Theses of the Ph.D. dissertation

Establishment of tissue culture methods for providing genetic transformation of cucurbits

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BUDAPEST

2009

PhD School

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

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INTRODUCTION AND RESEARCH GOALS

Cultivated or wild vegetables of the Cucurbita genus are spread on five continents. Due to their outstanding nutritional effects and manifold utilization possibilities they are grown in significant quantities worldwide. To satisfy the needs of producers and consumers, and to exploit the possibilities of continuously developing production technologies there is a constant need for newer varieties with the purpose of further enhancing excellent quality and nutritional benefits. By the improvement of sophisticated and quality food consumption the role of health-preserving effects and quality profiles are appreciated more and more. The breeding of muskmelon has a century-long history in Hungary. Hungarian varieties – old landraces, bred constant and hybrid varieties – possess valuable genetic resources and fine quality characteristics.

Most of the genetic transformation experiments conducted with practical purposes is directed to introduce resistance traits. It is possible to induce resistance to plant pests and pathogens (pathogen viruses, bacteria, fungi and insects), environmental stress factors (drought and salt, frost, heavy metal and general stress) and herbicides. There are transformant melon varieties which produce the coat proteins of CMV (cucumber mosaic virus) and ZYMV (zucchini yellow mosaic virus). In Cucurbita genus, there are also other various important breeding goals: improving quality, shelf-life and sugar content, enhancing health-preserving antioxidant capacity, salt and drought tolerance. Some papers were published on the improvement of shelf-life (via successful inhibition of ethylene synthesis), and enhancing of salt and drought tolerance. A further goal could be the modification of metabolic products to render the plant suitable to produce raw materials for food and pharmaceutical purposes (for example: essential amino acids, vegetable oils, carbohydrates, vaccines etc.)

Gene transfer methods are usually based on an effective *in vitro* plant regeneration system. In case of melon, there have been some reports on both direct and callus-induced plant regeneration and transformation via organogenesis or embryogenesis, and even on the engineering of transgenic plants primarily in foreign varieties (Fang and Grumet, 1990, Bordas et al., 1997, Curuk et al., 2005). The efficiency of these methods however, is not satisfying (Atares et al., 2004). The available results definitely prove genotypic and environmental dependency, meaning that a successful method does not provide satisfying results in case of another genotype or in another laboratory (Curuk et al., 2005). Therefore, the importance of testing the *in vitro* responsiveness of all genotypes has been grown.

The primary objective has been to test the *in vitro* regeneration ability of melon varieties grown domestically and abroad and some other Cucurbita varieties (zucchini, pattypan squash and winter squash); and to elaborate an appropriate *in vitro* regeneration method for the selected varieties. Another aim was to provide a decent basis for the establishment of a transformation protocol, and that useful genes could be transferred into the varieties showing good regeneration ability.

<u>In order to achieve the goals listed above, the following tasks had to be completed:</u>

1. Appropriate seed sterilization procedure had to be elaborated.
2. Testing the responsiveness of the nine melon and three squash varieties on various solid culture media, the suitable responding varieties had to be selected for later experiments. In liquid culture, on a known culture medium the varieties were intended to be ranked based on their responsiveness.
3. Responsiveness of particular plant parts (cotyledon, leaf, hypocotyl, decapitated hypocotyl) had to be determined on solid culture medium with the application of the best responding varieties.
4. Following the selection of appropriately responding varieties and plant parts, the effect of plant growth regulators on regeneration had to be evaluated in detail; and in an optimization experiment a satisfactory auxin-cytokinin ratio to be determined for the induction of organogenesis or embryogenesis from explants on solid and liquid media.
5. The necessary length of induction period had to be determined on solid culture medium and in liquid culture
6. The effect of agar and phytagel on regeneration and on the number of regenerated and developed shoots had to be determined.
7. In liquid culture, the effects of various cultivation parameters, such as the various applied vitamins, carbon sources (sucrose, glucose, and maltose) and pH on the induction of embryogenesis had to be determined.
8. In case of liquid cultures, the effect of the age of the seeds (by applying young and stored seeds) used to initiate the cultures on somatic embryogenesis had to be determined.
9. Composition of induction media and solid media for further cultivation and transfers, and the length of cultivation had to be determined for the success of raising the shoots.
10. The final task has been to establish efficient induction and plant regeneration systems and protocols on both solid culture medium and in liquid culture.

Since it is known that the efficiency of regeneration systems decreases during transformation processes, – due to several factors, primarily because of *Agrobacterium* infection and the applied selection agents – in the possession of an appropriate regeneration system, the efficiency of the established system in the transformation system had to be determined, and the unfavourable effects to be eliminated as possible.

To accomplish these goals, the completion of the following tasks was necessary:

1. Examine the interactive effect of infection and cocultivation on regeneration and on the efficiency of bacteria elimination.
2. Determining the appropriate concentration of antibiotics that on one hand assures the elimination of bacteria used for infection and the efficient selection of transformants on the other.
3. Test the effect of agar and phytagel solidifying the medium on the process. Evaluate the efficiency of the system based on the number of rooted shoots and raised plants, namely elaborating a system providing plants utilizable in practice.

MATERIALS AND METHODS

Melon regeneration initiated on solid culture medium

The efficiency of several sterilization methods has been examined. Regeneration ability of Muskotály, Ezüstananász, Javított Zentai, Topáz, Hógolyó, Tétényi csereshéjú, Magyar kincs, Fortuna and Hale's Best varieties has been tested on 10 different media. Many combinations of culture media and growth regulators have been tested, applying Murashige and Skoog (1962) medium (MS) as a basal medium in every case. In the experiments, the cotyledon, hypocotyl (cut into three segments) of 2-, 4-, 8- and 14-day-old seedlings, and in case of 14-day-old seedlings the first foliage leaves have been used as initial materials of regeneration (Hógolyó and Hale's Best). In later experiments the 4-day-old cotyledons of Hógolyó and Hale's Best varieties cut into two segments have been examined on 25 different media combinations. In the regeneration medium 6-benzylaminopurine and indole-3-acetic acid have been applied in concentrations between 0 and 1.2 mg/l with the addition of identical amounts (0.26 mg/l) of abscisic acid. In the case of Hógolyó melon variety a comparative experiment has been conducted in order to determine whether the agar (8 g/l) or phytagel (2.5 g/l) influences the regeneration efficiency on regeneration medium. The differentiated leafy

„shoot-bunches" were transferred to rooting medium divided into individual shoots (regulator-free MS medium). Following appropriate root induction, plants were transferred to sterile peat-soil (1:1) mixture. After acclimatization the grown plants were transferred into a greenhouse.

Melon regeneration initiated in liquid culture

Two types of culture media have been applied for somatic embryogenesis: a liquid induction medium which induced the formation of embryos, and then a solid medium for the further development of embryos. In the regeneration experiments conducted in liquid medium, the responsiveness of the six Hungarian melon varieties has been tested first on an MS medium containing 0.1 mg/l 6-benzylaminopurine and 2 mg/l 2,4-D.

Following pre- experiments the growth regulator composition of the induction medium has been examined in many combinations, and the pH, vitamin content and carbon source of the liquid medium have been modified. In case of induction media two different pH levels (pH 5.4 and 4.6) have been examined. In case of vitamins the quantity of MS vitamins (normal and double quantity) has been modified and the B5 vitamin (pantothenic acid) has also been tested. As carbon source of the media, glucose, sucrose and maltose have been used. As a solid culture medium, a regulator-free MS medium has been used with 30 g/l sucrose carbon source in every case. Agar has been used in three concentrations (0.5; 1 and 2%).

Growth regulator combination experiments have been conducted with the Muskotály variety. In the experiment, various combinations of 2,4-D (0, 2, 5 and 10 mg/l) and 6-benzylaminopurine (0, 0.1, 0.5 and 1 mg/l) have been applied on liquid MS medium. The full content of the Erlenmeyer flask has been transferred to solid culture medium after different induction periods (7, 14, 28 and 34 days). Regeneration ability of fresh seeds derived from matured melons and commercially available seeds stored for one year has been compared by Muskotály melon variety.

Squash regeneration

The regeneration ability of 'Óvári fehér', 'Black Beauty' and 'Nagydobosi' squash varieties has been studied on seven different regeneration MS media (2,4-D, kinetin, 6-benzylaminopurine, indole-3-acetic acid). Then a growth regulator optimization experiment with multiple repetitions has been conducted with all three varieties, in which indole-3-acetic acid in 0-0.9 mg/l and 6-benzylaminopurine in 0-1.2 mg/l concentrations have been applied. Cotyledons of 7-10-day-old plants after opening or some days later have been used in a state

of 1-2 leaves. The shoots grown on cotyledon segments have been transferred to a rooting medium, then the rooted plantlets have been acclimatized in a sterile peat-soil mixture (1:1) on 25 °C with 100% relative humidity. Grown plants have been transferred to greenhouses after conditioning.

Melon and squash transformation

Before starting transformation experiments, tests have been carried out to determine the sensitivity to antibiotics (kanamycin, augmentin, cefotaxime, and carbenicillin) of 'Hógolyó' and 'Hale's Best' melon varieties and the 'Nagydobosi' winter squash variety. In case of melon, the 4-day-old, cotyledon segments cut around have been infected with *Agrobacterium tumefaciens* suspension for various periods. During cocultivation, infected cotyledon segments have been transferred to a light chamber, and after cocultivation, they have been transferred to the selection medium. During selection, explants infected with LBA4404 strain (plazmid pGA482) have been transferred to a selection, regeneration medium containing kanamycin. Augmentin, cefotaxime or carbenicillin has been used for the elimination of bacteria. In winter squash transformation experiments cotyledons have been segmented in the same way as in the regeneration experiments. Cotyledon segments of winter squash and melon varieties have been transferred into the bacterium suspension (cultured overnight) for various periods, then they have been dried on sterile paper and transferred to a regeneration medium without antibiotics. After a few days of cocultivation, for the elimination of *Agrobacteria* the leaf segments have been transferred to another medium, which contained kanamycin and cefotaxime beside the growth regulators. Regenerant plants have been transferred to a rooting medium. 1-2 leaves have been taken from the selected regenerant plants and the samples have been crumbled in a mortar with liquid nitrogen and put into individual Eppendorf tubes. DNA has been extracted with the Qiagen DNeasy Plant System Mini Kit DNA extraction system with the application of the process determined by the manufacturer (QIAGEN, 2000). The presence of the gene construction intended to be transferred has been checked by PCR technique with primers specific to the coat protein genes of CMV (cucumber mosaic virus) and ZYMV (zucchini yellow mosaic virus). DNA-sequences amplified during PCR reaction have been studied further by agarose gel electrophoresis.

RESULTS AND CONCLUSIONS

When the objectives of *in vitro* regeneration and transformation of Hungarian varieties have been set, survey on literature data revealed that a suitable protocol is not available for this task, and many questions must be answered till such a protocol can be established. A large number of papers deal with the regeneration and transformation of Cucurbits, mainly in case of cucumber (*C. sativus*) and melon (*C. melo*), but far less publications discuss squash varieties. Published results did not mention any method which could have been generally applied for Cucurbitaceae. The main objective of these publications – just like in this present work – has been to establish an effective *in vitro* regeneration system and produce transformants. Experiments conducted to achieve this goal mainly discussed basic questions, e.g. which plant part should be the source material and what kind of medium and growing conditions should be applied (Moreno et al., 1985; Kathal et al., 1988; Niedz et al., 1989; Gray et al., 1993). According to Nunez-Palenius et al. (2008) the most important factor determining the success of regeneration is the selection of genotype. The reason for this – among others – is the great morphological and genetic variability of melon varieties. The regeneration ability of commercially available varieties varies greatly even if identical conditions and regeneration methods are applied (Gray et al., 1993; Ficcadenti and Rotino, 1995; Molina and Nuez, 1995a; Kintzios and Taravira, 1997; Galperin et al., 2003a). But according to some authors, the way of regeneration is also genotype-dependant. Oridate et al. (1992) and Gray et al. (1993) found that somatic embryogenesis in melon varieties of *reticulatus* took place more frequently than in the case of varieties belonging to the *inodorus*. Additionally, Oridate et al. (1992) pointed out significant differences in the regeneration ability of the 18 commercially available varieties examined. Gray et al. (1993) tested a regeneration method established for a specific variety on 51 commercially available varieties. The responsiveness of the varieties ranged between 5-100% per cotyledon segment (0.1-20.2 embryos/cotyledon segment). Ficcadenti and Rotino (1995) studied 11 melon varieties (*reticulatus, inodorus*). The number of shoot primordia developed via organogenesis per cotyledon segment ranged between 6.0 and 17.3 in case of varieties belonging to the *reticulatus* variety type, while the regeneration ability of *inodorus* types showed results between 12.2 and 14.2 (shoot primordia/cotyledon segment). The reproducibility of published results in other laboratories is quite uncertain even in case of successful systems. The reasons for this are that the genotype-dependency of regeneration ability is strong among varieties and even in seed units, the methods of evaluation and measurement are inadequate, the lack of

appropriate statistical evaluations lead to inaccurate conclusions (Molina and Nuez, 1995b). The majority of authors agree that regeneration can be most effectively initiated from the cotyledon (Gaba et al., 1996; Liborio-Stipp et al., 2001; Galperin et al., 2003a; Gaba et al., 2004; Nagesha et al., 2007). More recent studies proved that the hypocotyl (Curuk et al., 2003) is more reliable than the cotyledon in case of producing diploid regenerants where poliploidia is quite prevalent (Ezura et al., 1992; Adelberg et al., 1994). 6-benzylaminopurine is generally applied for the induction of organogenesis (Debeaujon and Branchard, 1993; Adelberg et al., 1994). The application of 2,4 D synthetic auxin worked well in the initiation of embryogene cultures (Tabei et al., 1991; Debeaujon and Branchard, 1993; Gray, 1996; Guis et al., 1997; Ezura and Akasaka-Kennedy, 2004).

Melon on solid medium

Following international practice, seed coats were removed from the seeds in the experiments, since melon seeds are often infected inside the seed coat. Various methods have been tested as seed disinfection protocol. In accordance with other authors' results (Yadav et al., 1996; Liborio-Stipp et al., 2001; Yalcin-Mendi et al., 2004) the application of 15 % Clorox gave the best results.

In the beginning, the regeneration ability of the four varieties (Magyar kincs, Javított Zentai, Hógolyó, Tétényi csereshéjú) has been studied on three different culture media. The medium containing indole-3-acetic acid and kinetin (MD1) applied successfully by Moreno et al. (1985) was not efficient in the case of the four Hungarian varieties when the regeneration had been initiated from cotyledons. Only white callus has been formed on the medium containing 2,4 D and 6-benzylaminopurine (MD2) formulated by Branchard and Chateau (1988) for the Cantaloup charentais variety, on which according to the former results, embryos and plantlets could have been regenerated. The application of the medium (MD3) containing naphtyl acetic acid and 6-benzylaminopurine (Roustan et al., 1992) resulted green callus formation on the cotyledons of the Magyar kincs variety, while the rest of the species formed white callus. On the MD4 medium, where successful plant regeneration had formerly been described from the cotyledons of Hale's Best variety (Niedz et al., 1989; Fang and Grumet, 1990), and on the MD5 medium (Bársony et al., 1999) – containing similar concentrations of growth regulators – direct organogenic shoot induction could have been observed on the edge of cotyledon segments of Hógolyó and Hale's Best varieties.

Testing the responsiveness of the nine varieties on five different solid media (MD6, MD7, MD8, MD9, MD10) the Hógolyó, Magyar kincs, Javított Zentai and Hale's Best

varieties showed good, while the Tétényi csereshéjú variety showed poor responsiveness. These results indicate the greatly different responsiveness and growth regulator requirements of varieties, which are supported by earlier results (Debeaujon and Branchard, 1993; Molina and Nuez, 1995a). All the media contained 6-benzylaminopurine, since the 6-benzylaminopurine in itself proved to be sufficient for the induction of regeneration in case of several varieties (Kathal et al., 1988; Gaba et al., 1996, Liborio-Stipp et al., 2001). Most varieties showed regeneration on the media containing 1 mg/l 6-benzylaminopurine (MD10) and 0.5 mg/l 6-benzylaminopurine (MD9). However, plant development stopped after induction in many cases. The addition of auxin promoted the organogenesis of only a few varieties, while inhibited it in case of others. On the medium containing 2,4 D no regenerants at all could have been observed. Ficcadenti and Rotino (1995) successfully regenerated plants with the addition of 6-benzylaminopurine (0.6 mg/l) and abscisic acid (0.26 mg/l), while without the addition of abscisic acid, significantly less shoots could have been observed. This contradicts to the statement (Kathal et al., 1988; Gaba et al., 1996, Liborio-Stipp et al., 2001) that 6-benzylaminopurine is sufficient in itself for efficient organogenic shoot induction. Thus these results have been combined with the results of Niedz et al. (1989) and the quantity of indole-3-acetic acid added to the medium has been increased while that of the abscisic acid has not been modified (MD6 medium). This has been the medium on which the most plantlets have been regenerated among all the applied media in the case of Hale's Best and Hógolyó melon varieties. The regeneration ability of the Hale's Best variety has been proven similarly to the results of other authors (Trulson and Shahin, 1986; Niedz, et al., 1989; Yadav et al., 1996).

In the experiments with the objective to select the appropriate plant tissue it has been appointed that the cotyledon is the most effective initial material regarding regeneration, both in the case of Hógolyó (0.89 shoots/cotyledon segment) and Hale's Best (1.03 shoots/cotyledon segment) varieties. This result supports the ability of cotyledon for regeneration, as formerly described by other authors (Gaba et al., 1996; Liborio-Stipp et al., 2001; Galperin et al., 2003a; Gaba et al., 2004; Nagesha et al., 2007). Regarding the age of the selected plant material it has been observed that – similar to the results of other authors (Niedz et al.,1989; Ficcadenti and Rotino 1995; Gaba et al., 1996; Ben Amor et al. 1998; Curuk et al., 2003) – the most regenerant plants can be obtained from the cotyledons of four-five-day-old seedlings. Many authors do not even study the effect of explant age on the regeneration, they determine an ideal seedling age based on the results of other studies or determine just an interval and describe the state of the applied plant part. These studies

compare other variables in their experiment, like e.g. Ficcadenti and Rotino (1995), who also used 4-5-day-old cotyledons in the experiments, but studied the effect of basal medium, various genotypes, three different growth regulators and solidifying materials (agar or phytagel) on the efficiency of regeneration. The hypocotyl is also suitable for being used as an initial material, but its efficiency is inferior compared to cotyledon, and this result is in accordance with literature data. Plantlets have been successfully regenerated from the proximal zone of the hypocotyl in case of Hale's Best (0.33 shoot/hypocotyl segment) and Hógolyó (0.13 shoot/hypocotyl segment) varieties, just like Kathal et al. (1986). Shoot induction from leaves could not have been observed in the case of the studied Hógolyó and Hale's Best varieties, in contrast with other varieties mentioned in literature (Kathal et al., 1988; Tabei et al., 1991).

The hypocotyl decapitation method described for eggplant (Fári et. al., 1995) has been applied for the first time in case of Hógolyó (0.31 shoots/hypocotyl) and Hale's Best (0.45 shoots/hypocotyl) melon varieties. For the first time in case of melon, shoots have been regenerated on the cut surface of the hypocotyl, and obtained plantlets from every second seed in average. This result however, lags behind in efficiency compared to cotyledon.

In case of the two selected varieties a growth regulator optimization experiment has been conducted with 25 different combinations of 6-benzylaminopurine and indole-3-acetic acid. Most authors studied varieties only on simple growth regulator series (Liborio-Stipp et al. 2001) or tested only one growth regulator composition (Molina és Nuez, 1995a). Complex growth regulator optimization studies have been conducted only by a few authors (Moreno et al., 1985; Roustan et al., 1992). In case of Hógolyó variety, as a result of the optimization experiment, the most developed shoot primordia suitable for transfer have grown on the medium containing 0.9 mg/l 6-benzylaminopurine, 0.6 mg/l indole-3-acetic acid and 0.26 mg/l abscisic acid (MDOP1).

In case of the Hale's Best variety, the most efficient shoot regeneration has been observed on the medium containing 0.6 mg/l 6-benzylaminopurine, 0.9 mg/l indole-3-acetic acid and 0.26 mg/l abscisic acid in the growth regulator optimization experiment. In this case two or even three shoot primordia have appeared on one cotyledon segment, however only one or two shoots could have been regenerated per cotyledon segment (MDOP2).

Comparing the solidifying materials, in case of the Hógolyó variety the application of phytagel proved to be more effective than agar, because the regeneration has been faster. There has not been significant difference in the number of regenerated and grown shoots per cotyledon segment. This contradicts the results of Ficcadenti and Rotino (1995) where

significantly more shoots per cotyledon could have been induced on an MS medium containing agar, with identical composition of growth regulators. Yadav et al. (1996) however could have obtained significantly more plants on a medium containing phytagel. The authors mentioned above took into consideration only the number of grown up plants as a basis of comparison, but none of them compared the media containing agar or phytagel on the basis of the period required to grow up plants. This is the first report to appoint the favourable effect of phytagel on the growing period of the Hógolyó melon variety.

Altogether 6272 cotyledon segments have been transformed from the Hógolyó variety in the experiments, and 206 rooted regenerant plants have been selected, thus the efficiency of regeneration after transformation was 0.032 shoots/cotyledon segment. In case of Hale's Best variety, 12,000 cotyledon segments have been transformed and 230 rooted regenerant plants have been selected; this means 0.02 rooted regenerant plants per cotyledon segment. The efficiency of this result is similar to those in literature (1.9-3.2% transformation efficiency).

It has been concluded on the basis of the results of transformation experiments that regeneration is heavily inhibited by transformation in which *Agrobacterium* has been applied, and in a lesser extent by the applied antibiotics as well. Decrease in the number of supposedly transformant individuals which can be grown up could have been observed in every development phase. This decrease is the effect of the transformation process itself, which is caused by the stress of *Agrobacterium* infection and the applied antibiotics and other selection agents. In further steps, the decrease between the phases can be definitely attributed to the efficiency of the selection system, since those shoot primordia which initiated not from transformant cells could not develop further. This means that the goal of selection has been achieved: plants have been obtained only from transformant cells or shoot primordia. The efficiency of the selection has been well demonstrated by the experiment where the control plant of identical age can be seen positioned with supposedly transformant plants passing selection.

According to the results the transformation system decreased the efficiency of regeneration in the extent described in literature. Of course, differences are caused by the application of individual varieties and various selection agents.

Melon regeneration in liquid culture

Many authors reported successful regeneration in liquid culture via somatic embryogenesis (Oridate and Oosawa 1986; Akasaka-Kennedy et al., 2004; Ezura and Akasaka-Kennedy 2004). During the testing of responsiveness of melon varieties, Muskotály

(10.5 plantlets/seed) and Hógolyó (8.5 plantlets/seed) varieties responded best to the treatments, therefore these varieties have been selected for the most detailed examinations during experiments. Because of its good responsiveness, it is also planned to involve the Ezüstananász (4.5 plantlets/seed) variety in future regeneration experiments.

In the plant growth regulator optimization experiment it has been intended to obtain the answer for the following questions. Could the efficiency of somatic embryogenesis be enhanced by adding large amount of 2,4-D? How decisive is the role of the amount of 6-benzylaminopurine during induction, and how does the length of incubation period influence the development of cultures? The suitability of 2,4-D and 6-benzylaminopurine to induce somatic embryogenesis has already been proved (Oridate and Oosawa, 1986; Trulson and Shahin, 1986; Branchard and Chateau, 1988; Debeaujon and Branchard, 1988; Kintzios and Taravira, 1997). During the experiments the conclusion has been drawn that media of 2 mg/l 2,4-D content were the most appropriate in regard of plant regeneration. By these media, both 21 and 34 days of shaking cultivation delivered good results, and the latter yielded the most plantlets. Therefore, later longer shaking periods have been also tested to enhance efficiency. At higher 6-benzylaminopurine concentrations, the green colour of seed segments was more vivid and also their size was larger. However, 6-benzylaminopurine concentrations higher than 0.1 mg/l in later experiments have not been applied, since high level of cytokinines could also be responsible for the vitrification of samples, and this process must be avoided in any case (Jámborné Benczúr, 2005).

The significance of the age of seeds during regeneration and also the correlation between the age of seeds and the efficiency of somatic embryogenesis has been examined. Cultures initiated from seeds of freshly collected, mature melons regenerated better during the treatments than those initiated from stored seeds. At the same time however, the liability to vitrification was also higher in the case of fresh seeds. This means that the number of regenerated plantlets can be increased by the application of fresh seeds, but regarding the quality of the regenerated plantlets, dry seeds performed better. Cultures initiated from stored seeds in a media of 1% agar content, 22.25 plantlets developed per seed on average, while in case of fresh seeds this amounted only to 14.75. In media containing 2 % agar, stored seeds yielded 19 plantlets/seed on average, while fresh seeds yielded 46.25 plantlets/seed.

Based on the comparison of the 6 different media applied in the study, it can be stated that the addition of different vitamins had not fulfilled the expectations. No significant change in the efficiency of regeneration was found by the comparison of MD11 medium (with elevated MS-vitamin level) and MD12 medium (normal vitamin content) both in the case of

Muskotály and Hógolyó varieties. Regarding the number of regenerated plantlets no significant difference could be found between MD14 (normal vitamin content) and MD16 (containing vitamin B5 – pantothenic acid) media.

Evaluating the experiment from the aspect of the selected carbon source, it can be stated that media containing sucrose (MD11, MD12, MD13) are more favourable compared to those containing only glucose (MD14, MD15) or mixed carbon source (MD16) for the embryogenesis induction in melon. This statement is valid for both the Muskotály and Hógolyó varieties. Among the three applied carbon source, glucose gave the poorest performance: the least number of regenerated plantlets were observed on MD14 and MD15 media. MD16 gave better results, but it also lagged behind compared to the yields of media containing only sucrose.

In case of melon, type and concentration of carbohydrates heavily influence somatic embryogenesis (Oridate and Yasawa, 1990; Debeaujon and Branchard, 1992; Gray et al., 1993; Guis et al., 1997). Gray et al. (1993) found that the sugar concentration of induction and growth media explicitly determine the efficiency of somatic embryogenesis. Finally they also determined that 3 % sucrose produced a greater explant response than lower or higher.

Guis et al. (1997) produced plantlets from cotyledons on solid medium via somatic embryogenesis. They reported significant differences in the number of grown plantlets when applying sucrose, glucose or maltose carbon sources. Contrary to our results, media containing glucose performed better than those with sucrose in their experiments, and media containing maltose only inhibited somatic embryogenesis.

The role of the length of induction period on different media and varieties has also been studied during our experiments. To assess this, not only one transfer has been applied, but the developed embryos have been continuously passed at regular intervals from the liquid medium to the solid medium. In case of Hógolyó and Muskotály varieties (primarily at MD13 samples) it can be observed that after the 28-day incubation period yielding relatively high number of plantlets, the number of new plantlets decreased in the following 35-day period, and the number of new plants started to increase again following 43 days of shaking.

A possible explanation for this is that embryos do not form in a definite moment, but continuously, in many stages. In many cases, high plantlet numbers after the 50th day could not be observed, so it is not worth to maintain the cultures after this incubation period. With regard on shaking period, the behaviour of Ezüstananász variety was slightly different form that of the two other varieties. At this variety the highest number of plantlets was observed after 35 days, the number of newly regenerated plants decreased after 43 days of incubation,

and after 50 days of induction it was negligible. This can probably be explained with the difference in the vegetation periods of the examined varieties: while Hógolyó and Muskotály varieties have medium-long vegetation periods, Ezüstananász has a short vegetation period (Balázs, 1994).When adjusting pH, the initial hypothesis was – based on results obtained by different plants on liquid medium, and results of fermentor cell cultures – that by setting a low pH in the liquid medium an optimal environment for cell proliferation and embryo induction can be created. Following this stage, the higher pH of the solid medium promotes embryo formation and the differentiation of the embryos to intact plants. The pH of MD13 medium was adjusted to 4.6 during autoclaving, in contrast with the 5.4 pH of MD11 medium, which was advantageous for the seed cuts, since these seeds were immediately placed into a favourable environment. These results are in accordance with those obtained at other plants (Martin and Rose 1975; Skirvin et al. 1986; Kovács et al. 1995) and show that low pH (4.5-4.6) promotes cell proliferation also in the case of melon, but inhibits differentiation, while higher pH (4.8-5.2) promotes embryo development. Since the pH of cultures automatically adjusts to pH 4.5-4.6 in the beginning, it is reasonable to initiate cultivation on a low pH.

When varieties were compared regarding the number of regenerated plants, the Muskotály variety showed the best results (with a total of 1222 regenerated plants during the experiments). Altogether the Hógolyó and Ezüstananász varieties also proved to be suitable for the induction of somatic embryogenesis, so it is worth to deal with all three varieties in the future. Among the applied media, definitely the MD13 proved to be the best, so it can be worth to initiate transformation experiments, possibly with all three varieties.

Squash varieties

The results obtained from the regeneration experiments are generally in accordance with those already published by other authors, where cotyledons were good initial materials (Jelaska, 1972; Katavic et al., 1991; Chee, 1992; Gonsalves et al., 1995; Abrie and van Staden, 2001; Lee et al., 2003; Urbanek et al. 2004; Zang et al., 2008). In case of Cucurbits, the best respondent is the proximal part of the half-cut cotyledon (Lee et al., 2003).

In contrast with the results of Gonsalves et al. (1995), experiments showed that the different applied concentrations of 2,4-D were not effective. It has been found that treatments with both 6-benzylaminopurine only, and also when complemented with indole-3-acetic acid were effective in regard of regeneration. In a concentration of 1 mg/l 6-benzylaminopurine, in itself has been successfully applied by other authors in case of many varieties (Ananthakrishnan et al., 2003; Lee et al., 2003; Kathiravan et al., 2006; Ananthakrishnan et al., 2007; Amutha et

al., 2009). Zang et al. (2008) however significant differences in the number of regenerated shoots per cotyledon segments could not have been showed when using culture media with 0.5, 1 and 2 mg/l 6-benzylaminopurine content. In the present experiments the best results in case of winter squash have been obtained with the combination of 6-benzylaminopurine and indole-3-acetic acid. Urbanek et al. (2004) applied cytokinin and auxin combined in case of oilseed pumpkin and they used seven-day-old cotyledons; but they applied naphthyl acetic acid in their experiments and obtained regenerant plants via embryogenesis. Similarly to the present experiments, Ananthakrishnan et al. (2003) regenerated plants via organogenesis. Kathiravan et al. (2006) obtained results of 1.2 - 3.9 shoot/cotyledon segment studying 15 different squash varieties.

It is generally known that responsiveness is heavily genotype-dependent; therefore the present results can be regarded as new and justifying former experiments in respect of the studied varieties. Root induction of the newly formed shoots was quite difficult except in the case of zucchini, where adventitious shoot induction often took place without the use of a rooting medium. After root induction, the incubation of plants did not cause any problems, therefore it is especially important to find a more effective root induction method, e.g. by modifying the growth regulator concentration or by the application of other growth regulators. In case of transformation, it has been found that the determination of the length of cocultivation period is quite problematic, since a short cocultivation greatly decreases transformation rate, while a long cocultivation period renders the elimination of *Agrobacteria* difficult.

SUMMARY OF NEW SCIENTIFIC RESULTS

New results obtained with melon

1. This is the first report on *in vitro* regeneration of the plants of Javított Zentai, Muskotály, Tétényi csereshéjú and Magyar kincs varieties from cotyledon, on solid medium.
2. This is the first report on regenerating plants of Hógolyó and Hale's Best varieties from hypocotyl and decapitated hypocotyl explants on solid medium.
3. In plant growth regulator optimization experiments, most shoots available for transplantation developed on the solid culture medium with 0.9 mg/l 6-benzylaminopurine and 0.6 mg/l indole-3-acetic acid content (MDOP1) in case of Hógolyó melon variety; while the Hale's Best melon variety produced the most transplantable shoots on the solid culture medium containing 0.6 mg/l 6-benzylaminopurine and 0.9 mg/l indole-3-acetic acid (MDOP2).
4. This is the first report on regenerating plants via somatic embryogenesis from the seeds of Muskotály, Hógolyó and Ezüstananász varieties in liquid culture.
5. By plant growth regulator optimization experiments conducted with the Muskotály variety, the appropriate concentration of liquid medium (0.1 mg/l 6-benzylaminopurine and 2 mg/l 2,4-D) has been determined.
6. Comparing the six types of liquid media applied in our examinations, it has been appointed that the addition of the various vitamins did not influence the induction of embryogenesis by the Muskotály, Hógolyó and Ezüstananász varieties.
7. Examining the effect of various carbon sources applied in the liquid media it has been appointed that media containing sucrose were more advantageous for the induction of embryogenesis in melon (Muskotály and Hógolyó varieties).
8. This is the first report to appoint that low pH has a favourable influence on the induction of somatic embryogenesis, while high pH positively affects plant differentiation in melon (Muskotály, Hógolyó and Ezüstananász varieties).
9. Examining the effect of age of seeds of the Muskotály variety on somatic embryogenesis, it has been appointed that cultures initiated from young seeds produce more plants in liquid culture.
10. Plants have been successfully grown on selection media, on which the control plants definitely perished. Presumptively transformant plants have been successfully rooted

on selection medium (Hógolyó and Hale's Best varieties) and bloomed as well when transplanted to greenhouses.

New results obtained with squash

1. This is the first report on *in vitro* plant regeneration initiated from cotyledon in case of Nagydobosi winter squash and Óvári fehér pattypan squash varieties.
2. In transformation experiments regenerant Nagydobosi winter squash plants have been successfully grown on selection medium. Presumptively transformant plants have been successfully rooted on selection medium and bloomed as well when transplanted to greenhouses.

MOST IMPORTANT PUBLICATIONS FROM THE SUBJECT OF THE DISSERATION

Journal articles

Journals with IF

Kiss-Bába E., Pánczél S., Velich I., Bisztray G. D. (2010) Influence of genotype and explant source on the *in vitro* regeneration ability of different melon varieties. Acta Biologica Hungarica (in press)
IF: 0.619 (2008)

Journals without IF

Kiss-Bába E., Pánczél S., Simonyi K., Bisztray Gy. D. (2010) Investigations on the regeneration ability of squash cultivars. Acta Agronomica Hungarica (in press)

Bársony Cs., Bisztray Gy., **Bába E.** and Velich I. (1999) Shoot induction and plant regeneration from cotyledon segments of the muskmelon variety „Hógolyó". International Journal of Horticultural Science 5, (1-2): 61-64 p.

Bába E., Zarka V., Deák T., Pedryc A., Velich I., Bisztray Gy. D. (2002) Molecular diversity of Hungarian melon varieties revealed by RAPD markers. International Journal of Horticultural Science 8, (3-4): 11-13 p.

Book chapters

Kissné Bába E. Bisztray Gy. D. (2005) Sárgadinnye, In: Jámborné Benczúr E., Dobránszki J. (szerk.): Kertészeti növények mikroszaporítása. Mezőgazda Kiadó. 147-149 p.

Bisztray Gy. D. **Kissné Bába E**. Zarka V. (2005) Tök, In: Jámborné Benczúr E., Dobránszki J. (szerk.): Kertészeti növények mikroszaporítása. Mezőgazda Kiadó. 149-153 p.

Kissimon J., **Kissné Bába E**. (2005) Fotoszintetikus sajátosságok változtatása a mikroszaporítás során, In: Jámborné Benczúr E., Dobránszki J. (szerk.): Kertészeti növények mikroszaporítása. Mezőgazda Kiadó. 87-95 p.

Conference proceedings, abstracts

International conference (full paper)

Bába E., Zarka V., Pedryc A., Velich I., Bisztray Gy. D. (2003) The Use of RAPD markers for distinction of Hungarian melon and watermelon cultivars. 4th International Conference of PhD students in Miskolc Agriculture section 175-180 p.

Kiss-Bába E., Pánczél S., Zarka V., Bisztray Gy. D., Velich I. (2004) Regeneration ability of some Hungarian melon varieties, In: A. Lebeda and H.S. Paris (Eds.) Progress in Cucurbit Genetics and Breeding Research. Proceedings of Cucurbitaceae 2004 8th EUCARPIA Meeting on Cucurbit Genetics and Breeding. (July 12-17, Olomouc, Czech Republic) 437-439 p

Deák T., Seregély Zs., Kaffka K.J., **Bába E.,** Zarka V., Bisztray Gy.D. (2004) Distinction of melon genotypes using NIR spectroscopy. In (Daevis and Garrido-Varo ed.): Near Infrared Spectroscopy: Proceedings of the 11th International Conference. NIR Publications, Charlton Mill, UK. 385-388. p

Kiss-Bába E., K. Simonyi, V. Zarka, I. Tóbiás., I. Velich, Gy. D. Bisztray (2006) Experiments on transformation and *in vitro* regeneration of squash varieties. 5th In Vitro Culture and Horticultural Breeding Symposium, (12-17. September 2004., Debrecen, Hungary) ISHS Acta Horticulturae 725 Vol. 2. 791-794 p.

Hungarian Conference (abstract)

Bába E., Velich I., Bisztray Gy. (2000) A sárgadinnye *in vitro* regenerációja sziklevélből. Lippay János & Vas Károly Tudományos Ülésszak, Élettani és Genetikai Kutatások Kertészeti alkalmazása Szekció, (2000. november 6-7, Budapest). Összefoglalók 136-137 p.

Bisztray Gy., Bársony Cs., **Bába E**., Borókai R., Zarka V., Szabados A., Velich I. (2000) A görög és sárgadinnye *in vitro* regenerációja. VI. Növénynemesítési Tudományos napok (2000. március 8-9. Budapest) Összefoglalók 33 p

Bába E., Bisztray Gy., Velich I. (2001) A sárgadinnye *in vitro* regenerációja sziklevélből. VII. Növénynemesítési Tudományos napok (2001. január 23-24. Budapest) Összefoglalók 70 p.

Bába E., Zarka V., Bisztray Gy. D., Velich I. Tóbiás I. (2003) Transzformációs kísérletek a vírusrezisztencia kialakítására sárgadinnyénél. IX. Növénynemesítési Tudományos napok (2003. március 5-6. Budapest) Összefoglalók 75 p.

Kissné Bába E. Zarka V., Bisztray Gy. D., Velich I., Tóbiás I. Transzformációs kísérletek sárgadinnyén. (2003) „Lippay János - Ormos Imre - Vas Károly" Tudományos Ülésszak, Biotechnológiai, alkalmazott genetikai és növényélettani Szekció, (2003. november 6-7. Budapest). Összefoglalók: 100-101 p.

Simonyi K, **Kissné Bába E**., Bisztray Gy., Velich I. (2004) Kabakosok *in vitro* regenerációja sziklevélből. X. Növénynemesítési Tudományos Napok (2004. február 18-19. Budapest) Összefoglalók 148 p.

Kissné Bába E., Simonyi K. Pánczél S., Tóbiás I., Velich I., Bisztray Gy. D. (2005) Transzformációs kísérletek kabakosoknál. XI. Növénynemesítési Tudományos Napok (2005. március 3-4. Budapest) Összefoglalók 101 p.

Bisztray Gy. D. **Kissné Bába E**., Sz. Nagy L., Bodor P., Simonyi K. Deák T., Pánczél S., Bacsó R. Zarka V., Oláh R. Tóbiás I., Balog I., Velich I. (2005) Molekuláris növénynemesítési módszerek alkalmazása kabakosoknál és szőlőnél. XI. Növénynemesítési Tudományos Napok (2005. március 3-4. Budapest) Összefoglalók 19 p.

Kissné Bába E., Gyurcsáné Millei Á., Pánczél S., Velich I., Bisztray Gy. D. (2006) A regeneráció hatékonysága embriogenezis és organogenezis útján sárgadinnyénél XII. Növénynemesítési Tudományos Napok (2006. március 7-8. Budapest) Összefoglalók 136 p.

Pánczél S., **Kissné Bába E**, Bisztray Gy. D. (2007) Magyar sárgadinnye fajták *in vitro* regenerációja szomatikus embriógenezis útján. XIII. Növénynemesítési Tudományos Napok (2007. március 12. Budapest) Összefoglalók 115 p.

Pánczél S., **Kissné Bába E**., Gell Gy., Balázs E. (2008) A hipokotil *in vitro* regenerációs képességének vizsgálata sárgadinnye és olajtök csíranövényeken. XIV. Növénynemesítési Tudományos Napok (2008. március, Budapest) Összefoglalók 77 p.