose and various combinations of growth regulators. The auxins, IBA, NAA, 2,4-D were used in concentrations of 0.1, 0.5, 2 and 10 mg l^{-1} kinetin or 0.5 mg l^{-1} BAP. Explants were kept under illumination of 7000 lux with a photoperiod of 16 h or continuous dark. After 40 d of cultivation pigments were extracted from calli by methanol acidified with 1 % HCl and measured by spectrophotometry. All extracts exhibited maximal absorbances at 530 nm. Although differences were noted between cultivars under the same conditions in general calli grown on media containing kinetin produced more anthocyanins than those on BAP. Two auxins, IBA and NAA, caused the production of higher pigment levels *per* callus but also higher production of anthocyanins *per* gram of callus tissue. The highest yield was achieved on the media with auxin concentration of 0.5 and 2 mg l^{-1} (from 0.93 to 4.99 A_{530} g^{-1} fresh mass depending upon cultivar used). 2,4-D as a growth regulator was less effective though anthocyanin synthesis was not mostly interrupted. Regarding genetic variability among different calli the cultivar Saint Laurent, generally produced the largest amount of anthocaynins. Synthesis of anthocyanins in the dark was not seen.

Do different plant materials require different levels of cytokinins in tissue culture?

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Biol. Plant. 34 (Suppl.): 554, 1992

Tissue culture of *Gossypium hirsutum* cv. Im 216 and Acala 44 grew differently on Schenk-Hildebrandt media. Analysis of endogenous cytokinins in 12-d old cotyledons showed, that plant tissue high in cytokinin does not require as much cytokinin in the culture media. The authors suggest that concentrations of cytokinins in synthetic media should be based on immunological tests.