SZENT ISTVÁN UNIVERSITY

THE ROLE OF CYTOKININS IN *IN VITRO* SHOOT REGENERATION IN APPLE

PHD THESIS

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ABBREVIATIONS

BA- 6-benzylaminopurine

BAR – 6-benzylaminopurine riboside

IAA – indole-3-acetic acid

IBA – indole-3-butyric acid

KIN – kinetin

KINR – kinetin riboside

MS Murashige and Skoog medium (1962)

NAA – α-naphtaleneaacetic acid

TDZ- thidiazuron

TOP – metatopolin

TOPR – metatopolin riboside

ZEA – zeatin

ZEAR – zeatin riboside

1. ANTECENDENTS AND AIMS OF THE WORK

Introduction

The apple (*Malus x domestica* Borkh.) has been growing for millennia and is one of the most important fruit crops in Hungary beside the grape. The primary aim of apple breeding is to develop new varieties with proper quality and good yield, suiting to integrated and environmentally sound cultural technology. Breeding of apples by traditional methods requires many years because of their long juvenile period, a high level of self-incompatibility, and the concomitant highly heterozygous nature of the genome. The use of biotechnological methods offers a way to bypass the disadvantages of sexual hybridization. Therefore *in vitro* regeneration of adventitious shoots is necessary for breeding plants via nonsexual methods (e.g. somaclonal variants, *Agrobacterium*-mediated transformation, *in vitro* mutageneseis). Several factors can influence the success of *in vitro* shoot regeneration (genotype, explant, environmental conditions).

Among phytohormones (or plant growth regulators; PGRs), cytokinins have proven to be the most important factors affecting shoot regeneration before and during regeneration process.

Problems and the aims of experiments

Regeneration of adventitious shoots is still difficult in many species and cultivars. The optimum conditions for shoot regeneration vary according to the genotype thus methods should be fitted for each genotype. Regeneration efficiencies for apples (up to 50% regeneration rate for several genotypes) reported till now could be further improved with new methods. Moreover, there are some cultivars without published regeneration methods.

Shoot regeneration experiments presented in this study were performed in the Plant Biotechnology Laboratory of Research Institute of Nyíregyháza (belongs to the Debrecen University CAAES RISF). Our aim study was to develop efficient adventitious shoot regeneration methods from leaf segments for some apple cultivars (’M26’ rootstock, ’Royal Gala’, Freedom’ and ’Húsvéti rozmaring’ scions). Several cytokinins were tested before and during regeneration process including cytokinins, which have not been tested yet in apple regeneration. Because of their chemical structure (hydroxilated or conjugated bezyladenine analogs) it was supposed that they have not so harmful side effects (hyperhydricity, dwarfing), which often occur when bezyladenine or TDZ are used. Significant effects of cytokinins may be related to the histological changes in induced tissues, but there is little information about them and they only referred to the effect of TDZ or BA. Relationships between cytokinins treatments and changes in induced tissues and the regeneration ability of explants can be revealed during examination several cytokinins in extended experiments.

Our aims in short as follows:

1. Studying the effects of cytokinins and their concentrations in the pre-treating media in order to develop efficient regeneration methods for ’M26’rootstock, ’Royal Gala’, ’Freedom’ and ’Húsvéti rozmaring’ scions.

2. Studying the effects of cytokinins and their concentrations in the regeneration media in order to develop efficient regeneration methods for above mentioned cultivars.

3. Studying the effects of cytokinins and their concentrations applied in the pre-treating media on histological changes of induced tissues and revealing relations between histological changes and regeneration ability of ‘Royal Gala’ leaf explants.

4. Obtaining intact plants from regenerated shoots of ’Royal Gala’.

2. MATERIAL AND METHODS

For regeneration experiments *in vitro* shoot cultures of ’Royal Gala’, ’Freedom’, ’Húsvéti rozmaring’ and ’M26’ were used as stock.

2.1. Pre-treatment experiments

Effect of pre-treatments was observed on media containing TOP, BAR, BA and KIN. They were added in concentrations 0.5; 1.0; 1.5 and 2.0 mg l^{-1} to the basal medium (MS, 3% sucrose, 0.7% agar-agar, 0.2 mg/l GA_3, 0.3 mg/l IBA). Moreover, effect of dual cytokinins was also tested (0.5 mg l^{-1} BA supplemented with KIN or TOP in the above mentioned concentrations). Cytokinin-free medium was used as control. Shoots were pre-treated for 3 weeks, after then two, fully expanded leaves were used for regeneration, explants were placed onto regeneration media with their abaxial side up (6 explants/petri dish). The regeneration medium contained MS salts, B5 vitamins, 100 mg l^{-1} myo-inozitol, 0.2 NAA, 0.25% Gelrite and 5.0 mg l^{-1} BA. Explants were incubated at 24.5 ºC for 3 weeks in darkness then they were exposed to 16 h photoperiod and light intensity was increased weekly (35, 70 és 105 μmols^{-1} m^{-2}).

2.2. Histological studies

Histological studies were performed on two, newly developed leaves of ’Royal Gala’ after each pre-treatment described in 2.1. section. Leaf samples were fixed in 5% glutaraldehyde for 2 hours followed by 1.5-hour treatment with osmium tetroxide and dehydration in a graded acetone series. After dehydration the samples were embedded in Spurr resin and stained with toulidin blue. Cross sections were made from leaves using an ultramicrotome (Reichert) in BCE Budai Campus, Centre Laboratory.

2.3. Regeneration experiments

After selection of the best pre-treatments the regeneration ability of each genotype were tested on regeneration media containing TDZ, BA, BAR, TOP, TOPR, ZEA, ZEAR, KIN and KINR in concentrations 0.5; 2.0; 3.5; 5.0 and 6.5 mg l^{-1}. Further concentration (8.0 mg l^{-1}) was also tested in the cases of ribosides. Medium with 5.0 mg l^{-1} BA was used as control.

2.4. Rooting experiments

These experiments were conducted with 'Royal Gala' shoots regenerated on media with 0.5 mg l^{-1} TDZ; 5.0 mg l^{-1} BAR; 5.0 mg l^{-1} BA and 6.5 mg l^{-1} TOPR. Rooting was attempted directly after regeneration shoots were transferred onto root induction medium (1/2 MS salts, 100 mg l^{-1} myo-inozitol, 0.5 mg l^{-1} B_1 vitamine, 2% sucrose, 0.7% agar-agar, 2.0 mg l^{-1} IBA) for 5 days, then shoots were passed onto root elongation medium (1/2 MS salts, 50 mg l^{-1} myo-inozitol, 3% saccharose, 2.0 ml l^{-1} Wuxal®, 0.7% agar-agar). After 2 two weeks rooted shoots were acclimatized by Bolar *et al*. (1998).

In further experiments the regenerated shoots were subcultured on media as follows: **A**: hormone-free, **B**: 0.5 mg l^{-1} BAR+0.3 mg l^{-1} IBA+ 0.2 mg l^{-1} GA_3; **C**: 0.5 mg l^{-1} BAR+0.3 mg l^{-1} IBA+ 0.5 mg l^{-1} GA_3) before rooting. Regenerated shoots were also subcultured on proliferation medium (1.0 mg l^{-1} BAR+0.3 mg l^{-1} IBA+ 0.2 mg $l^{-1}$$GA_3$) for 4 weeks then newly developed shoots were rooted.

2.5. Parameters observed and statistical methods

During regeneration experiments the percentage of regenerated shoots (regeneration %) and that of hyperhydrated cultures (hyperhydricity %), and the number of regenerated shoots/explants were observed. Organogen indices were calculated as follows:

OI= (Regenertion %- hyperhydricity %) x shoot number/100

This index was based on calculation of Famiani *et al.* (1994) but the length of shoots was excluded and the rate of hyperhydricity was included because of its importance.

In the rooting experiments the rate of rooted shoots, the number and the length of roots (mm) and surviving rates were recorded.

Data of regeneration experiments were analyzed by ANOVA followed by Tukey-test using SPSS 9.0 for Windows to detect differences between concentrations. For comparing combinations to the control treatment least significant difference (LSD) method was used.

3. RESULTS

3.1. Role of cytokinins applied in pre-treatments in shoot regeneration

The number of shoots per explant could significantly be increased by pre-treatment on media containing cytokinins in the cases of ’Freedom’, ’Royal Gala’ and ’Húsvéti rozmaring’ scions compared to the control (cytokinin-free treatments) (Table 1).

Table 1 The best result achieved by pre-treatments considering the number of shoots per explant

Cultivar	Regenerated shoots/explant	Pre-treatment (mg l^{-1})	Control mean
’M26’	3.8	BAR (0.5)	3.4
’Freedom’	2.5*	BA+TOP (0.5+1.5)	1.9
’Royal Gala’	8.9*	TOP (0.5)	5.4
’Húsvéti rozmaring’	3.3*	KIN (1.5)	2.3

*significantly differ from control

Considering the regeneration rate the best results were also achieved by pre-treatments on media containing cytokinins, although differences were only significant in the cases of ’Freedom’ and ’Húsvéti rozmaring’ (Table 2).

Table 2 The best result achieved by pre-treatments considering the regeneration rate

Cultivar	Regeneration rate (%)	Pre-treatment (mg l^{-1})	Control mean (%)
’M26’	96.6	TOP (1.0)	91.9
’Freedom’	84.7*	BA+TOP (0.5+1.0)	57.5
’Royal Gala’	100	TOP (0.5-1.5) BA (1.5) BAR (0.5) KIN (0.5; 1.5-2.0)	95.4
’Húsvéti rozmaring’	94.9*	KIN (1.5)	65.5

* significantly differ from control

The sensitivity of cultivars to hyperhydricity was different. The rate of hyperhydricity was influenced by pre-treatments in the case of’M26’: presence of TOP, BA, BAR and BA+TOP significantly decreased the rate of hyperhydrated cultures (Figure 1).

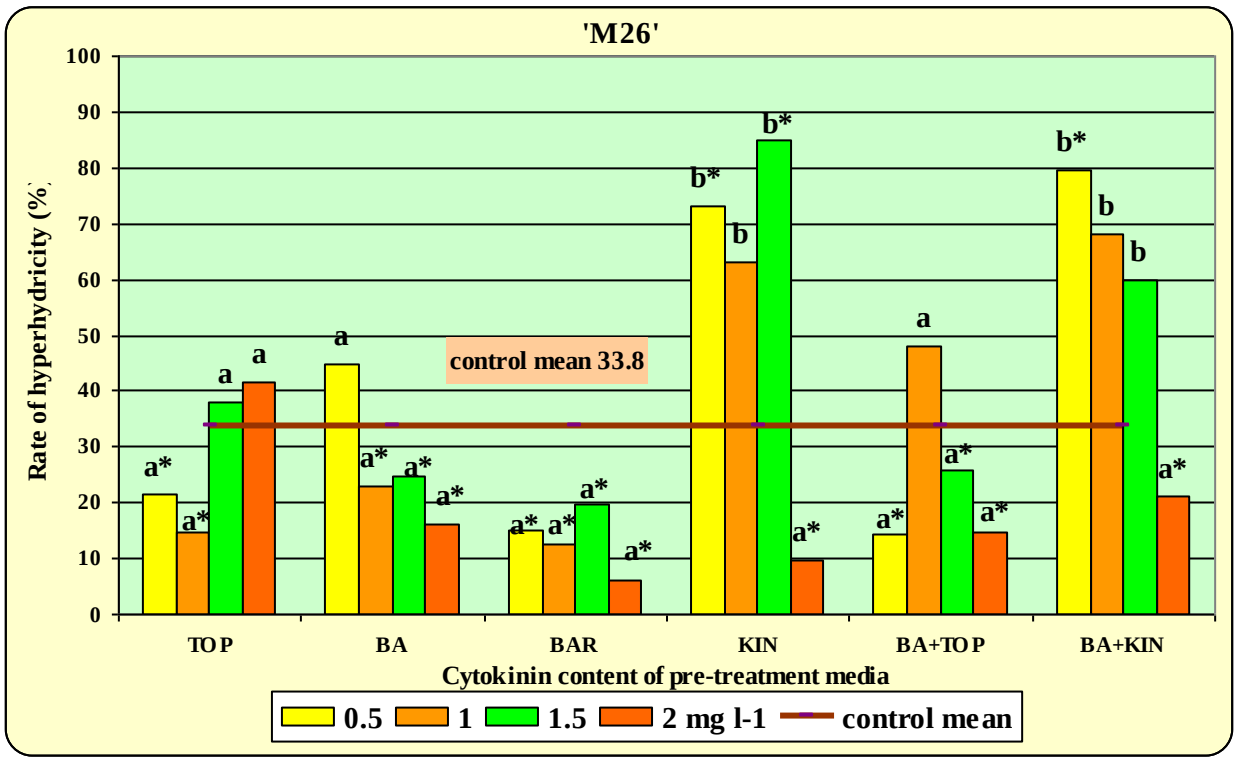


Figure 1 The rate of hyperhydricity aftre pre-treatments in ’M26’ rootstock. The small letters mean the homogenous groups within a citokinin according to Tukey’test, while * means separation from control by LSD ($p<0,05$)

Very low hyperhydricity rates were found in the case of ’Freedom’, it was less than 1% in control treatment. The rate of hyperhydricity was significantly decreased by pre-treatment on medium with 0.5 mg l^{-1} BA+1.5-2.0 mg l^{-1} TOP in the case of ’Royal Gala’ (from 32.9% in control to 2%).

Considering the calculated organogen index the best pre-treatments were selected as follows (Table 3):

Table 3 The best OI values obtained by pre-treatments

Cultivars	Organogen index (OI)	Pre-treatment (mg l^{-1})	Control (OI)
’M26’	2.3	TOP (1.0)*	1.4
		BAR (0.5)	
’Freedom’	1.9	BA+TOP (0.5+1.5)	1.0
’Royal Gala’	7.7	TOP (1.0)	3.4
’Húsvéti	2.0	KIN (1.5)*	1.3
rozmaring’	2.1	KIN (1.0)	
	2.2	TOP (0.5)	

*selected for further experiments as the best pre-treatments

When we had to select from more than one OI values, other parameters were also considered such as regeneration rate (‘M26’) or beside the regeneration rate the shoot number per explant (’Húsvéti rozmaring’).

3.2. Histological changes induced by cytokinins applied in pre-treatments

Cytokinins and their concentrations altered significantly the histological structure of newly developed leaves of ‘Royal Gala’. Anatomy of leaves developed on citokinin-free medium was similar to that of *in vivo* leaves. The mesophyll consisted of one cell layer palisade and spongy parenchyma with large intercellular spaces. Well-developed vascular bundle could be observed (Figure 2).

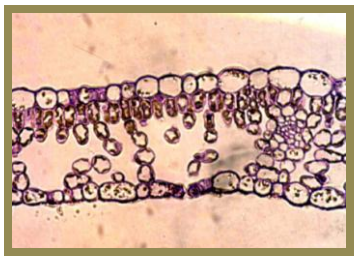


Figure 2 Cross-sections of leaf after pre-treatment on cytokinin-free medium (x400)

Regeneration ability of ‘Royal Gala’ leaves was increased by pre-treatments with TOP and KIN thus histological changes induced by these cytokinins will only be detailed here.

When pre-treating medium contained TOP, the compact structure of mesophyll without intercellular spaces and with immature vascular bundle was observed. In the case of the lowest concentration, two rows of palisade cells developed, however, when the TOP concentration increased the mesophyll became more and more homogenous and the palisade and spongy parenchima layers were hardly distinguishable. In the case of 2.0 mg l^{-1} TOP more differentiated leaf structure could be observed: the palisade and spongy parenchima cells were distinguishable (Figure 3).

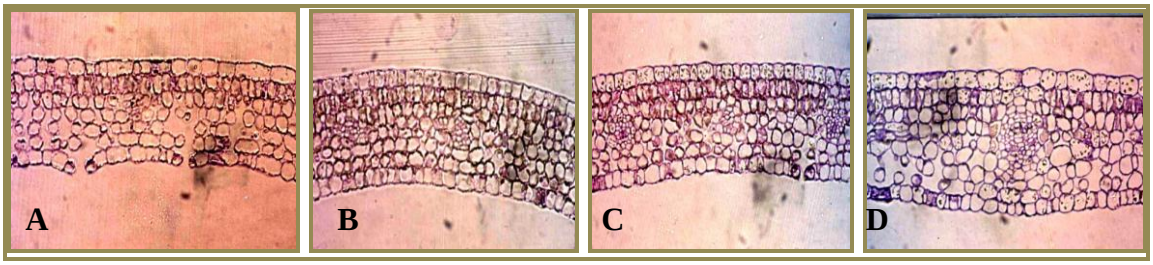
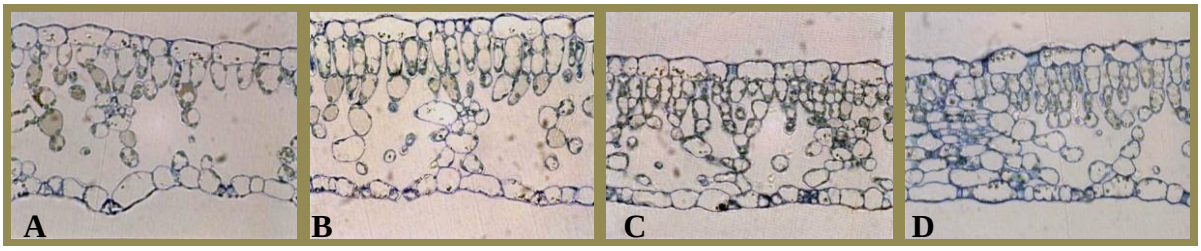


Figure 3 Cross-sections of leaves after pre-treatments with **A:** 0.5 mg l^{-1}, **B**: 1.0 mg l^{-1}, **C**: 1.5 mg l^{-1}, **D**: 2.0 mg l^{-1} TOP (x400)

When pre-treating medium contained KIN, large cells developed in both epiderm and mesophyll and they were vacuolized. At the level of 0.5 mg l^{-1} the mesophyll was greatly disorganized. The palisade parenchima was well-distinguishable, large intercellular spaces could be observed. The spongy parenchima contained few cells, very large intercellular spaces were observed (Figure 4).



4. ábra Cross-sections of leaves after pre-treatments with **A:** 0.5 mg l^{-1}, **B**: 1.0 mg l^{-1}, **C**: 1.5 mg l^{-1}, **D**: 2.0 mg l^{-1} KIN (x400).

3.3. Role of cytokinins applied in regeneration media in shoot regeneration

The number of regenerated shoots per explant was significantly increased compared to control treatment (5.0 5 mg l^{-1} BA) in the cases of ’M26’ and ’Royal Gala’ (Table 4).

Table 4 The best results of regenerated shoot per explant

Cultivar	Regenerated shoots/explant	Treatment (mg l^{-1})	Control mean
’M26’	3.2*	BAR (6.5) TOPR (8.0)	2.0
’Freedom’	1.8	TDZ (5.0)	1.3
’Royal Gala’	11.1*	TDZ (0.5)	4.6
’Húsvéti rozmaring’	3.1	BAR (6.5)	2.7

* significantly differed from control

The best regeneration rate was achieved by control treatment in the case of ’Húsvéti rozmaring’, while they were significantly increased by other treatments in the case of ’M26’ and ’Freedom’. Responses of ‘M26’ to TDZ 0.5 mg l^{-1}, BA 2.0-3.5 mg l^{-1}, BAR above 5.0 mg l^{-1}, TOP 3.5-5.0 mg l^{-1}, and ZEAR of 6.5 mg l^{-1} concentrations were also significantly better than for control.

In the case of ’Royal Gala’ the majority of treatments were less efficient than control excluding TDZ treatments (Table 5).

Table 5 The best results achieved by treatments considering the regeneration rate

Cultivar	Regeneration rate (%)	Treatment (mg l^{-1})	Control mean (%)
’M26’	84.9*	BAR (8.0)	46.4
’Freedom’	59.7*	TDZ (5.0)	11.4
’Royal Gala’	100	TDZ (2.0; 3.5)	94.9
’Húsvéti rozmaring’	81.5	BA (5.0)	81.5

*differed significantly from control

Hyperhydicity could not be seen on media containing KIN and its rates were very low on media with TOP, TOPR, ZEA and KINR in the case of ’M26’. The rate of hyperhydricity was only notable on medium containing 5.0 mg l^{-1} TDZ in ‘Freedom’ (23.1%). The rate of hyperhydricity was the highest (88.2%) on medium with 2.0 mg l^{-1} TDZ in the case of ’Royal Gala’, while other cytokinins decreased the hyperhydricity. TOP, ZEA, KIN and their ribosides decreased significantly the hyperhydricity in ’Húsvéti rozmaring’ (Figure 5).

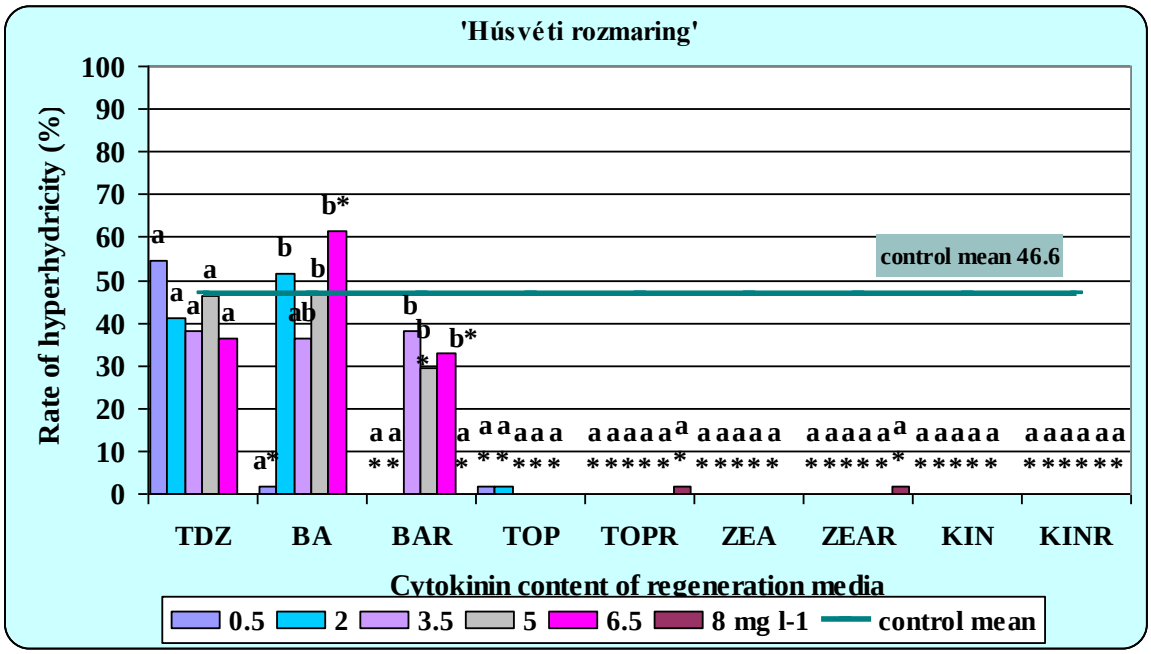


Figure 5 The rate of hyperhydricity of ‘Húsvéti rozmaring’ on regeneration media with different cytokinins. The small letters mean the homogenous groups within a citokinin according to Tukey’test, while * means separation from control by LSD ($p<0,05$)

The highest OI value (1.74) was obtained on medium with 8.0 mg l^{-1} TOPR for ’M26’ rootstock, moreover, applying BAR and TOP in regeneration media also resulted in higher OI than control treatment. Regeneration on medium with 5.0 mg l^{-1} TDZ proved to be the most efficient for ‘Freedom’

scion (OI=0.65). Excluding TDZ treatments each cytokinin resulted in lower OI than control in ‘Royal Gala’. The best OI could be obtained by control treatment in the case of ’Húsvéti rozmaring’. Based on OI we propose the use of treatments for shoot regeneration listed in the Table 6.

Table 6 The best cytokinin content of regeneration media for different cultivars considering organogen indices

Cultivar	Organogen index (OI)	Treatment (mg l^{-1})	Controll mean (OI)
’M26’	1.7	TOPR (8.0)	0.9
’Freedom’	0.7	TDZ (5.0)	0.2
’Royal Gala’	7.0	TDZ (0.5)	3.8
’Húsvéti rozmaring’	1.2	BA (3.5)	1.0

3.4. Rooting and acclimatization of regenerated shoots

When shoots were rooted directly after regeneration only shoots developed on media with BA and BAR rooted (10 and 25%, respectively) but their acclimatization was not successful. Rooting after subculture of shoots on hormone-free medium was also not efficient (max. 4%). Subculture of shoots on media with halved citokinin or elevated GA_3 content resulted in still low rooting ability (max. 36%).

When regenerated shoots were subcultured on proliferation medium for 4 weeks and newly developed shoots were rooted, the rooting capacity was significantly increased. Shoots regenerated on medium with BAR showed the best rooting rate (76%) while rooting ability of shoots developed on medium with TOPR was the lowest (32%). However, after subcultures all rooted shoots were acclimatised and survived with maximum efficiency (100%).

3.5. New and novel scientific results

1) We have foremost studied the effect of BAR, TOP and TOPR applied in pre-treating media on shoot regeneration of apple and proved their effectiveness.
2) We have foremost revealed the relation between the changes in leaf tissues induced by cytokinins applied in pre-treatment media and regeneration ability of apple leaf explants.
3) We have foremost studied the effect of BAR, TOP and TOPR applied in regeneration media on shoot regeneration of apple and proved their effectiveness.
4) The rate of hyperhydricity was significantly decreased by applying BAR and TOP, thus the effectiveness of the regeneration was increased.

5) In our experiments the number of regenerated shoots was significantly increased in the case of ’Royal Gala’ compared to results have so far reported in literature.
6) In our experiments the regeneration rate was significantly increased in the case of ’M26’ compared to results have so far reported in literature.
7) We have worked out efficient regeneration methods for ’Freedom’ and ’Húsvéti rozmaring’.
8) We have worked out a new organogen index based on modification of the index reported by Famiani *et al.* (1994), which can be used for comparing the effectiveness of different regeneration methods.
9) We have foremost studied the post-effect of cytokinins applied in regeneration media on rooting ability of apple regenerants and proved the enhancing effect of BAR.

4. CONCLUSIONS AND PROPOSALS

Responses of ’M26’ apple rootstock and ’Freedom’, ’Royal Gala’, ’Húsvéti rozmaring’ scions to cytokinins (applied in the pre-treating and regeneration media) during shoot regeneration were studied.

The regeneration ability of ’Royal Gala’ was prominent: 100% regeneration rate often was observed, similarly to the rates reported by Sriskandarajah (1990) and Korban *et al.* (1992). However, the number of regenerated shoots per explant achieved in our experiments was much more (8.9) than the best result published so far (4-6) (Yao *et al*., 1995).

High regeneration rate was also obtained in ’M26’ rootstock (96.6%), which surpassed the best results published so far (50-60%) (Famiani *et al*., 1994; Caboni *et al*., 2000).

Regeneration ability of ’Húsvéti rozmaring’ was also good (94.9%), while lower regeneration rate was achieved in ’Freedom’ (84.7%). Regeneration performance of both latter cultivars has not been published yet.

The regeneration capacity of explants was affected by cytokinin content of pre-treatment media and changes could be detected in the regeneration rate, number of shoots, and the rate of hyperhydricity although differences were not always significant.

The regeneration rate of ’Freedom’ and ’Húsvéti rozmaring’ was significantly increased by TOP, BAR, KIN, BA+ KIN applied in the pre-treating media compared to cytokinin-free control.

The number of regenerated shoots was also significantly increased by TOP, BAR, KIN, BA+TOP applied in the pre-treating media in the case of ’Royal Gala’. Gercheva *et al.* (2009) also reported that regeneration rate of ’Chadel’ was also higher after culture on medium with BA than after culture on hormone-free medium.

The rate of hyperhydricity varied between both pre-treatments and genotypes. Sensitivity of ‘Freedom’ was very low, while hyperhydricity often occurred in ‘M26’. However, BAR, BA, TOP and BA+TOP applied in pre-treatment media decreased significantly the rate of hyperhydricity in the latter cultivar (below 22% compared to 33.7% in control). BA+TOP applied in the pre-treatment medium also decreased the hyperhydricity of ’Royal Gala’ (down to 2% compared to 32.9% in control).

Several authors reported the stimulation effect of TOP and its derivates in micropropagation experiments (Bogaert *et al*., 2006; Bairu *et al*., 2007, Bairu *et al.,* 2009; Malá *et al*., 2009; Amoo *et al.,* 2010). However, they have not been yet tested in adventitious shoot regeneration of woody plants, excluding *Citrus* sp. (Niedz and Evens, 2010).

Summarizing these results, it can be concluded that TOP applied in the pre-treating media increased the morphogenic activity of leaf explants in three genotypes from four tested cultivars.

The following pre-treatment media are proposed: 1.0 mg l^{-1} TOP for ’M26’, 0.5 mg l^{-1} BA+1.5 mg l^{-1} TOP for ’Freedom’, 1.0 mg l^{-1} TOP for ’Royal Gala’, 1.5 mg l^{-1} KIN for ’Húsvéti rozmaring’.

Significant differences were found in the anatomical structure in leaf tissues induced by pre-treatment. Anatomical structure of leaf developed on cytrokinin-free medium was similar to the structure of *in vivo* woody plant leaves (Kiss *et al*., 1999) although cell walls are thinner. Pre-treatment of shoots on medium with TOP induced juvenile structure, which characterized the young *in vitro* leaves. Leaves developed on pre-treating medium containing KIN were the thickest and the mesophyll was greatly desorganized. Since the ‘Royal Gala’ showed the best regeneration parameters after pre-treatment on media with TOP and KIN, the earlier presumption i.e. the good regeneration ability is related to juvenile or undifferentiated structure of leaves (Zhu and Welander, 2000; Welander és Maheswaran, 1992) have been confirmed. Further directed experiments should be performed to explain the presumed relationships between the functional, histological, and molecular changes observed prior to and/or during *in vitro* regeneration in apple.

Both the amount and type of cytokinins applied in the regeneration media have played important role in the expression of regeneration capacity of explants.

Responses of genotypes to cytokinins were different, although KIN and ZEA and their ribosides were the least efficient for induction of adventitious shoots in most genotypes. Although the primary role of endogenous ZEA in cell differentiation of *in vitro* apple and pear organogenesis has been proved (D’Angeli *et al*., 2001, Caboni *et al*., 2002), applying alone ZEA exogenously did not enhance shoot regeneration (De Bondt *et al*., 1996, Ling *et al*., 2002).

Applying TDZ as a cytokinin-like substance can be very effective, especially in the case of recalcitrant genotypes but it can have undesirable side effects, such as hyperhydricity or dwarfing.

In this study TDZ has been proven to be highly efficient to increase the shoot number in ’M26’ and ’Royal Gala’. Regeneration rate was also increased by TDZ in ‘M26’ and ‘Freedom’.

However, the rate of hyperhydricity was also the highest on media with TDZ in ‘M26’ (20-60%), ’Royal Gala’ (35-88%) and ’Freedom’ (23%), while these rates on control medium were the following: 1.6; 13.1 and 0%, respectively. 100% hyperhydricity was observed on TDZ containing media by others (Caboni *et al*., 1996; Höhnle és Weber, 2010).

Korban *et al*. (1992) found 1.1-4.4 mg l^{-1} TDZ concentration range to be optimal for ‘Royal Gala’. In our study all concentrations of TDZ resulted also in high regeneration rates and shoot number but the rate of hyperhydricity was very high when more than 0.5 mg l^{-1} TDZ was applied. That is why we propose addition of 0.5 mg l^{-1} TDZ to the regeneration medium for this cultivar similarly to application proposed by Sriskandarajah *et al*. (1990) (0.66 mg l^{-1} TDZ).

In general 5.0 mg l^{-1} BA was found to be the most efficient for ’M26’ rootstock (Famiani *et al*., 1994, Ferradini *et al*., 1996) although 1.0 mg l^{-1} BA has also been proven to be usable (Predieri és Fasolo, 1989). In our experiments BA was also more efficient than TDZ considering the OI values, but the best results were achieved applying cytokinins, which have not been tested in apple regeneration so far. BAR added to regeneration medium resulted in the best regeneration rate (84.9%), while the most efficient regeneration medium containing 8.0 mg l^{-1} TOPR (the best OI value).

The most responsive cytokinin was the BA for ’Húsvéti rozmaring’ (82%), although the rate of hyperhydricity was also the highest (61.5%). The most shoots per explant (3.2) developed on media supplemented with BAR.

Addition of TOP, TOPR and ZEA to the regeneration medium decreased significantly the rate of hyperhydricity in ’Royal Gala’, while TOP, KIN, ZEA and their ribosides also decreased significantly the hyperhydricity in ’Húsvéti rozmaring’.

The following citokinin contents are proposed in the regeneration media: 8.0 mg l^{-1} TOPR for ’M26’, 5.0 mg l^{-1} TDZ for ’Freedom’, 0.5 mg l^{-1} TDZ for ’Royal Gala’and 3.5 mg l^{-1} BA for ’Húsvéti rozmaring’.

Although micropropagated ’Royal Gala’ shoots have very good rooting ability (100% rooting rate), regenerants rooted directly after regeneration showed poor rooting ability and acclimatization of rooted shoots was not successful. The rooting ability of regenerants was also lower than that of parent-line in James and Dandekar’s experiments (1991). The best results (76%) were achieved when shoots regenerated on media supplemented with BAR were subcultured on proliferation media for 4 weeks and newly developed shoots were rooted.

The utilization of results

Utilization of such results in practice can greatly help the breeding work of apple, since effective shoot regeneration will enhance the chances of recovering shoots formed from transformed cells, somaclones and *in vitro* induced mutations. Efficient regeneration methods can also be utilized in micropropagation of apple since the propagation rate can significantly be increased by shoot regeneration on leaf explants.

Scientific utilization of results includes the development of regeneration system for 'Húsvéti rozmaring' and 'Freedom' and utilization of new results obtained with TOP, TOPR and BAR in regeneration experiments conducted with other species.

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