Influence of aromatic cytokinins on shoot multiplication and their after-effects on rooting of apple cv. *Húsvéti rozmaring*

INTERNATIONAL JOURNAL OF HORTICULTURAL SCIENCE

AGROINFORM Publishing House, Hungary

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Key words: 6-benzylaminopurine riboside, 6-benzylaminopurine, kinetin, meta-topolin

Abbreviations: 6-benzylaminopurine riboside – BAR, 6-benzylaminopurine – BA, kinetin – KIN, meta-topolin – TOP, indole-3-butyric acid – IBA, gibberellic acid – GA_3

Summary: Different aromatic cytokinins (BA, BAR, TOP and KIN) were tested alone or in combination for the shoot proliferation response of '*Húsvéti rozmaring*' apple scion. The best multiplication rate was achieved by dual cytokinin application (1 mg l^{-1} BA + 1.5 mg l^{-1} KIN). The rooting capacity was affected considerably by the position of shoots: transfer of the three-week-old shoots to the same or other proliferation medium in vertical position inhibited the following rooting totally. Post-effects of different cytokinins (BA and TOP) on subsequent rooting could be detected: BA increased the number of roots markedly, while TOP resulted in significantly longer roots.

Introduction

'*Húsvéti rozmaring*' ('Rosemary of Eastern') was the one of the most popular old apple varieties (scions) in Hungary. Nowadays, it is current mostly in home orchard and it can serve as a source of resistance in apple breeding, because it has good tolerance against different diseases. It has very good post-harvest characteristic, because it can be stored at 10 °C till Easter time.

The micropropagation procedure involves the culture of explants, production of shoots by adventitious or axillary shoot proliferation and then induction of these shoots to form adventitious roots (*Broome & Zimmerman*, 1984). A lot of factors, such as type and concentration of growth regulators, macro- and micronutrients in the medium, light, temperature etc., have been reported affecting shoot proliferation, root induction and development (*Jones*, 1976, *Snir & Erez*, 1980, *James & Thurbon*, 1981, *Jones*, 1992, *Karhu*, 1995, *Modgil et al.*, 1999). Different cultivars of apple do not respond in the same way during micropropagation to the *in vitro* conditions, especially to the different growth regulators. Therefore the *in vitro* procedures described earlier could not be extrapolated with the same success to other genotypes (*Huth*, 1978, *James & Thurbon*, 1979, *Zimmerman & Fordham* 1985, *Abdul-Kader* et al., 1991, *Karhu & Zimmerman*, 1993, *Karhu*, 1995, *Modgil* et al., 1999).

Data concerning *in vitro* propagation of this cultivar were not found. This study was undertaken to investigate the influence of different types of cytokinin on shoot proliferation and the after-effects of some cytokinins on rooting capacity of '*Húsvéti rozmaring*' and this report describes an effective *in vitro* method for rapid multiplication of this apple scion-variety.

Material and methods

Explants from field grown trees were collected and surface sterilized. First step of sterilization was the washing in tap water with Tween-20 for one hour. After washing shoot apices with a small piece of stem were sterilized in 70% ethanol for 3 minutes, then in 0.1% $HgCl_2$ for 5 minutes. After surface-sterilization shoot tip explants were washed three times in sterilized distilled water and inoculated on initiation medium based on half-strength MS-medium *(Murashige & Skoog,* 1962) supplemented with 0.5 mg l^{-1} BAR, 0.1 mg l^{-1} IBA and 0.5 mg l^{-1} GA_3. To prevent the phenol oxidation, initiation medium was supplemented with antioxidants (0.15 g l^{-1} citric acid and 0.1 g l^{-1} ascorbic acid) and adsorbents (1 g l^{-1} polyvinil pyrrolidone and 2.5 g l^{-1} activated charcoal and 40% perlite). After one-week-culture explants were placed on solid medium with the same compounds as the initiation medium.

Shoots were subcultured and multiplied at 4-week intervals on full-strength MS-medium supplemented with 100 mg l^{-1} myo-inositol, 0.7% agar-agar, 3% saccharose and 0.5 mg l^{-1} BA, 0.1 mg l^{-1} IBA and 0.2 mg l^{-1} GA_3, to get sufficient number of shoots for multiplication experiments.

Shoot multiplication experiments

Shoot multiplication experiments were carried out on MS-medium supplemented with 100 mg l^{-1} myo-inositol, 0.7% agar-agar, 3% saccharose, 0.3 mg l^{-1} IBA, 0.2 mg l^{-1} GA_3 and different concentrations and types of cytokinins. Response to different types and concentrations of BA, BAR and TOP alone and response to BA in combination with KIN were tested. *(Table 1).*

Table 1 Type and concentrations of cytokinins in the culture media used in the shoot multiplication experiments

Media	Concentrations of cytokinins (mg l^{-1})			
	BA	BAR	TOP	KIN
M–1	0.5	–	–	–
M–2	1.0	–	–	–
M–3	–	0.5	–	–
M–4	–	1.0	–	–
M–5	–	–	0.5	–
M–6	–	–	1.0	–
M–7	1.0	–	–	1.0
M–8	1.0	–	–	1.5

All media were autoclaved for 20 min at 121 °C and 10^5 Pa. The pH of the medium was adjusted to 5.8 before autoclaving. Shoot cuttings of 35–40 mm were transferred to different media in order to induce shoot proliferation. Four explants were placed horizontally on 40 ml of medium in Kilner-jar (400 ml, 75 mm inside diameter and 85 mm long).

Cultures were grown at 22±2 °C with 16 h photoperiod provided by warm-white lamps (Tungsram) at a PPF of 105 Mmol s^{-1} m^{-2} for four weeks. Each treatment consisted of at least 20 replicates and experiments were repeated three-times.

After four weeks of culture, the length of newly developed shoots were measured and the rate of shoot multiplication was determined by counting the number of new shoots per explant. The statistical analysis was made according to the Tukey's test by the statistical programme SPSS 9.0 for Windows.

Rooting experiments

After-effects of the last proliferation conditions, including different cytokinins in media combined with two positions of shoots on the substrate were examined on the rooting capacity. Explants were placed horizontally on the substrate as applied in shoot multiplication experiments. In the pre-treatments the cultures were grown on the same medium for four weeks (P-1 and P-4) or newly developed three-week-old shoots were cut and placed vertically on the same or other media (P-2, P-3 and P-5) as explained in *Table 2.* Cultures were grown under the same conditions used in shoot multiplication experiments. After the different pre-treatments the shoots 1.5 to 2.5 cm in length were inserted to a root induction medium. The medium contained MS salts at half strength supplemented with 100 mg l^{-1} myo-inozitol, 0.5 mg l^{-1} vitamin B_1, 20.0 g l^{-1} sucrose, 1.0 mg l^{-1} indole-3-butyric acid (IBA), and 7.0 g l^{-1} agar-agar. The pH was adjusted to 5.5 prior to autoclaving. Cultures were incubated at 26 °C in total darkness for a week. Then shoots were transferred to a root elongation medium, which contained MS salts at half strength supplemented with 50 mg l^{-1} myo-inozitol, 30.0 g l^{-1} sucrose, 2.0 ml l^{-1} Wuxal and 7 g l^{-1} agar-agar. Subsequently, the cultures were incubated under the same conditions for shoot multiplication experiments. Rooting percentage, number of roots per rooted shoot and the length of roots were observed after three weeks and rooted shoots were planted in Jiffy-7 pellets after removing the medium. Previously, Jiffy-7s were soaked in a sterile solution containing MS major- and minor elements and Fe 0.1 strength and 0.15% Previcur to prevent fungal contamination. Acclimatization was made according to *Bolar* et al. (1998). Each treatment was replicated at least 30 times.

Table 2 Culturing of shoots on different media and in different position (pre-treatments) before rooting

Pre-treatments	Weeks			
	1st	2nd	3rd	4th
P-1	medium M-8			
P-2	medium M-8			med. M-8, vertically
P-3	medium M-8			med. M-6, vertically
P-4	medium M-6			
P-5	medium M-6			med. M-6, vertically

Results and discussion

Shoot multiplication

Jones et al. (1977) reported that the type and concentration of cytokinin affected the shoot multiplication of apple considerably. Similarly, in our experiments, both

the multiplication rate and the length of newly developed shoots varied with the type and concentration of cytokinins *(Table 3)*. When BA was applied alone as exogenous cytokinin, increasing the concentration of BA from 0.5 mg l^{-1} to 1.0 mg l^{-1}led to a slight increase in multiplication rate (4.4 to 4.7-fold), although the differences were statistically not significant.

Table 3 Effect of different cytokinins on the rate of shoot multiplication (shoots per explant) and on the shoot length after 4-week-culture.+

Type and concentration of cytokinin (mgl^{-1})	Multiplication rate	Shoot length (mm)
0.5 BA	4.4 b	42.1 ab
1.0 BA	4.7 b	39.5 a
0.5 BAR	3.1 a	50.7 c
1.0 BAR	3.1 a	45.7 bc
0.5 TOP	2.3 a	50.0 c
1.0 TOP	2.8 a	51.2 c
1.0 BA + 1.0 KIN	5.3 c	44.4 b
1.0 BA + 1.5 KIN	6.2 d	44.8 b

+: The small letters mean the homogenous groups according to Tukey's test.

The favourable effect of BAR on shoot-proliferation has been reported earlier *(Dobránszki* et al., 2000 a,b), which may be due to the presence of a riboside at the N^9-position protecting the cytokinin against N^9-glucolysation *(Werbrouck* et al., 1996). The multiplication rate could be increased markedly for some apple genotypes using the BAR *(Dobránszki* et al., 2000 a,b), or using the meta-topolin (hydroxylated BA analogues) in other plant species *(Werbrouck* et al., 1996). In contrast, very low multiplication rate could be reached, only, by application of BAR or TOP regardless of their concentrations in this cultivar.

In this study, the BA was the best of tested cytokinins for '*Húsvéti rozmaring*' considering the rate of multiplication. Although BAR and TOP decreased the multiplication rate in the concentrations tested, they increased the shoot length significantly. There was a strong negative correlation (r^2= 0.869) between shoot length and number as reported earlier for apple shoot-tip culture by *Skirvin* et al. (1986). Plantlets developed on media containing meta-topolin seemed to be more vigorous and had larger leaves with dark-green colour.

The combination of BA with KIN, especially with the higher concentration of KIN, was the most favourable for shoot multiplication in '*Húsvéti rozmaring*'. Application of 1.0 mg l^{-1} BA combined with 1.0 or 1.5 mg l^{-1} KIN caused a significantly higher multiplication rate of 5.3 and 6.2 shoots per explant, respectively, in comparison with the 4.7-fold multiplication rate achieved on media containing 1.0 mg l^{-1} BA alone. This finding is in agreement with earlier results of other apple genotypes reported by *Modgil* et al. (1999) and *Dobránszki* et al. (2000 b). The length of shoots developed on this medium was higher significantly than those developed on a medium containing 1.0 mg l^{-1} BA alone.

Rooting capacity

Both positions of shoots and type of cytokinin during the pre-treatments had significant after-effect on rooting capacity. The rooting percentage was affected only by the position of shoots during pre-treatments, while different cytokinins in the last proliferation media resulted in the same percentage with different characters of the root system *(Table 4)*.

Table 4 Rooting characteristics of shoots after different pre-treatments.+

Characteristics	Pre-treatments				
	P-1	P-2	P-3	P-4	P-5
Rooting percentage	88.6	0	2.0	88.6	6.0
Number of roots per shoots	6.4 c	0	1.0 a	4.5 b	1.0 a
Length of roots	15.3 b	0	6.0 a	22.0 c	8.0 a
Rate of callus development	++	+++	+++	+	++
Rate of explants with new shoots (%)	0	45.7	42.9	0	0
New shoots per explants	0	2.0	2.7	0	0

+: The small letters mean the homogenous groups according to Tukey's test.

Vertical position of shoots prior to root induction treatment inhibited the rooting considerably (P-2, P-3 and P-5 treatments). The most of these shoots showed high capacity to form calli on the base of shoots without any roots although rooting in a very low rate with weak root development could be observed. Moreover, the BA applied during pre-treatments enhanced the proliferation of these shoots more than 40% of them produced in average 2–3 newly developed shoots per explant originating from treatments P-2 and P-3. When shoots were grown on media containing only TOP during pre-treatments, they produced large and dark-green leaves but shoot-proliferation could not be observed (P-5).

Webster & Jones (1991) found differences in rooting percentage between shoots originating from different content of BA in media. In our study the rooting percentage was the same (88.6%) for shoots originating from treatments P-1 and P- 4. Shoots grown on media containing BA (P-1) formed more roots per shoots than shoots from media containing TOP (P-4). In contrary, the lengths of roots were shorter on shoots from P-1, than shoots from P-4. There was a significant negative correlation between the length of roots and the number of roots per rooted shoots.

Rooted shoots of P-1 and P-4 treatments and rootless shoots from remaining treatments were planted to Jiffy-7 pellets to test the rate of survival. The rate of shoot growth was very low during the first four weeks and at the end of the 5th week the rate of survival was 100% in treatments P-1 and P-4 but shoots from the other treatments died.

Our results suggested that the KIN applied together with the BA had a synergistic effect on shoot number without decreasing the length of shoots thus it can be used, ideally, for *in vitro* proliferation of '*Húsvéti rozmaring*'. Moreover, the citokinin content of the last proliferation medium could affect the rooting capacity of shoots. Although the method

described here allows an effective micropropagation procedure of *Husvéti rozmaring*', further improvement in rooting percentage could be achieved the optimalization of the rooting procedure.

Acknowledges

The authors wish to say thank you to Mrs. Bohács Jánosné for her technical assistance. This work was supported by OTKA (Hungarian Sci. Res. Fund), project No. T-030103.

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