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Effects of aromatic cytokinins on structural characteristics of leaves and their post- effects on subsequent shoot regeneration from in vitro apple leaves of 'Royal Gala'

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Summary: The effects of different aromatic cytokinins applied in different concentrations and combinations were investigated on the histology of *in vitro* apple leaves and their post-effects on subsequent shoot regeneration from these leaves were studied. Great differences in the anatomical structure of leaves could be detected originating from media containing different types and concentrations of aromatic cytokinins. The number of regenerated shoots per explant and the organogenetic index were used for the evaluation of the post-effect of aromatic cytokinins on shoot regeneration. The histological structure of leaves used for regeneration and their regeneration response showed a good correlation. When the pre-treatment caused a juvenile-like or less-differentiated structure, the number of regenerated shoots per explant increased and often vitrification also decreased and consequently the organogenetic index also increased. A strong interaction between cytokinin-content (type and concentration) of the pre-treatment medium and that of the regeneration medium could also be detected.

Key words: apple, cytokinin, leaf histology, pre-treatment, shoot regeneration

Introduction

Several authors have described anatomical changes of leaves grown *in vitro* compared to the structure of leaves grown in glasshouse or under field conditions. Presently, there is limited knowledge about the structural and functional changes in *in vitro* plantlets or organs caused by different *in vitro* conditions (temperature, light, etc.) or different components of media, such as the different cytokinins. Anatomical changes could be observed in different plant organs, but one of the most important organs is the leaf because the structural and/or functional changes may significantly affect the success of acclimatization or may modify the organogenetic potential of the leaf tissue and consequently the efficacy of regeneration. (*Schmidt & Waldemaier,* 1992; *Kiss* et al., 1994; *Jámbor-Benczúr* et al., 2001; *Kiss* et al., 1997). *In vitro* pre-treatments of source shoots with TDZ enhanced the organogenetic potential of leaves of *Rubus* hybrids (*Swartz* et al., 1990). In *Malus,* both TDZ and BA applied in pre-treatment media were effective for stimulating shoot organogenesis from leaves (*Fasolo* et al., 1989).

Cytokinins are N^6-substituted adenines with growth regulatory activity in plants and they can be grouped into two main classes: isoprenoid and aromatic cytokinins, which differ from each other in their biochemistry, receptors, biological activity and metabolism (*Strnad* et al., 1997).

Considering natural cytokinins, benzyladenine (6-benzylaminopurine, BA), or sometimes kinetin (6-furfurylaminopurine, KIN) are used in apple tissue culture systems. BA is a generally used cytokinin in apple micropropagation or regeneration systems but it might have several side effects, such as difficulties in rooting, toxicity, hyperhidricity, short shoots, callus formation, etc. These harmful effects may be due to the N^7- and N^9-glucolisation or alanin conjugation resulting in biologically inactive but chemically very stable derivatives and their slow release from their derivatives (*Jones* et al., 1977; *Lane* 1978; *Werner & Boe,* 1980; *Sriskandarajah & Mullins* 1981; *Webster & Jones,* 1991; *Werbrouck* et al., 1995). The use of hydroxylated benzyladenine analogues, such as meta-topolin (N^6-meta-hydroxy-benzyladenin, TOP) could be an alternative way to avoid the side effects of benzyladenine. The hydroxylated BA analogues have a different metabolism from that of BA (*Strnad* et al., 1997).

The aim of this work was to study the effects of different aromatic cytokinins or their combinations on the histology of *in vitro* apple leaves and their post-effects on subsequent shoot regeneration from these leaves.

Material and method

Plant material

In vitro shoot cultures of apple (*Malus* x *domestica* Borkh.) cultivar Royal Gala (Tenroy) were used in the experiments. Shoot explants were placed horizontally on shoot proliferation medium consisting of MS (*Murashige & Skoog*, 1962) basal medium supplemented with 100 mg l^{-1} myo-inositol, 0.7 % agar-agar, 3 % sucrose, 1.0 mg l^{-1} 6-benzyladenine riboside (BAR), 0.3 mg l^{-1} indole-3-butyric acid (IBA) and 0.2 mg l^{-1} gibberellic acid (GA_3). The pH of the medium was adjusted to 5.8 before autoclaving and it was autoclaved for 20 min at 121 °C and 10^5 Pa in a ST-124/2 autoclave. The cultures were grown at 22 °C with 16 h photoperiod provided by warm-white lamps (Tungsram F74) at PPF of 105 µmol s^{-1} m^{-2}. Shoots were subcultured at 4-week intervals for the regeneration experiments.

Pre-treatments of shoots with different cytokinins

The effects of a 3-week-long culture period of shoots on media with different types and concentrations of aromatic cytokinins were studied on the histology of newly-developed, fully expanded leaves and on the regeneration potential of these leaves. For the experiments, 3-week-old shoots having 5–7 leaves with a length of about 35–40 mm were placed on the different media consisting of MS basal medium supplemented with components used in shoot proliferation medium with only the cytokinin supply being different. The media contained different types and concentrations of cytokinins. 6-benzylaminopurine (BA), 6-furfurylaminopurine (KIN) and N^6-meta-hydroxy-benzyladenin (TOP) were applied in 0.5, 1.0, 1.5 and 2.0 mg l^{-1} (≈from 2 to 9 µM) concentrations, respectively and TOP or KIN was applied in the above mentioned four concentrations together with 0.5 mg l^{-1} BA (BA+TOP and BA+KIN treatments). Treatments were carried out in Kilner-jars (400 ml volume, 75 mm inside diameter and 85 mm length) and five shoots were placed vertically on 40 ml of the medium in each jar. Cultures were grown under the same environmental conditions as used during shoot multiplication.

After a 3-week-long culture period (pre-treatment) the upper two, fully expanded young leaves were collected for histological studies. Leaf samples for light microscopy were fixed in 5 % glutaraldehyde for 2 hours followed by a 1.5-hour-treatment with osmium tetroxide and dehydrated in a graded acetone series. After dehydration, samples were embedded in a Spurr resin and stained with toluidin blue. The cross-sections from leaves were made by ultramicrotome (Reichert).

Regeneration from leaves

After a three-week-long culture period on media with different types and concentrations of aromatic cytokinins, the upper two, fully-expanded young leaves were used for regeneration. Petiole and tip of leaves were removed and leaves were cut transversely into two stripes (of about 5 mm width). All cutting were made in a solution of citric acid (0.15 g l^{-1}) and ascorbic acid (0.1 g l^{-1}). Leaf explants were placed then with the adaxial side onto two regeneration media (36 explants per combination of pre-treatment and regeneration). Regeneration media consisting of MS salts, B_5 vitamins supplemented with 100 mg l^{-1} myo-inositol, 0.25 % gelrite, 3 % sucrose, 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} (2.3 µM) TDZ (R1) or 5.0 mg l^{-1} (22.2 µM) BA (R2), respectively. Explants were incubated in the dark at 24.5 °C for 3 weeks, then in the light at 22 °C with a 16-hour photoperiod for another 4 weeks. The light intensity was increased weekly: it was 35 µmol s^{-1} m^{-2} during the first week, 70 µmol s^{-1} m^{-2} during the second week and 105 µmol s^{-1} m^{-2} from the third week on. After seven weeks, the regeneration percent (=number of explants with regenerated shoot, R%), the number of regenerated shoots per explant (SN), and the vitrification percent (=the number of explants with vitrified shoots, V%) were recorded. The organogenetic index (OI) was calculated from these data (OI=[R% – V%] x SN / 100) as described earlier (*Dobránszki* et al., 2002). The data were analysed statistically by one- and two-way-ANOVA followed by Tukey's test by using the *SPSS 9.0 for Windows* software. The values of OI and SN were used for the evaluation of the post-effect of aromatic cytokinins on the shoot regeneration from the treated leaves.

Results and Discussion

Effects of different cytokinins on leaf histology

The main effects of cytokinin-treatments on the anatomical structure of the first leaf are presented in Figures 1–6. The structure of the second leaf was similar to that of the first one after each treatment because they also developed during the 3-week-long cytokinin-treatment.

Cross section of leaves harvested from the cytokinin-free medium showed a well-differentiated structure with a single cell layer of epidermis and high number of closed stoma on

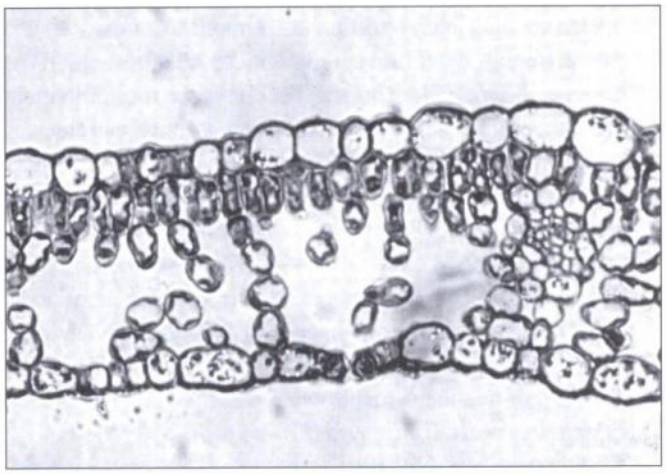

Figure 1. Light micrographs of the 1st leaf after culturing shoots on cytokinin-free medium. X 400.

the abaxial epidermis. The spongy parenchyma contained very large intercellular spaces similar to the structure of *in vivo* leaves described in other woody plants (*Kiss* et al., 1999), but the cell walls were thinner. The vascular bundle was also well developed *(Figure 1)*.

Leaves from the BA-containing media showed a structure like mature *in vitro* leaves (*Kiss* et al., 1999; *Jámbor-Benczúr* et al., 2001). The adaxial and abaxial epidermis consisted of one cell layer and differed from each other and the mostly opened stomata often rose above the surface of the abaxial epidermis. One row of elongated palisade cells could be observed. Intercellular spaces of spongy parenchyma were much smaller than in the cytokinin-free treatment *(Figure 2A, 2B)*. With the increase of BA-concentration, the parenchyma cells became increasingly roundish with less differentiated structure *(Figure 2C, 2D)*.

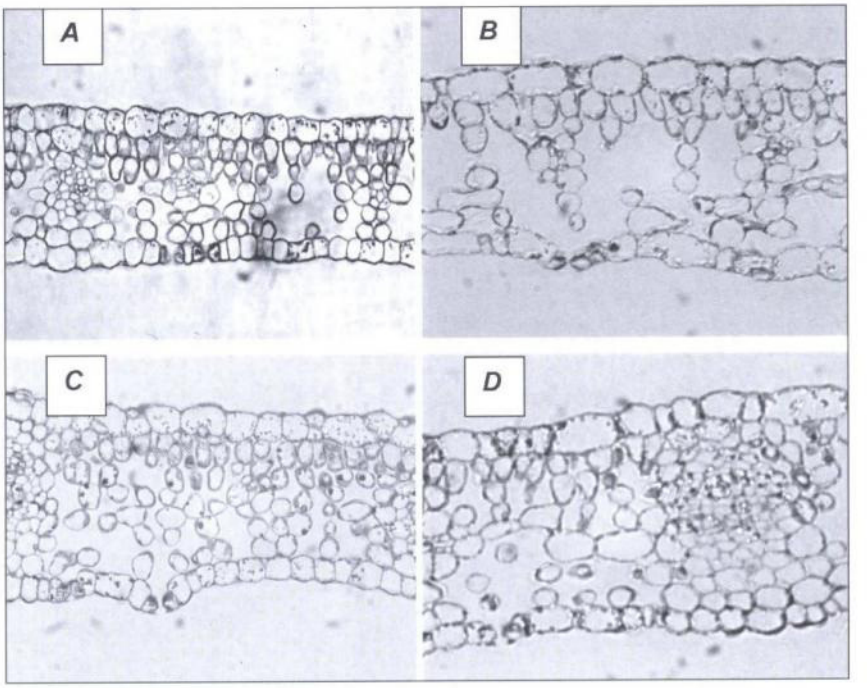

Figure 2. Cross section of the 1st leaf after culturing on media containing BA (6-benzilamino-purin) in **(A)** 0.5 mg l^{-1} (2.2 µM), **(B)** 1.0 mg l^{-1} (4.4 µM), **(C)** 1.5 mg l^{-1} (6.7 µM) and **(D)** 2.0 mg l^{-1} (8.9 µM) concentrations, respectively. X 400.

Transverse section of the first and second leaves from shoots treated with TOP in a concentration range of 0.5 to 1.5 mg l^{-1} showed a structure similar to immature *in vitro* leaves with very few immature stomata on the abaxial epidermis. The structure of palisade and spongy parenchyma is compact. Parenchyma cells have a dense cytoplasm and immature vascular bundles. The mesophyll was homogenous; the palisade and spongy mesophyll layers were hardly distinguishable *(Figure 3A-C)*. At 2.0 mg l^{-1} TOP, the palisade and spongy parenchyma cells were distinguishable and two rows of palisade cells were detected *(Figure 3D)*.

When BA and TOP were applied together in the medium *(see in Figure 5)*, we could observe, that at 0.5 mg l^{-1} BA + 0.5 mg l^{-1} TOP the structure of leaves was similar to that of the mature *in vitro* leaves *(Figure 5A)*. However, with the increase of TOP concentration, the anatomical structure of leaves became increasingly juvenile similarly to the effect of TOP applied alone, which was the most expressed at 0.5 mg l^{-1} BA +1.5 mg l^{-1} TOP treatment *(Figure 5C)*.

Application of KIN resulted in an unorganised, spongy mesophyll with large intercellular spaces and with increased cell area. Cells were roundish with large vacuoles. At 0.5 and 1.0 mg l^{-1} KIN, a single row of palisade parenchyma with spaces between cells could be observed *(Figure 4A, 4B)*. However, at 1.5 and 2.0 mg l^{-1} KIN, two rows of palisade cells could be detected *(Figure 4C, 4D)*.

When BA and KIN were applied together, the cell area decreased compared to the effects caused by KIN. The cells of epidermis and parenchyma are roundish with large vacuoles and little grade of organisation could be detected in

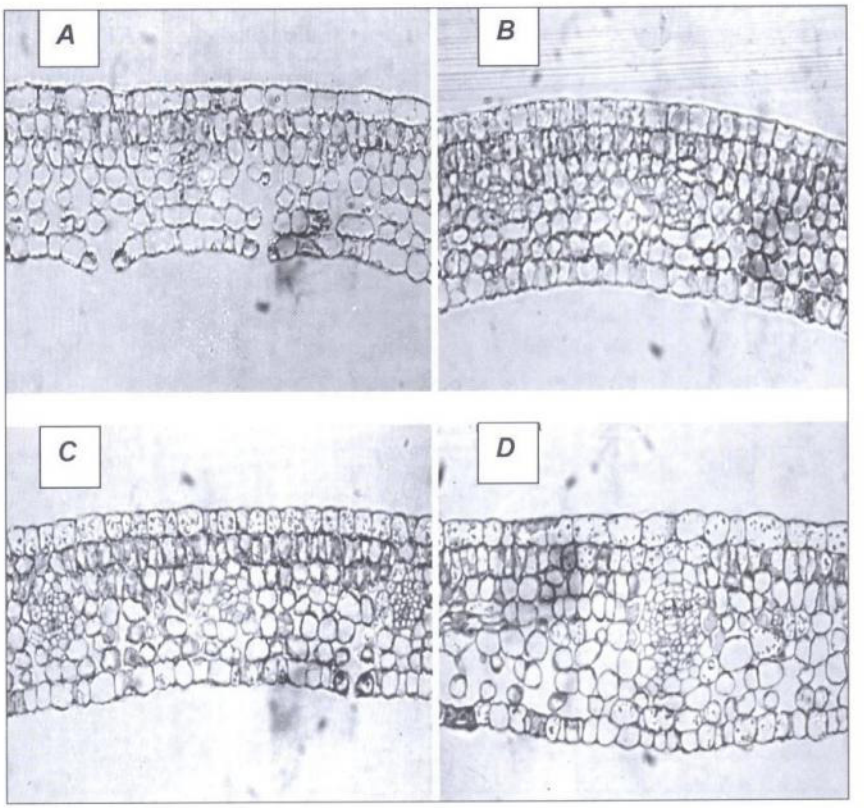

Figure 3. Cross section of the 1st leaf after culturing on media containing TOP (6-(3-hidroxi-benzilamino)-purin) in **(A)** 0.5 mg l^{-1} (2.1 iM), **(B)** 1.0 mg l^{-1} (4.1 iM), **(C)** 1.5 mg l^{-1} (6.2 µM) and **(D)** 2.0 mg l^{-1} (8.3 µM) concentrations, respectively. X 400.

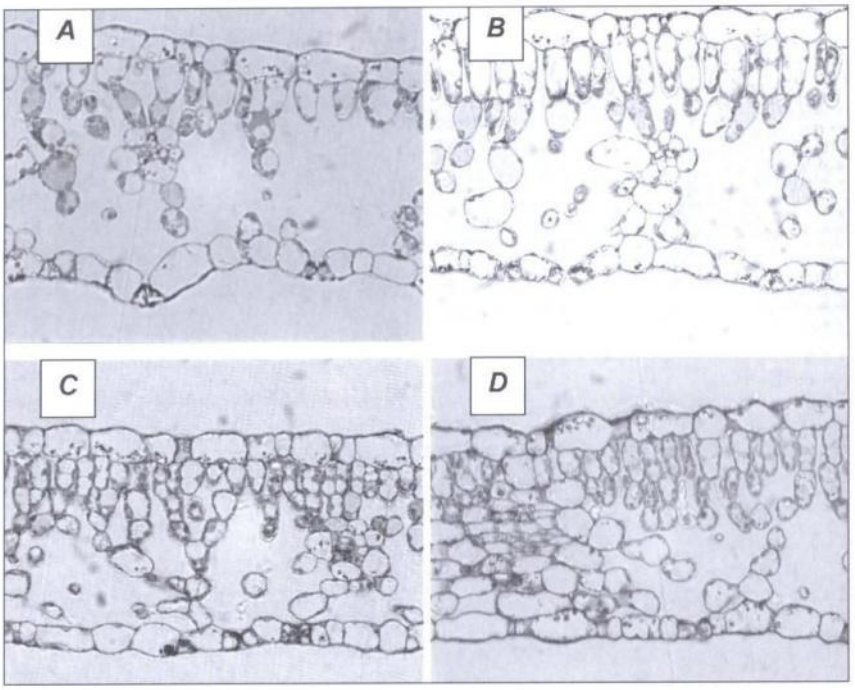

Figure 4. Cross section of the 1st leaf after culturing on media containing KIN (6-furfurilamino-purin) in **(A)** 0.5 mg l^{-1} (2.3 µM), **(B)** 1.0 mg l^{-1} (4.6 µM), **(C)** 1.5 mg l^{-1} (6.9 µM) and **(D)** 2.0 mg l^{-1} (9.3 µM) concentrations, respectively. X 400.

spongy parenchyma *(Figure 6)*. At 0.5 mg l^{-1} KIN, a single row, at a higher KIN concentration two rows of palisade parenchyma with spaces between cells could be observed, respectively. The spongy parenchyma contains very few, roundish cells and great intercellular spaces. Stomata are opened and raised above the surface of the abaxial epidermis at lower KIN concentrations (0.5 and 1.0 mg l^{-1}) *(Figure 6A, 6B)*. At higher concentrations (1.5 and 2.0 mg l^{-1}), however, they are mostly closed similarly to the observation after applying only KIN as cytokinin in the medium *(Figure 6C, 6D)*.

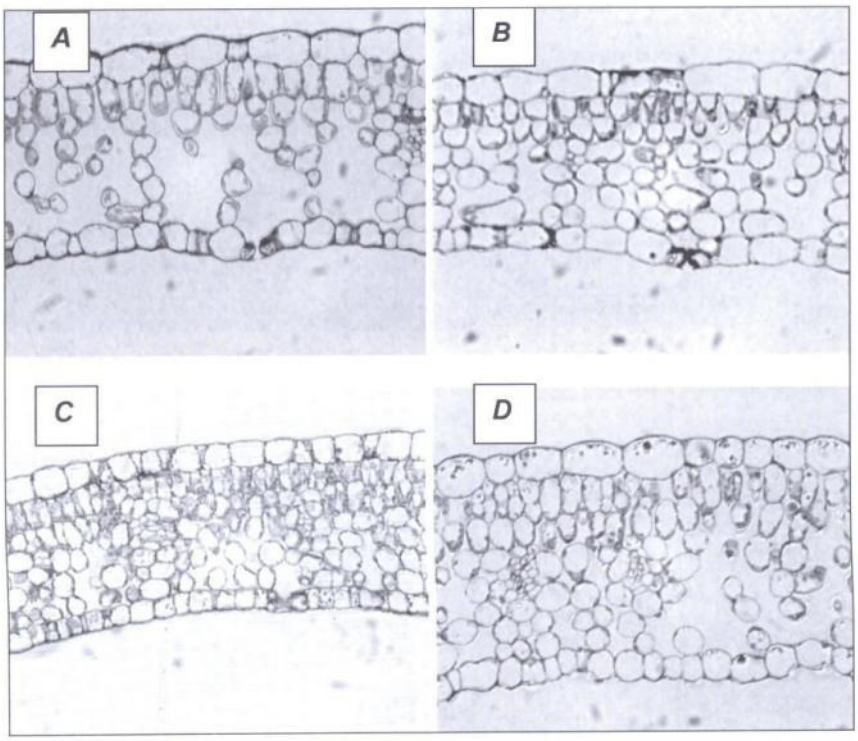

Figure 5. Cross section of the 1st leaf after culturing on media containing 0.5 mg l^{-1} (2.2 µM) BA and TOP (6-(3-hidroxi-benzilamino)-purin) in **(A)** 0.5 mg l^{-1} (2.1 µM), **(B)** 1.0 mg l^{-1} (4.1 µM), **(C)** 1.5 mg l^{-1} (6.2 µM) and **(D)** 2.0 mg l^{-1} (8.3 µM) concentrations, respectively. X 400.

Post-effects of cytokinins on regeneration from leaves

Analysing all the data from both regeneration media, two-way ANOVA proved a significant effect ($P<0.5$) of different concentrations of cytokinins applied in pre-treatment media and in regeneration media on the number of regenerated shoots and on the vitrification percent and also the interaction was proved to be significant. Cytokinin-content of the regeneration media modified the regeneration percent significantly. However, the pre-treatments did not cause any significant differences regarding the regeneration percent. The organogenetic index calculated from these three parameters mentioned above showed important differences depending both on pre-treatments and on the regeneration media. The main effects of pre-treatments on the number of regenerated shoots and on the organogenetic index are presented in Figure 7. Pre-treatments with any concentrations of TOP or 0.5 -1.5 mg l^{-1} KIN or 0.5 mg l^{-1} BA + 1.5 - 2.0 mg l^{-1} KIN resulted in higher SN than in the pre-treatment on cytokinin-free medium. OI was the highest (>6.0) after pre-treatment with 0.5 –1.5 mg l^{-1} TOP, 0.5–1.5 mg l^{-1} KIN, or 0.5 mg l^{-1} BA + 2.0 mg l^{-1} KIN, respectively.

Post-effects of different cytokinins applied before regeneration on SN and OI after regeneration separately on R1 and R2 regeneration media are presented in Table 1. If shoots were cultured on a cytokinin-free medium before regeneration no significant differences were detected in SN between the effect of R1 and R2 regeneration media. However, important differences could be observed considering OI; it was nearly fourtimes higher when the regeneration medium contained BA as a cytokinin-source (R2) because of the much lower vitrification (data not presented).

Considering any concentrations of TOP applied in pre-treatments, we found significantly more shoots when regeneration occurred with TDZ on R1 medium. The highest SN was achieved on R1 medium after pre-treatments with 0.5 – 1.0 mg l^{-1} TOP, but OI was the highest after pre-treatments with

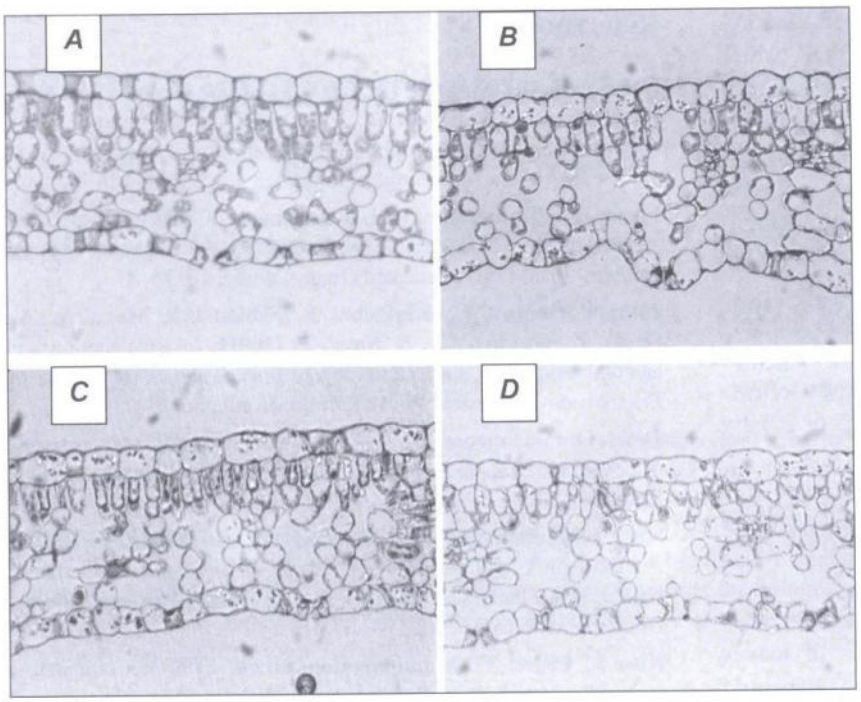

Figure 6. Cross section of the 1st leaf after culturing on media containing 0.5 mg l^{-1} (2.2 µM) BA and KIN (6-furfurilamino-purin) in **(A)** 0.5 mg l^{-1} (2.3 µM), **(B)** 1.0 mg l^{-1} (4.6 µM), **(C)** 1.5 mg l^{-1} (6.9 µM) and **(D)** 2.0 mg l^{-1} (9.3 µM) concentrations, respectively. X 400.

1.0 –1.5 mg l^{-1} TOP due to the fact, that after the lowest TOP concentration vitrification was too high (66.5%), but after 1.5 mg l^{-1} TOP it decreased almost to one half (36.2%). The decrease of vitrification is probably one of the most important post-effects of TOP. When regeneration occurred on BA-containing regeneration media (R2), both SN and OI were the highest after 0.5 mg l^{-1} TOP pre-treatment.

When shoots were pre-treated with BA before regeneration, the highest SN could be recorded on R1 medium after 0.5 – 1.0 mg l^{-1} BA pre-treatments but – because of the 100% vitrification - OI was zero after the 0.5 mg l^{-1} BA pre-treatment.

When the BA and TOP were combined in the pre-treatments, the highest SN and OI was detected on R2 medium after pre-treatment with 0.5 mg l^{-1} BA + 2.0 mg l^{-1} TOP. When regeneration occurred on TDZ-containing R1 medium, the highest SN was recorded after 0.5 mg l^{-1} BA + 1.5 mg l^{-1} TOP pre-treatment, but OI was zero because of the

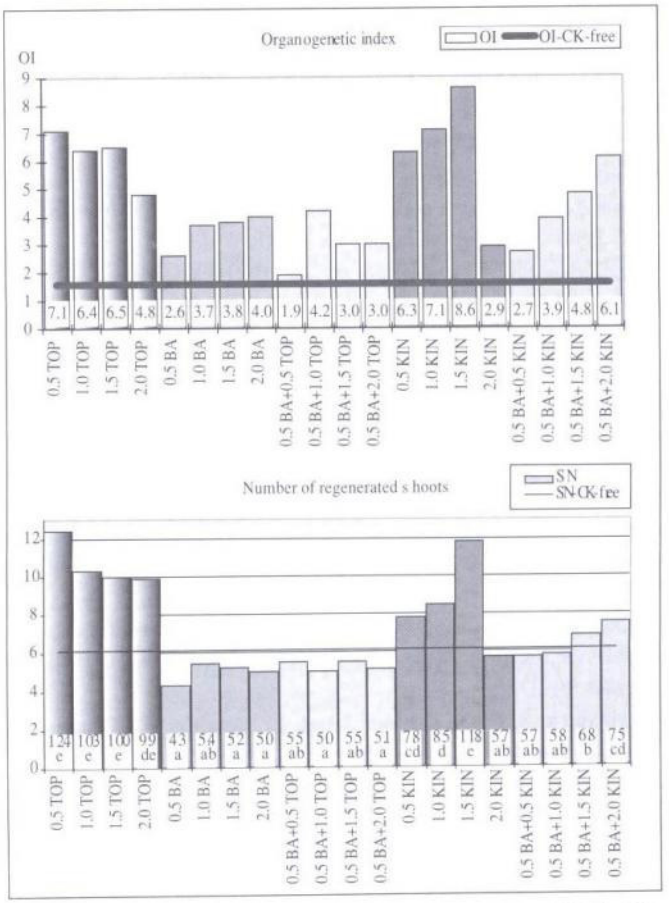

Figure 7. Main effects of different cytokinins on the organogenetic index (OI) and on the number of regenerated shoots per explant (SN) considering the data from both regeneration media. (The different small letters in the columns represent the significant differences ($P<0.05$) between the treatments.)

Table 1. Post-effects of cytokinins on the organogenetic index (OI) and on the number of regenerated shoots per explant (SN) after regeneration on regeneration medium with 0.5 mg l^{-1} (2.3 µM) TDZ (R1) or 5.0 mg l^{-1} (22.2 µM) BA (R2), respectively.*

Cytokinin content in the medium (mg l^{-1})	SN		OI	
	R1 medium	R2 medium	R1 medium	R2 medium
cytokinin-free medium	6.4 bcd, A	5.9 def, A	0.7	2.6
0.5 TOP	15.1 ij, B	8.9 g, A	5.1	7.7
1.0 TOP	14.1 ij, B	6.4 f, A	7.5	4.6
1.5 TOP	12.5 hi, B	7.5 f, A	8.0	5.0
2.0 TOP	13.3 i, B	6.5 f, A	4.0	4.1
0.5 BA	11.9 ghi, B	4.6 abc, A	0	3.2
1.0 BA	6.9 bcde, B	4.4 abc, A	3.7	3.3
1.5 BA	5.5 bcd, A	5.0 bcde, A	4.6	3.3
2.0 BA	4.9 b, A	5.1 bcde, A	4.3	3.9
0.5 BA + 0.5 TOP	5.2 bc, A	5.7 cdef, A	0	3.1
0.5 BA + 1.0 TOP	5.4 bc, A	4.8 abcde, A	4.9	3.8
0.5 BA + 1.5 TOP	7.1 bcde, B	4.3 abc, A	0	3.8
0.5 BA + 2.0 TOP	2.3 a, A	6.9 f, B	0	6.4
0.5 KIN	9.4 efg, B	6.6 f, A	8.9	4.6
1.0 KIN	10.6 fgh, B	7.1 f, A	10.6	5.2
1.5 KIN	17.1 j, B	8.6 g, A	14.7	5.6
2.0 KIN	4.6 ab, A	6.3 ef, B	0	5.1
0.5 BA + 0.5 KIN	7.3 bcde, B	4.8 abcd, A	1.8	2.8
0.5 BA + 1.0 KIN	8.4 def, B	4.2 abc, A	3.1	3.6
0.5 BA + 1.5 KIN	11.6 ghi, B	3.8 ab, A	7.1	2.9
0.5 BA + 2.0 KIN	13.4 i, B	3.6 ab, A	11.9	2.7

*: Different small letters in the columns represent the significant differences ($P<0.05$) between the pre-treatments, different block capitals in the rows indicate differences between regeneration media in the same pre-treatment.

high vitrification (100%). It may be hypothesized, that BA repressed the vitrification decreasing effect of TOP, which was detected when TOP was applied alone in pre-treatment.

When regeneration was induced with TDZ (R1), the SN and OI was higher after 0.5 –1.5 mg l^{-1} KIN pre-treatment compared to the regeneration with BA (R2), but a reverse situation could be detected after pre-treatment with 2.0 mg l^{-1} KIN. The best regeneration result was observed after 1.5 mg l^{-1} KIN on both regeneration media.

After combined application of BA and KIN in the pre-treatment, SN and OI were always higher when TDZ was used for regeneration (R1). The most favourable post-effects were obtained in the cases of 0.5 mg l^{-1} BA + 1.5 or 2.0 mg l^{-1} KIN.

Conclusions

In this paper we have demonstrated, that great differences could be detected in the anatomical structures of leaves originating from media containing different types and concentrations of aromatic cytokinins.

Considering the subsequent regeneration, we observed that the regeneration response of leaves was significantly better after pre-treatments with 0.5 –1.5 mg l^{-1} TOP, 0.5 –1.5 mg l^{-1} KIN, or 0.5 mg l^{-1} BA + 2.0 mg l^{-1} KIN, respectively.

The histological structure of leaves used for regeneration and their regeneration response showed a good correlation. When the pre-treatment caused a juvenile-like or less-differentiated structure, the number of regenerated shoots per explant increased and often also the vitrification decreased and consequently, the organogenetic index also increased. A strong interaction between cytokinin-content (type and concentration) of the pre-treatment medium and that of the regeneration medium could also be detected. These results indicate, that the type and concentration of cytokinins applied before the regeneration phase could modify the organogenetic potential of apple leaf tissues, and therefore also the regeneration process. This suggests that anatomical changes detected after pre-treatment of shoots with different types and concentrations of cytokinins may indicate functional differences in the leaf tissue.

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