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Photo-Inactivation of Indolebutyric Acid During Rooting *In Vitro*

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We have initiated a research programme on the factors that determine the success of rooting of microcuttings *in vitro*. This research is carried out in *Malus* and encompasses physiological, hormonal and molecular regulation. Here we report on the role of indolebutyric acid (IBA).

The effect of IBA depends upon the length of application and upon the concentration. It is concluded that it is not the concentration but the dose of the hormone taken up which is decisive for the number of roots formed. IBA is rapidly conjugated in the tissue, probably to IBA–aspartate. In contrast to various reports in literature, we observed no conversion of IBA into indoleacetic acid (IAA). A product of photo-inactivation of IBA closely co-chromatographed with IAA. In the medium, photo-inactivation of IBA occurred rapidly: at 80 $\mu mol\ s^{-1}\ m^{-2}$ after 32 h, 30% or 88% of the IBA had disappeared in the absence or presence of riboflavin (1 $mg\ l^{-1}$), respectively. In the dark, no photo-inactivation was observed. Promotion of rooting by darkness is possibly caused in part by a lack of photo-inactivation of IBA. On tlc-plates, photo-inactivation of IBA was very rapid.

Flow Cytometric Selection of *Tagetes* Cell Lines with an Enhanced Thiophene Production

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Selection of high-producing cell lines is an important strategy in developing methods for the industrial production of useful secondary metabolites by cultured plant cells. A flow cytometer (FCM) enables rapid analysis and subsequent sorting of large amounts of cells. FCM selection requires individual cells, for example protoplasts, to produce a fluorescent secondary metabolite. Aggregated cell suspensions of *Tagetes patula* produce various thiophenes (natural biocides). When exposed to UV light thiophenes are highly fluorescent. Therefore, *T. patula* cell suspensions represent an attractive model system. From these cultures *c.* 4% of the cells yielded protoplasts. The thiophene level of the protoplasts was low (*c.* 10% of the concentration in whole cells), but still measurable with an FCM.

It has been suggested that enhancement of production is mainly a question of enhancing the number of productive cells within a cell population (Hara, Y. *et al. Planta Med.* 1989, **55**: 151–154). Continuous white light appeared to decrease the thiophene content in the aggregates as well as in the medium of *T. patula* cell suspensions. Dark- and light-grown cell suspensions, with respectively high and low thiophene content, have been analysed with the FCM. When various cell lines were compared, the mode of fluorescence distribution shifted towards a higher intensity in cell lines with a high average thiophene concentration. Also, the range of intensities increased. These results indicate that differences in thiophene level of cell suspensions are caused by variation of the thiophene levels of individual cells and not by variation of the number of productive cells.

Cell suspensions regenerated from protoplasts with a high thiophene content will be tested for stability of growth and thiophene production using HPLC and FCM. Repeated selection will probably be necessary to maintain a high yield. So far our results indicate that the time-consuming regeneration of selected protoplasts will remain the bottleneck for selection of high-producing cell lines of *Tagetes*, especially when repetitive selection is envisaged.

Distribution of Calmodulin and Membrane-Bound Calcium During Somatic Embryogenesis of Carrot (*Daucus carota* L.)

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The development of a plant embryo requires co-ordinated cell division and cell differentiation, which are both influenced by several external and internal signals that must be translated into an appropriate action. Ca^{2+} and the Ca^{2+}-binding protein calmodulin (CaM) are important in signal transduction in plants. Localization studies of Ca^{2+} and CaM during somatic embryogenesis may lead to a better understanding of the mechanisms controlling embryogenesis. In this study the distribution of membrane-bound Ca^{2+} was examined by chlorotetracycline fluorescence. The

amount of membrane-bound Ca^{2+} was found to be low in pro-embryogenic masses (pems) and high in progressive stages of embryogenesis. In pems the level of total CaM also appeared to be low, as revealed by immunocytochemistry; it was distributed uniformly. In the different stages of embryogenesis CaM was mainly found in organelles surrounding the nucleus in the cells of the embryo protoderm. By conventional electron microscopy these organelles were revealed to be plastids. In contrast, activated CaM, visualized by fluphenazine fluorescence, was only present in the basal part of the developing embryo. It can therefore be concluded that, although CaM is present in all cells of the embryo protoderm, it is only bound to Ca^{2+} in the cells at the future root side of the embryo. From this it appears that in carrot a high concentration of membrane-bound Ca^{2+} and a distinct distribution of CaM are indicative of the development of cells from pems into somatic embryos.

An Embryogenic Suspension Culture of Maize

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An embryogenic suspension culture of maize was initiated from friable callus with high regenerative potential. The callus was derived from zygotic embryos of the commercial selection AE1, a kind gift of the Foundation of Plant Breeding, The Netherlands. The callus aggregates grown in darkness in liquid N6 medium supplied with 6 mM L-proline, 100 mg/l casein hydrolysate, 2% sucrose, 3% mannitol and 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) released cells in the medium. For the first 2 weeks only elongated cells were released. From the third week small isodiametric cells and clusters of such cells also appeared in the medium. In these clusters cells started to divide and groups of cells differentiated into globules, and subsequently into ovoids and polar somatic embryos which separated from the clusters. The cell clusters, therefore, can be designated as pro-embryogenic masses. When cultured without 2,4-D, the polar somatic embryos germinated, obtaining a root with root hairs. The shoot side became green and consisted of meristematic cells. However, under the conditions tested, no leaves developed. If the somatic embryos were kept in medium containing 2,4-D, they proliferated, forming callus aggregates again, which in fact consisted of aggregated globular stage somatic embryos. These aggregates, and their mechanically isolated subunits, could be grown into complete plants.

To explain this apparent contradistinction, we have two hypotheses. Either, the leaf primordia development is blocked by an inhibitor, which is overruled during callus proliferation, or, an essential substance, involved in leaf primordia formation, is produced by the aggregates under proliferating conditions.

Effects of Donor Plant Growth Conditions on Embryo Production in Microspore Culture of *Brassica oleracea*

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An efficient procedure of microspore culture has been established for different *Brassica oleracea* crops. In a study on effects of donor plant growth conditions on embryo production, Brussels sprouts plants, after vernalization, were placed in controlled growth rooms at 5, 10 and 15°C with a 16 h photoperiod of 50 $\mu E/m^2/s$. Broccoli plants were grown under 50, 100, and 200 $\mu E/m^2/s$ at 10°C during flowering. In culture, the microspores from all temperatures yielded embryos. Between 0·1 and 50 embryos per bud were obtained. With higher temperature, however, in Brussels sprouts the number of embryos produced were found to decrease (for 15°C and 5°C, 23 and 0·3 embryos per bud, respectively). This indicated that temperature had a quantitative rather than a qualitative effect. Light intensity did not clearly affect the ability of embryo production in broccoli (for 50, 100 and 200 $\mu E/m^2/s$, 9·9, 4·7 and 15·7 embryos per bud, respectively).

Synthesis and Accumulation of Thiophenes in Relation to Root Morphogenesis in *Tagetes*

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Thiophenes are heterocyclic aromatic compounds that accumulate in roots of *Tagetes* species. Higher concentrations of thiophene are present in detached roots cultured *in vitro*. The root cultures are very plastic: growth rate and branching pattern can be manipulated by changing the culture conditions and by transformation of the tissue with *Agrobacterium*. The plasticity creates the opportunity to study thiophene accumulation in relation to root morphogenesis. Root cultures in liquid medium accumulate thiophene in the exponential growth phase. All compounds formed are stable until they are broken down in the stationary phase. In cultures on solid medium, indoleacetic acid (IAA) influences the elongation,

branching, and thiophene content of the roots. The IAA concentrations optimal for elongation and thiophene accumulation are approximately equal but the maximum number of laterals is formed at a 100–1000 times higher concentration. After transformation with *Agrobacterium rhizogenes* the optima shift to 100 times lower values.

A characterization of the relationship between morphogenesis and secondary metabolism at the cellular and molecular level is now underway. Relevant aspects that are being investigated include: the localization of thiophene synthesis and accumulation in cells and tissues, the regulation of thiophene metabolism and the expression of the genes involved, and the coupling between root differentiation and thiophene synthesis in terms of gene regulation.

Pollen Development *In Vitro*

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The development of microspores into fertile pollen proceeds in the anther and is accompanied with specific changes at the structural, biochemical and molecular level. Some of the structural changes are pollen specific, such as the formation of the pollen wall, vegetative nucleus and generative cell. Changes in transcription, molecular level, could be demonstrated by analyses of the polypeptides derived after *in-vitro* translation of RNA extracted from a homogeneous population of developing tobacco pollen. These analyses showed the accumulation of different groups of mRNAs. A group was seen to be present transiently, for example around microspore mitosis, while another group arose during the latest phase of pollen development. Some of these latter mRNAs are pollen specific, as shown after differential Northern hybridization analyses with pollen-specific cDNA clones.

The developmental process in the anther is difficult to affect directly by changes of the growth conditions of the plant. This means that changes in gene expression affecting only pollen fertility are difficult to perform by manipulation of the intact plant.

Culturing of microspores or young pollen in a liquid medium into fertile or non-fertile pollen has the opportunity to regulate the processes involved without any influence of the surrounding tissue of anther or intact plant.

A homogeneous tobacco pollen population could be achieved by homogenization of harvested anthers of fixed flowerbud size. The microgametophytes were separated from vegetative tissue by filtration and centrifugation and cultured into fertile pollen in standard medium. A simple change in the composition of the standard medium yielded non-fertile pollen. The results of a culture of mid-binucleate tobacco pollen in standard medium showed a homogeneous development into fertile pollen within 3 days. The fertility was proved by sampling an aliquot of a pollen suspension on the stigma yielding a seed set, low at day 2 and normal at day 3. The germination of the developing pollen was tested *in vitro* and varied from 0 to 70% with a maximum at day 3 of culture. The protein synthesis changed qualitatively at the different days of development, as could be shown by labelling proteins during a 2-h period. A pollen-specific cDNA clone was hybridized with RNA from pollen developed *in vitro* and showed the same change of expression as RNA from pollen developed in the anther.

These results show that the development of tobacco pollen *in vitro* demonstrates a similar developmental process as in the anther for the tested physiological markers such as pollen-specific transcription, translation, percentage germination and fertility.