# SUMMARY OF THE PhD THESIS

# APPLICATION OF METHODS SUITABLE FOR IMPROVING THE EFFICIENCY OF *IN VITRO* PROPAGATION ON HORTICULTURAL PLANTS

**DR. ANNAMARIA MÉSZÁROS**

**BUDAPEST**
**2006**

**PhD School/Program**

| | |
|---|---|
| **Name:** | **PhD School of Horticultural Science** |
| **Field:** | **Biological Science** |
| **Head:** | **Prof. János Papp**<br>**CORVINUS UNIVERSITY OF BUDAPEST** |
| **Supervisor:** | **Prof. Ervin Balázs**<br>**Agricultural Research Institute of the HAS, Martonvásár** |

**The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.**

**Prof. János Papp**
**Head of PhD School**

**Prof. Ervin Balázs**
**Supervisor**

# THE ANTECEDENTS AND GOALS OF THE WORK

***Introduction***

The term micropropagation means a technological process consisting of several steps in order to produce numerous propagula from a chosen mother plant under *in vitro* conditions. Large scale technologies have been developed following long experimental procedures worldwide. In the period of 1970-1980 research was focused onto the methodology of steril culturing, and the propagation of economically important plant species. Trials with different nutrient media, investigation on growth regulator effects, experiments concerning interaction of genotype and environmental effects – some of the most important topics at this period. From the beginning of the nineties attention was payed to new technologies, scale-up and automatization.

It can be stated that in this field of science theory and practice forms a strong unity, both in improving the knowledge and in developing new technologies.

***Propoundment of problems and goals of the work***

1. Micropropagation means the multiplication of the initial plant material via shoot induction caused by cytokinins. The mode of action on plants expressed by different cytokinins can be different, influenced by the type of cytokinin, the genotype of the plant, the age and physiological status of the cultured tissues. Before starting the propagation of a given plant cultivar it is advisable to determine which is the cytokinin that is able to induce the largest amount of shoots from the apex or other tissue in the course of culture cycle.

In the first period of our work we have investigated the effect of cytokinins in the course of shoot induction in cultures of two plants exhibiting different growth habit. The perennial *Atropa belladonna* is an important medical plant, the woody ornamental *Populus alba* is valuable for its wood production.

In the course of experiments we intended to investigate the effective range of different cytokinins in which they are able to promote shoot induction and so to improve the efficiency of both propagation and regeneration. In paralel, the purpose of investigations was to determine the regeneration ability of different somatic tissues.

2. As a consequence of using micropropagation techniques in industrial scale it became evident, that increasing the concentration of growth regulators or application of high doses does not increase the multiplication rates in all cases, moreover it can lead to the deterioration of plant material. Therefore it is important to pay attention on methods, improving the efficiency of

propagation process either by increasing the number of end-products or improving the quality of plants thus improving the chance of acclimatization. Interest is focused nowadays onto natural substances, which exerted positive influence to the growth of different plants in field trials.
We have demonstrated earlier positive effects of triacontanol in the microporpagation process of *Melissa officinalis* and fruit rootstocks. Based on these results we intended to continue the experiments in order to prove triacontanol effects on other, economically important plants too.

The aim of the work was to determine in what extent can triacontanol influence growth and physiology of the cultures both in the multiplication and the rooting phase. In order to utilize results in the practice, different horticultural plants like as raspberry (*Rubus idaeus*), gerbera (*Gerbera jamesonii*) and asparagus (*Asparagus officinalis*) were choosed as objects of investigation.

3. Procedures of scale-up and automation can be important tools for further development of micropropagation methodology nowadays. For that purpose utilization of the advantages of liquid media can be ideal. Following investigations in the last decade several bioreactors, operating in different ways, were constructed. Most of these instruments are based on the principle of the temporary immersion, what means that cultures are growing in liquid media, but they are not completely or not constantly covered by it. In the first period liquid was stirred in the culture vessels or rotated together with it. Nowadays it is possible to change the gasous atmosphere of the headspace and to provide oxygen or $CO_2$ for the plants.

Our experiments were directed to the development of technology, and motivated by practical demands. The aim of the work was on the one hand to prove the usefulness of a novel type bioreactor and to compare it with similar equipments. For this purpose economically important plants, propagated in many countries: banana (*Musa nana*) and ananas (*Ananas comosus*) were choosed as model plants. On the other hand we intended to demonstrate the efficiency of liquid based system regarding both in quantity and quality of offsprings.

# MATERIALS AND METHODS

***Plants and media***

**1. Investigation of cytokinin effects in the course of shoot induction**

Shoot tips with 3-4 leaves, pieces of leaves, internodes, petioles and roots of sterile plants were used. Leaves were cut into three parts perpendicular to the midrib obtaining an apical, a middle and a basal piece. Pieces were placed to the media in different orientations: partly with the adaxial partly with the abaxial surface down. Pieces of the other tissues were cut to approx. 1 cm long and placed horizontally to the media. In all experiments plants of 3-4 weeks old cultures served as initial material.

The degree of shoot induction in cultures of Atropa was investigated on media containing KIN, 2iP and BAP in the concentration range of 0,5; 1,0 and 2,0 mg/l. Media used in experiments focused to shoot induction from somatic tissues contained 1,0, 2,0, and 5,0 mg/l BAP or 2, 5, and 10 mg/l KIN together with 0,2 or 1,0 mg/l IAA. Root pieces were placed to media containing 0,5 mg/l NAA or IAA, or 1,0 mg/l BAP + 0,1 mg/l NAA.

In shoot cultures of *Populus alba* cv. Silver the effect of cytokinins was tested in different concentrations. BAP and 2-iP was investigated in concentrations of 0,25; 0,5; 1,0; 2,0 mg/l, ZEA in 0,1; 0,2; 0,5; 1,0 mg/l and TDZ in 0,05, 0,1; 0,25; 0,5 mg/1. As a basic media the Murashige and Skoog (1962) formula was used with 20 g/l sucrose.

**2. Application of triacontanol in the micropropagation of horticultural plants**

Based on positive results with triacontanol obtained earlier by our working group we have decided to extend investigations to other, economically important plants, beeing propagated in industrial scale worldwide. In the present experiments a selected genotype of raspberry (*Rubus idaeus* cv. Malling Exploit), gerbera (*Gerbera Jamesonii* cv. coded as B5) and asparagus (*Asparagus officinalis* clone 1113) served as objects of examinations. In the rooting phase Murashige – Skoog basic media with N6 vitamins and without growth regulators were used in order to study the effects of TRIA in itself. In the multiplication phase routinely applied hormon combination for each plant was used as follows: for gerbera 3 mg KIN + 0,1 mg IBA; for raspberry 0,5 mg BAP + 0,1 mg IBA; for asparagus 2 mg KIN + 0,2 mg NES in one liter of media. Triakontanol was added in 2, 5, 10 and 20 µg/l concentration to the media of each plants.

## 3. Liquid culture system: trials with a novel type bioreactor in the propagation process

In the experiments presented below plants were propagated in liquid media in a new equipment, called as 3R bioreactor, developed on the basis of temporary immersion. Different culture systems were compared. In one treatment plantlets were grown in a closed reactor vessel without the change of internal air, while in the other treatment active ventillation was promoted by pumping filter-sterilized air into the headspace. Cultures grown on the usual solid multiplication media in small containers were considered as control.

Multiplication and shoot growth of three different plants: hosta (*Hosta tokudama* cv. Dew Drop), banana (*Musa nana* cv. Dwarf Cavendish) and ananas (*Ananas comosus* cv. Lucidus) was investigated in the course of propagation. In all treatments the usual multiplication media were used as follows. Banana:1 mg/l BAP + 0,1 mg/l IBA, ananas: 0,25 mg/l BAP + 0,1 mg/l IVS, hosta: ½ MS supplemented with 3 mg/l kinetin + 0,1 mg/l IBA.

### *Culture conditions*

For shoot tip cultures baby food jars of 0,25 l volume, for somatic tissues 10 cm diameter Petri dishes were used. In the experiments with triacontanol explants were placed into 0,5 l volume glass jars, covered with a metal cup that had a hole and a piece of spongy in it, in order to provide air exchange. Plants were cultured on side-illuminated growth shelves on 22±2$^{O}$C temperature, 16/8 hours photoperiod, with 80 μM/m$^2$/s light intensity.

In the experiments testing the operation of bioreactor, the modules eg. the cylinders of the reactor served as culture vessels. The equipment was settled in a laboratory on a constant temperature of 22$^{O}$C. Illumination was provided by the light tubes of the reactor and the photoperiod was controlled automatically. The upper level of the reactor contained cultures in the reactor moduls rotating along a vertical axis, while on the lower level traditional glass jars containing control cultures were placed.

### *Plant characteristics investigated*

Development of the plants was evaluated following a 4 weeks culture cycle. Growth parameters like as the number and length of the shoots, the number and length of the roots, fresh and dry weights mere measured. Photosynthetic data: chlorophyll content, values of the fluorescence induction kinetics and the intensity of phtosynthesis were recorded.

### *The methods of evaluation*

For determination of chlorophyll content a sample of 50 mg leaf tissue was homogenized with 6 ml 80% aceton. Following a centrifugation for 3 seconds on 4000g the absorption of the

supernatant was measured by the method of Arnon (1949). Photosynthetic activity was determined with data of fluorescence induction (Lichtentaler and Rinderle, 1988) measured by a Plant Efficiency Analyser (Hansatech Ltd., King's Lynn, UK). Data were analysed by a PEA Analyser computer program (Hansatech Ltd.). The intensity of photosynthesis was measured by an infra-red gasanalyzer **(**LI-6200, LI-COR Inc.). Leaf anatomy was investigated by Scanning Electron Microscope (magnification 1000x, 2000x) on thin layer and cross sections.

***Statistical eveluation of the data***

All investigations were based on three independent experiments. Every treatments were repeated at least three times and ten plants were inveastigated in one repetition. SPSS 7.0 computer program and t probe was used for the evaluation of data.

## RESULTS

### 1. Investigation of cytokinin effects in the course of shoot induction

Nutrient media for *Atropa* shoot tip cultures contained kinetin, 2iP and BAP in the concentration of 0,5; 1,0 and 2,0 mg/l. In case of other plant tissues 1,0, 2,0, and 5,0 mg/l BAP, or 2, 5, and 10 mg/l kinetin was added to the basic media together with 0,2 or 1,0 mg/l IAA. In the regeneration experiments starting from root pieces media containing 0,5 mg/l NAA- or IAA, as well as 1,0 mg/l BAP + 0,1 mg/l NAA were used.

According to our results in shoot tip cultures of *Atropa belladonna* BAP and 2-iP was able to promote shoot development in the concentration of 0,5-2 mg/l and 2 mg/l respectively. Kinetin proved to be ineffective. Shoot initiation from somatic tissues was elicited by BAP in the concentration range of 1-5 mg/l. The number of regenerated shoots varied between 2-3 without showing a correlation between the shoot number and BAP concentration. The level of regeneration occurring on the leaf pieces was not influenced by the orientation of the explants. Regarding polarity, leaf base parts showed the best regeneration capacity. Root pieces were able to develop new shoot without any cytokinins in the media. However the presence of BAP induced more shoots within a shorter period of time.

In case of *Populus alba* shoot tip cultures the effect of BAP, 2-iP ((0,25; 0,5; 1,0; 2,0 mg/l of each) and zeatin (0,1; 0,2; 0,5; 1,0 mg/1) was investigated. In cultures of somatic tissues TDZ was applied too, in concentrations of 0,05, 0,1; 0,25; and 0,5 mg/1. In the process of shoot induction originating from shoot tips and nodal segments BAP proved to be the most effective. He highest number of shoots developed in its lowest concentration. 2-iP showed a stimulatory effect only in the

concentration of 1 mg/l. Zeatin was not effective at all. In contrast, concerning leaf- and internode pieces, zeatin surpassed the effect of other citokinins. The highest shoot number was registered in the treatments with 0,5 mg/l ZEA. In case of leaf pieces the degree of shoot induction was influenced by the polarity of explants too. The best regeneration capacity was shown by the leaf bases, followed by the leaf middle and the leaf tip parts. TDZ induced the development of numerous small shoots or leaf bunches, that were not able to elongate even on hormon free media, thus proved to be useless for the further propagation. None of the cytokinins were able to promote regeneration on the root pieces.

According to our results in case of both plants it is possible to elaborate a stable and reproducible regeneration system, that can be the basis either of the large-scale production or the genetic manipulation.

**2. Application of triacontanol in the micropropagation of horticultural plants**

In the last years it became evident, that different substances, occurring naturally in plants, are exhibiting growth regulator effects. These properties make it reasonable to test them in the conditions of *in vitro* systems, in order to increase the efficiency of propagation.

Triacontanol ($CH_3(CH_2)_{28}CH_2OH$), a long-chain aliphatic alcohol was found in 1933 (Chibnall et. al.) as a part of the epicuticular waxes of alfalfa. Since then, its growth regulating habit have been shown in several greenhouse and field experiments.

Stimulating effects of triacontanol in the micropropagation process of *Melissa officinalis* and fruit rootstocks have been demonstrated at first by our group. Based on the positive results, we deciced to explore its application to other horticultural plants as well.

In the present experiments raspberry (*Rubus idaeus*), gerbera (*Gerbera Jamesonii*), and asparagus (*Asparagus officinalis*) plants served as objects of investigations. Murashige-Skoog medium (1962) was used supplemented with 0,5 mg/l BAP + 0.1 mg/l IAA in the case of raspberry, 3 mg/l KIN + 0,1 mg/l IBA in the case of *Gerbera*, and 2 mg/l KIN + 0,1 mg/l NAA for *Asparagus* in the multiplication phase. Basal medium without plant growth regulators was applied in the root induction phase, in order to test the effects of TRIA when applied alone. Both the multiplication and rooting media were supplemented with 0, 2, 5, 10 and 20 µg/l triacontanol.

Our results demonstrated, that, although in a different degree, triacontanol exhibited a positive influence in the case of all plants. Due to its effect, the number of shoots increased significantly in the multiplication phase. In raspberry cultures most of the new shoots developed at the highest (20 µg/l) triacontanol concentration. In case of *Gerbera* all concentrations in the range of 5-20 µg/l proved to be stimulatory. In contrast, the lowest (2 µg/l) concentration of triacontanol enhanced significantly the total shoot number of asparagus. Higher concentrations up to 20 µg/l did

not give any further increase. The length of shoots was not influenced by the treatments. Rise in the fresh weights was recorded in some cases, due to the elevated shoot number and development of callus.

In the rooting phase a stable elevation of both the proportion of rooted plants and the number of roots per plantlet was registered in all treatments. The rate of root induction in raspberry cultures was the highest on media supplemented with 10 and 20 µg/l triacontanol. In case of *Gerbera* significant increase of root number was evident even at the lowest TRIA concentration. The number of roots of *Asparagus* was significantly enhanced by 2µg/l triacontanol too and the higher concentrations did not result any further increase.

Photosynthetic system of the plants was positively influenced by the triacontanol treatments. A significant increase of chlorophyll content of *Gerbera* and *Asparagus* plants was registered both in the multiplication and the rooting phase even at the lowest concentration. In case of raspberry leaf chlorophyll content increased continuously and reached its maximum at 20 µg/l. Fv/Fm data of chlorophyll fluorescence indution kinetics showed a similar tendency.

Results of the present work clearly demonstrate, that triacontanol can be effectly applied in the micropropagation of different plants. However, it is necessary to determine the optimal concentration range, because it can be different depending on the plant species and nutrient media applied in the certain growth phases.

**3. Liquid culture system: trials with a novel type bioreactor in the propagation process**

In our experiments the operation of a new type bioreactor, called Revert Rotary Reactor (3R) have been tested.

The aim of the work presented here was to investigate the effect of culture conditions on *Hosta, Ananas* and *Musa* as model plants cultivated in different systems, thus to prove the usefulness of our bioreactor in the micropropagation process.

Three different culture systems were compared. In one treatment plantlets were grown in a closed reactor modul without the change of internal air, while in the other treatment active ventillation was promoted by pumping filter-sterilized air into the modul. Cultures grown on the usual solid multiplication media in small containers were considered as control.

The nutrient media were the same in all treatments. *Hosta* plantlets were propagated on half strength MS media with 3 mg/l kinetin. The culture media consisted of the MS formula with the addition of 1 $mgL^{-1}$ BAP + 0.1 $mgL^{-1}$ IBA for shoot multiplication in case of banana,. and 0.25 $mgL^{-1}$ BAP + 0.1 $mgL^{-1}$ IBA for propagation of *Ananas*.

Our results showed, that growth parameters were positively influenced by the liquid media. The number of shoots in the clusters increased significantly in the case of plants grown in the

aerated vessel. An increase of the multiplication rate was registered in the cultures grown in the closed vessel too, but the differences were not significant compared to the coltrol. No differences were found concerning length of the shoots except banana, but this deviation was caused more by the increase in the leaf size rather than in elongation of the shoot axis. Leaf area of *Hosta* shoots increased too in the liquid media. All of these changes were followed by the increase of fresh and dry weights. However data of dry matter content showed a wery small deviation between the treatments. It seems that the elevated biomass production is caused rather by the absorption of liquid than the increased accumulation of nutrients.

Differences were found in the photosynthetic characters between plants from the different treatments. The chlorophyll content was higher in the aerated vessel than in the other treatments. In case of *Hosta* clorophyll content and data of fluorescence induction kinetics were much lower in the closed system than that of the normal value. This shows that there is a certain inhibition in the function of the photosynthetic apparatus. Values of Fv/Fm ratio measured in the leaves of *Ananas* and banana in all treatments showed a level of 0.7-0.8 that is very close to the normal level of the *in vivo* plantlets. No photosynthetic activity but intensive respration was detected in banana plants grown on agar media. In contrast photosynthesis of plants grown in ventillated liquid has already evolved, its activity exceeded respiration.

Analysis of the leaf anatomy showed symptoms of hyperhydration (lack of palisade parenchyma, spongy mesophyll cells, large stomatas, thin layer of epidermis) in the case of plants cultured in the closed reactor modul. In contrast, the thicker epidermis, the functioning stomatas and the beginning of excretion of epicuticular waxes showed that the plants grown in the ventillated modul are much near to the autotrophic state. All the differences mentioned above suggest that the permanent ventillation and the absorption of nutrients from the liquid media are resposible for the favourable effects.

As a summary, we can conclude that our system allows an efficient nutrient uptake and a good aeration in the same time, thus provides good growing conditions and makes it possible to achieve high multiplication rate of good quality plants.

# NEW SCIENTIFIC RESULTS

- We have demonstrated the shoot induction effects of different cytokinins – KIN, 2-iP, BAP – in *Atropa belladonna* shoot cultures.
- It had been proved that all part of the plant is able to develop shoots in the presence of appropriate cytokinin, and that the degree of shoot induction is dependent on the type and concentration of cytokinin.
- We have demonstrated the shoot induction effects of different cytokinins – BAP, 2-iP, ZEA and TDZ – in *Populus alba* shoot cultures.
- It had been proved that all part of the plant, except the root, is able to develop shoots in the presence of appropriate cytokinin, and that the degree of shoot induction is dependent on the type and concentration of cytokinin.
- We were the first to point out that triacontanol influences growth and development, as well as photosynthetic activity in cultures of *Gerbera*.
- We were the first to point out that triacontanol influences growth and development, as well as photosynthetic activity in cultures of raspberry.
- We were the first to point out that triacontanol influences growth and development, as well as photosynthetic activity in cultures of the monocot *Asparagus*.
- We have successfully propagated different plants by the application of a novel type bioreactor.
- We have proved that the liquid state of media is favourable for the propagation of *in vitro* cultures.
- We have established, based on physiological survey data, that the content of headspace in the growing vessel, the oxygen supply influences significantly both the propagation rate and the photosynthetic capacity of the plants.
- We have demonstrated, based on anatomical investigations, that tissues of plants developed in the vented liquid are very close to the appearance of the autotrophic stage, therefore their acclimatization can be more efficient.

# CONCLUSIONS AND SUGGESTIONS

**1. Investigation of cytokinin effects in the course of shoot induction**

Shoot tip cultures are widely used in different physiological and morphogenetical investigations. Besides, they are the main objects of the commercial micropropagation, as they can assure a high level genetic stability. Data concerning effects of different cytokinins are originated mainly from investigations on shoot induction in shoot tip cultures. According to the observations not all of the cytokinins proved to be effective in the case of a given species or genotype.

In our experiments kinetin was not able to induce multiplication in *Atropa belladonna* shoot tip cultures, in contrast with 2-iP and BAP. The effective concentration of BAP was found in the range of 0,5-1 mg/l, the concentration of 2 mg/l resulted the decrease of shoot number. In contrast, 2-iP proved to be favourable in 2 mg/l concentration. No data were found concerning micropropagation of *Atropa belladonna*, because *in vitro* cultures are generally used to investigate the composition of active ingredients of the species. The role of hormon composition of the media on the alcaloid content of cultures was investigated by Benjamin et al. (1987). Concerning the morphogenetic changes observed, they have noted that BAP caused ”enormous” shooting in the concentration range of 1-5 mg/l, but 10 mg/l concentration inhibited growth. Kinetin had no effect on shoot development, however in some cases improved root and callus development. Zárate et al. (1997) investigated the effect of BAP in 0,25-2 mg/l concentration in *Atropa baetica* shoot cultures. They observed that the changes in shoot number is dependent more on the length of the culture period than the hormon concentration. Following a 17 days culture period an average of two new shoots was recorded. Following 24 days the number of shoots varied between 2,8-3,4 and after the 31. day it exceeded the value of 5,4-5,8.

In shoot cultures of *Populus alba* ’Silver’ BAP proved to be the effective cytokinin too. The highest shoot number (3,9) was induced by the lowest (0,25 mg/l) concentration. At higher concentration the shoot number remained under 2. Similar experiences were published by Welander et al. (1989). In cultures of *Populus wilsocarpa* in the concentration of 0,1 mg/l BAP resulted in an average of 5 shoots. The number of shoots decreased to 2,7 at the concentration of 0,5 mg/l. Effect of different cytokinins in cultures of 4 populus clones was investigated by Rutledge and Douglas (1988). BAP was found to be the most effective in case of three genotypes (3-5 shoots) when applied in the concentration of 0,25 mg/l. Zeatin in 1 mg/l concentration resulted in 6 new shoots in the case of one genotype, and the shoot numbers varied between 2-3,5 in case of the other two genotypes. 2-iP was not able to induce shoot development at all.

The source of explant is an important factor in the course of shoot induction from somatic tissues. Morphogenetic capacity of explants originated from different organs or tissues of differens organs are generally different. The active concentration of cytokinins, beeing able to induce morphogenesis, is often dependent on the origin and size of explant (George, 1993).

Leaves or pieces of leaves are often used for adventitious shoot induction and multiplication in case of numerous species e.g. *Begonia, Betula, Chrysanthemum, Fragaria, Malus, Saintpaulia* (George, 1993). Leaf pieces of *Atropa belladonna* were also used as initial materials (Eapen et al., 1978., Tóth et al., 1991.) cultured on BAP containing media. However no data were published concerning BAP concentrations or shoot numbers. Results of our own experiments showed that shoot induction occurred on the leaf pieces cultured on media with 1-2 mg/l BAP.

Concerning shoot regeneration from leaves of *Populus alba* no data were found in the literature. We have demonstrated, that shoot development can be induced on leaf pieces of genotype 'Silverleaf' too. Zeatin in 0,5 mg/l concentration proved to be the most effective. Similarly, in the experiments of Park and Son (1988) with leaf explants of a *P. nigra* X *P. maximoviczii* clone zeatin exhibited the highest efficiency in the regeneration, followed by BAP, KIN and 2-iP. The efficiency of shoot induction can be influenced by numerous factors such as the polarity of the plant organ (Welander, 1988). Our results showed that in case of *Atropa* polarity had no effect, but in case of *Populus* the level of shoot differentiation was dependent on it. Most of the shoots initiated on the leaf-base pieces, the less on the leaf-tips. It can be suggested, that the interaction of genotype and the endogenous hormon content of the explant plays an important role too.

Root pieces of Populus were not able to react to any cytokinin treatments. In contrast, shoot regeneration occurred on *Atropa* root explants even without cytokinin although the regeneration rate increased significantly on BAP containing media. This is in accordance with the statement of George (1993)who claimed that the the presence of cytokinin positively influences morphogenetic events in root. It seems that differentiation of buds starts in darkness as well but shoot development is completed only by light. Our observations are strengthened by Pierik's (1987) suggestion, that the adventitious shoot induction takes places in the darkness but its incidence is higher in the light.

It is necessary to mention our experiences regarding the application of TDZ. According to Huetteman és Preece (1993) plants developed on media containig TDZ often showed irregular morphology. Investigations on *Populus* explants proved that its effect is favourable in the initial stage of regeneration, but inhibitory in the elongation of shoot axis. Therefore it is advisable to transfer the explants onto media containing lower concentration of TDZ or different cytokinin following shoot induction in order to elongate and grow up the shoots.

Our results established a basis to elaborate an efficient regeneration system suitable for either mass propagation or genetic modification. It would be interesting to widen the concentration

range of the mentioned cytokinins as it is possible that in case of 2-iP higher concentrations in case of BAP and ZEA lower concentrations are more favourable.

**2. Application of triacontanol in the micropropagation of horticultural plants**

In the last years investigations were focused on natural plant substances and have proved their growth regulator effect. This property makes reasonable their application in the course of *in vitro* propagation in order to improve the efficiency of the process. Triacontanol, a long chain alcohol ($CH_3(CH_2)_{28}CH_2OH$) was discovered as a component of plant epicuticular waxes and isolated by Chibnall et al. (1933) from alfalfa. Since then several experiments proved its growth promoting effect in greenhouse or field trials.

Beneficial effects of TRIA in the micropropagation of lemon balm (*Melissa officinalis L.*) and rootstock varieties of apple and sour cherry have been demonstrated earlier by our working group. Based on the results we have decided to extend the investigations to other plant species too.
Growth stimulating effects of TRIA have been proved in the present experiments as well. Comparing our observations with those of other authors or with our previous results it can be stated that effects of TRIA exhibited *in vitro* are different according to the plant species. Generally it stimulated rooting when applied alone, without hormones. The number of roots increased significantly even at the lowest (2 µg/l) concentration. In case of raspberry and gerbera enhancement of the values reached the peak at the highest (20 µg/l) concentration. Similar tendency have been found earlier in case of JTE apple variety. In contrast in cultures of asparagus the highest root number - more than double of the control – was registered at the lowest concentration and a stepwise decrease was observed at the higher concentrations. This phenomenon strongly resemled to the one, observed previously with balm and sour cherry (Tantos et al., 1999, 2001). Fraternale et al. (2002; 2003) came to the same result in rooting experiments with *Bupleurum* and *Thymus.* TRIA in 2 µg/l concentration proved to be stimulatory but in higher concentrations inhibitory effects were observed. No correlation was found between root length and TRIA concentration.
In combination with cytokinins triacontanol stimulated shoot development and enhanced multiplication. In case of raspberry number of new shoots increased continuously and reached the maximum at 20 µg/l, similarly to results with *Thymus mastichina* (Fraternale et al., 2003). In cultures of gerbera the highest number of shoots was induced by 5 µg/l, of JTE apple 10 µg/l TRIA.
In case of asparagus, lemon balm and sour cherry the range of 2-5 µg/l concentration promoted shoot development, higher concentrations were not able to induce further increase. According to the paper of Reddy et al. (2003) enhanced multiplication rate of *Capsicum frutescens* and *Decalepis hamiltonii* occured in the lower concetration range. The fact, that TRIA was found to be efficient in different concentrations in case of different plants can be explained by the effect of genotype, the

hormon content of the media and the endogenious hormon level of the explants. In the experiments of Tantos et al., (2001) favourable effects were observed at the higher concetration of cytokinin when TRIA was applied in multiplication media containing different concentrations of BAP. This indicates that multuplication rates were enhanced primarily by the cytokinin concentrations. TRIA in low concentrations can be effective because plants are extremely sensitive to it. Triacontanol or its breakdown compounds or a secondary messenger is moving quickly in the plant while influencing enzimes of carbohydrate metabolism (Ries et al., 1977) and growth.

We have observed the continuous elevation of chlorophyll content paralelly with the growth of TRIA concentration in all cultures in the multiplication phase. The same phenomenon was reported by Reddy et al., (2003) on *Decalepis hamiltonii* plants.

In the rooting phase chlorophyll contents were increased even by the lowest concentration of TRIA and the values did not grow further at the higher concentrations. Influence of TRIA on the photosynthetic apparatus was not unambiguous. In case of gerbera photosynthetic activity of control plants proved to be satisfactory, so TRIA could not manifest its efficiency. In contrast, obvious differences were measured in cultures of raspberry 'Malling Exploit'. In this case photosynthetic activity of the control plants proved to be low, but TRIA was able to restore the normal value of Fv/Fm ratio in the treated plants.

According to our experiences application of triacontanol can be recommended in the micropropagation process of different plants especially in case of hard-to-propagate types or in photoautotrophic systems.

**3. Liquid culture system: trials with a novel type bioreactor in the propagation process**

The aim of the experiments presented here was to investigate the effect of culture conditions on model plants cultivated in liquid systems, thus to prove the usefulness of our bioreactor in the micropropagation process. The apparatus differs from the other TIS (Temporary Immersed System) based ones in two basic traits. The culture vessels e.g. the reactor modules are rotating in horizontal position, the plants are in the liquid culture medium and on the stainless steel mesh screen, alternately (periodical 'emergence-submersion' phases). The level of liquid remains stable in the bottom of the modul so the plants are falling into it in the submersion phase and coming above in the emerging phase (Fári et al., 2003)

We also intended to prove that the liquid system does not impair the quality and survival of plantlets. According to our preliminary results both ananas and banana can be propagated in stationary liquid media, but the height of the overlayer is crutial regarding efficiency of the method.. When cultures were completely covered by the liquid, a decrease in the multiplication rate and the

appearance of hyperhidration was usually observed. The highest shoot number was obtained with a thin layer of liquid, covering approx. one third of the shoot length. (Mészáros, 1986, Mészáros and Molnár, 1988).

It became evident, that growth of the cultures and quality of the plants can be strongly influenced by altering culture systems. The highest multiplication rates and the best quality shoots were obtained from the aerated vessels. Our results are in consonance with data of the literature.

In case of ananas a threefold increase of shoot number and shoot fresh mass was obtained from the aerated moduls in contrast with the solid media, similarly to the observation of Escalona et al. (1999). In their experiments three different cultivation systems – MS media + 2,1 mg/l BAP + 0,3 NAA in solidified form, liquid culture and temporary immersion - were compared. Cultures originated from the TIS system showed better quality and higher multiplication rates, three times and four times higher than in the stationary liquid or on agar media respectively. Data of fresh weights followed the same tendency, although no differences were found in the dry weights of liquid cultures. According to Escalona et al. (2003) photosynthetic characters, like as chlorophyll content and induction kinetics, did not differ in the solid or liquid systems.

Firoozabady and Gutterson (2003) used the same 10 liters volume glass jar in different modes of operation. In the upright position of the jar cultures were submerged to the liquid media and the air was bubbled through the liquid. This position proved to be unfavourable for the plants, as they showed necrosis and decrease of the multiplication. When the jar was turned to its side and rotated periodically the quality of plants improved but the rate of propagation was still not satisfactory. The best results were obtained in case of upright position combined with periodical immersion. This scheme, the so called ”twin flask” system consisted of two jars connected with tubing. From the media reservoir flask liquid was pressed to the flask containing plant cultures through a pump. This set-up, also called as PIB (periodically immersed bioreactor) or TIB (temporary immersed bioreactor) was used by Escalona et al. (1999) too. According to their results propagation rates increased and plant quality characters improved significantly in these treatments.

In experiments focused to the propagation of banana, results showed that development of explants is strongly influenced by the mode of application of liquid media. Different multiplication rates were measured in the pioneer investigations of Alvard et al. (1993) when applied autoclavable filter units of the Nalgene Co. as reactor vessels. Practically no multiplication occured in cultures grown in the stationary liquid or on the membrane rafts. On the solid media and in the aerated liquid multiplication rates varied between 2,2 and 3,1 while temporary immersion resulted in the highest (5,2) rates. In the latter treatments both development and quality of the leves appeared to be normal. Only some small leaves developed on the shoots grown on the membrane rafts. In case of cultures

submerged into the stationary liquid the lack of leaves and appearance of necrosis was usually observed. These findings showed that the lack of oxygen in the headspace is strongly inhibiting development and multiplication.

According to our own results culturing in the aerated reactor moduls resulted in a twofold shoot number and improved growth habit when compared to the control solid media. Both the photosynthetic parameters and the anatomical structure of the leaves proved that the shoots developed in the reactor modul are near to the autotrophic status due to the regular air exchange (Mészáros et al., 2004a).

Experiments with *Hosta* cultures showed that this species is very sensitive to the lack of oxygen. Shoot numbers and fresh weights were doubbled following a culture cycle in the aerated modules. Paralelly, data of chlorophyll content and induction kinetics showed normal values, very similar to those, measured on plants grown on the agar media. Electronmicroscopic pictures of leaf sections also strengthened this observation. In contrast, parameters of photosynthetic capacity indicated the presence of inhibition in the environment of the rotated modules Mészáros et al., 2004b). Till now papers of Adelberg et al., (2005) are available concerning micropropagation of *Hosta* varieties in liquid culture. Their apparatus combines the advantages of thin layer liquid and shaking. Both factors made it possible that the explants were not completely covered by the liquid. Their system resulted in better multiplication than on the solid media in baby food jars.

All the advantages arguing besides widening the application of bioreactors were demonstrated in our experiments. The system makes it possible to produce large quantities of plants in a relatively small area. Plants are in close contact with the nutrients so their development is accelerated and the rate of shoot induction is improved. Simultaneously, the air exchange, the continuous oxygen supply gives a positive influence on the photosynthetic capacity and anatomical structure, so, that the plants are able to turn to the autotrophic status and show a better survival than the control ones.

## LITERATURE CITED


Adelberg, J.. Efficiency in thin-film liquid system for *Hosta* micropropagation. (2005) Plant Cell Tiss. Org. Cult. 81:359-368.

Alvard, D,. Cote, F., Teisson, C. (1993) Comparison of methods of liquid culture for banana micropropagation. Plant Cell Tis. Org. Cult. 32 : 55 - 60.

Arnon, D. (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24:1

Benjamin, B. D., Roja, P. C. Heble, M. R., Chadha, M. S. (1987) Multiple shoot culture of *Atropa belladonna*: effect of physico-chemical factors on growth and alkaloid formation. J. Plant Physiol.129:129-135.

Chibnall, A. C., Williams, Latner, A. L., Pipes, A. H. (1933) The isolation of n-triacontanol from lucerne wax. Biochem J. 27: 1885-1888.

Eapen, S. Rangan, T. S., Chadha, M. S., Heble, M. R. (1978) Morphogenetic and biosynthetic studies on tissue cultures of *Atropa belladonna* L. Plant Sci Lett 13:83-89.

Escalona, M., Lorenzo, J.C., González, B., Daquinta, M., González, J.L., Desjardins, Y., Borroto, C.G. (1999) Pineapple (*Ananas comosus* (L.) Merr.) micropropagation in temporary immersion systems. Plant Cell Rep. 18:743-748.

Escalona, M., Samson, G., Borroto, C.G., Desjardins, Y. (2003) Physiology of effects of temporary immersion bioreactors on micropropagated pineapple plantlets. In Vitro Cell Dev. Biol – Plant: 39:651-656.

Fári, M.G., Kertész, T., László, M., Varga, Zs. (2003) Design of a revert rotary plant micropropagation bioreactor –'3R' system:application for research, possibilities of scaling-up and limitations. In: Proceedings of the 1st International Congress on Bioreactor Technology in Cell, Tissue Culture and Biomedical Applications, Tampere, Finland, 14-18 July.

Firoozabady, E., Gutterson, N. (2003) Cost-effective *in vitro* propagation method for pinapple. Plant Cell Rep. 21(9): 844-850.

Fraternale, D., Giampieri, L., Ricci, D., Rocchi, M.B.L. (2002) Micropropagation of *Bupleurum fruticosum*: The effect of triacontanol. Plant Cell Tis. Org. Cult. 69 135-140.

Fraternale, D., Giampieri, L., Ricci, D., Rocchi, M.B.L., Guidi, L., Epifano, F., Marcotullio, M.C. (2003) The effect of triacontanol on micropropagation and on secretory system of *Thymus mastichina*. Plant Cell Tis. Org. Cult. 74 : 87-97.

George, E. F. (1993) Plant propagation by tissue culture. Part I.-II. Exegetics LTD.

Huetteman, C., Preece, J.E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plan Cell Tiss. Org. Cult. 33:105-119.

Lichtentaler, H.K., Rinderle, U. (1988) The role of chlorophyll fluorescence in the detection of stress condition in plants. CRC Crit. Rev. An. Chem., Vol 19, 1. p.: ssi S29-S85.

Mészáros, A. (1986) Meriklón technológiák. Kézirat. Rozmaring Nyomda, Budapest

Mészáros, A., Molnár, Gy. (1988) Technológiai előirat a hálózati laboratóriumok számára. Kézirat. Rozmaring Nyomda, Budapest

Mészáros, A., Domokos-Szabolcsy, É., Halász, K., Kálai, K., Fári, M.G. (2004a) An efficient micropropagation system: 3R bioreactor. Acta Horticulturae (in press)

Mészáros, A., Jámbor-Benczúr, E., Fári, M.G. (2004b) Micropropagation of *Hosta tokudama* in bioreactor culture. Acta Horticulturae (in press)

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-479.

Park, Y.G., Son, H.S. (1988) *In vitro* organogenesis and somatic embriogenesis from punctured leaf of *Populus nigra x Populus maximoviczii*. Plant Cell Tiss. Org. Cult. 15: 95-105.

Pierik, R.L.M. (1987) *In vitro* culture of higher plants. Plant Cell Tiss. Org. Cult. 81:359-368.

Ries, S. K., Wert, V. F., Sweeley C. C. (1977) Triacontanol: a new naturally occurring plant growth regulator. Science 195: 1339-1341.

Tantos, Á., Mészáros, A., Kissimon, J., Horváth, G., Farkas, T. (1999) The effect of triacontanol on micropropagation of balm, *Melissa officinalis* L. Plant Cell Rep. 19 : 88-91.

Tantos, Á., Mészáros, A., Farkas, T., Szalai, J., Horváth, G. (2001) Triacontanol supported micropropagation of woody plants. Plant Cell Rep. 20 : 16-21.

Tóth, E. T., Onisei, T., Amariei, D., Lazurca, D. (1991) Variability in tissue culture regenerated plants of *Atropa belladonna*. Acta Hort 289:269-270.

Welander, M., Jansson, E., Lindquist, H. (1989) *In vitro* propagation of *Populus x wilsocarpa* – a hibrid of ornamental value. Plant Cell Tiss. Org. Cult. 18:209-219.

Zárate, R., Cantos, M., Troncoso, A. (1997) Induction and development of adventitious shoots of *Atropa baetica* as a means of propagation. Euphytica 94:361-366.

## PUBLICATIONS RELATED TO THE PRESENT WORK

### Journal articles

Tantos, Á., **Mészáros, A.,** Horváth, G., Farkas, T. (1999) The effect of triacontanol on micropropagation of *Melissa officinalis L.* Plant Cell Reports, 19: 88-91.

Tantos, Á., **Mészáros, A.,** Farkas, T., Szalai, J., Horváth, G. (2001) Triacontanol supported micropropagation of woody plants. Plant Cell Reports, 20 : 16 - 21.

Kissimon, J., Tantos, Á., **Mészáros, A.,** Jámbor-Benczúr, E., Horváth, G. (1999) Alterations in growth parameters, pigment content and photosynthetic functions under the stress condition of in vitro culture. Z. Naturforsch. 54c. 834-839.

Tantos, Á., **Mészáros, A.,** Farkas, T., Kissimon, J., Horváth, G., (2000) Triacontanol supported micropropagation of horticultural plants. Plant Physiol. Biochem. 38:256

**Conference proceedings**

**Mészáros, A.** (1986) A szövettenyésztési eljárások alkalmazása a szaporítóanyag termelésben. In: Napjaink biotechnológiája, OMIKK-OMFB, Budapest, pp.43-52.

**Mészáros, A.,** Tantos, Á., Kissimon, J., Jámbor-Benczúr, E., Horváth, G. (1998) Triacontanol as a potential growth regulator in tissue cultures. IX International Congress on Plant Tissue and Cell Culture, Jerusalem, Israel. Book of Abstracts pp. 94.

Kissimon, J., Tantos, Á., **Mészáros, A.,** Jámbor-Benczúr, E., Horváth, G. (1998) Alteration in photosynthetic functions under different *in vitro* culture conditions. Int. Workshop on Stress Synergism in Plants, Abiotic and Biotic Stress in Photosynthesis, 23-26. Aug. Tata, Hungary. Program and Abstracts pp.48

Tantos, Á., **Mészáros, A.,** Farkas, T., Kissimon, J., Horváth, G., (2000) A triakontanol mint természetes növekedésszabályozó néhány kertészeti növény mikroszaporításában. Lippay János Tud. Ülésszak Közleményei pp. 178-179.

Fári, M. G., **Mészáros, A.** (2003) Bioreaktorok a növényi szövettenyésztésben. 'Lippay János–Ormos Imre–Vas Károly' Tudományos Ülésszak, november 6-7. Budapest. Összefoglalók pp. 80-81.

**Mészáros, A.,** Jámbor-Benczúr, E. Fári, M.G. (2004) Micropropagation of *Hosta tokudama* in bioreactor culture. COST 843 WG3 Meeting, 8-12 September, Debrecen, Hungary. Scientific Programme and Abstracts.

**Mészáros, A.,** Domokos-Szabolcsy, É. Halász, K. Kálai, K. Fári, M.G. (2004) An efficient micropropagation system: 3R bioreactor. 5th International Symposium on In Vitro Culture and Horticultural Breeding, 12-18. September, Debrecen,Hungary. Book of Abstracts and Programme pp. 193.

**Mészáros, A.,** Kálai, K., Halász, K. (2005): Growth promoting effects of triacontanol in the micropropagation of horticultural plants. COST 843 Final Conference. 2-5. July, Stara Lesna. Book of Abstracts p. 85-86.

**Book chapters**

**Mészáros, A.** (1987) Szövettenyésztés. In: A gerbera termesztése (szerk. di Gleria E., Tusnádi, Cs.K.) Mezőgazdasági Kiadó, Budapest, pp. 101-107.

**Mészáros, A.** (1990). A növényi mikroszaporítás gyakorlata. Folia Biotechnologica (OMIKK-OMFB): pp. 1-47.

**Mészáros, A.** (1993) Gerbera. In: Dísznövények mikroszaporítása (Szerk: Jámborné, Benczúr E.) Kertészeti és Élelmiszeripari Egyetem jegyzetei pp. 47-50.

**Mészáros, A.** (1999) Mikroszaporítási módszerek a gyümölcs-faiskolai termesztésben. In: Gyümölcsfaiskola. (Szerk.: Hrotkó, K.) Mezőgazda Kiadó, Budapest, pp.: 217-228.

**Mészáros, A.** (2004) Trópusi gyümölcsök: banán, ananász. In: Kertészeti növények mikroszaporítása. (Szerk: Jámborné, Benczúr E., Dobránszki J.) Mezőgazda Kiadó, Budapest,

**Other professional papers**

**Mészáros, A.** (1986) Meriklón technológiák. Kézirat. Rozmaring Nyomda, Budapest

**Mészáros, A.,** Molnár, Gy. (1988) Technológiai előirat a hálózati laboratóriumok számára. Kézirat. Rozmaring Nyomda, Budapest.