

Role of Cytokinins and Explant Type in Shoot Multiplication of Buckwheat (*Fagopyrum esculentum* Moench) *in Vitro*

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ABSTRACT

The main objective of micropropagation after *in vitro* culture establishment is to work out an efficient and reliable method for shoot multiplication. The effect of isoprenoid (2-iP) and aromatic cytokinins (BA, BAR, KIN, TOP) were tested in experiments aimed on shoot multiplication. Beside the evaluation of the effects of different types and concentrations (1 to 20 μM) of cytokinins, the influence of explant type (shoot tip, nodal segment) on efficient multiplication was also assessed. Both type and concentration of applied cytokinins and the type of explants had a strong effect on the shoot multiplication and on growth type of *in vitro* shoots. A very tight relationship between the cytokinins and explant types was observed. If meta-topolin was applied between 5 and 15 μM and shoot tips were used as initial explants, high multiplication (3.05-3.49 shoots and 13.26-14.42 nodes per explant) could be achieved and the mean length of shoots was satisfactory (> 40 mm). Results obtained may contribute to the development of an efficient micropropagation protocol for common buckwheat.

Keywords: growth type, micropropagation, nodal segment, shoot tip, type of cytokinins

Abbreviations: 2iP, 6-(3,3-dimethylallyl-amino)purine; BA, 6-benzylaminopurine; BAR, 6-benzylaminopurine riboside; IAA, indole-3-acetic acid; KIN, 6-furfurylamino purine or kinetin; TOP, 6-(3-hydroxybenzylamino)purine or meta-topolin

INTRODUCTION

Common buckwheat has been grown widely in Asia but it has been of secondary importance in many countries of the northern hemisphere (Campbell 1997; Zeller 2001; Tetsuka and Uchino 2005; Senthilkumaran *et al.* 2008). However, buckwheat is one of the best sources of high quality and easily digestible protein and it is a good source of minerals (Edwardson 1996); because of high content of rutin and tannins it has high antioxidant activity; moreover, it is cholesterol-free and virtually fat-free as well, therefore it suits well to a modern, low calorie and high nutrition diet (Francischi *et al.* 1994).

An effective micropropagation method for buckwheat (*Fagopyrum esculentum* Moench) is a necessary precondition

for the application of different biotechnological methods in its breeding. It enables rapid multiplication of plants, propagation of pathogen-free material, and it may be used to recover and conserve gene resources. At present, limited information is available about micropropagation of common buckwheat (Nešković *et al.* 1990; Romchatngoen *et al.* 1998; Kločová and Gubišová 2008) (Table 1).

In micropropagation either propagation from axillary and/or terminal buds or direct organogenesis is favoured in order to avoid the callus phase and therefore decrease somaclonal variation. For rapid multiplication a protocol based on axillary and/or apical meristem shoot proliferation is preferable. After having *in vitro* axillary shoot culture, there are two methods for the multiplication of *in vitro* plants from *in vitro* axillary shoots: (1) shoot tip culture, where the

Table 1 Works on shoot multiplication of common buckwheat.

Initial explant	Subculture period	Basal medium	Applied cytokinin	Gelling agent	Number of new shoots	Number of new nodes	References
Shoot tip	4 weeks		2.2 mg/l BA		4		Nešković <i>et al.</i> 1990
Shoot section	6 weeks	MS in half strength	20 μM BA	1.5 g/l Gellan gum	20		Romchatngoen <i>et al.</i> 1998
Nodal segment	4 weeks	MS	0.5 mg/l BA	8 g/l agar	1.86	3.88 ^a	Kločová and Gubišová 2008
			1.0 mg/l BA		2.74	4.46 ^a	
		B5	-				4.50
		MSB ^b	-				4.74
		MS	-				4.71
		MS	-		8 g/l agar		4.16 ^a
			-		2.5 g/l Phytigel		3.70 ^a
	-		4 g/l Agar + 1.25 g/l Phytigel		3.54 ^a		
	MS	Two-step method (1 mg/l BA for 4 weeks then on PGR-free medium)	8 g/l agar			14.05 ^a	
Nodal segment	4 weeks	MS	5 μM BA	8 g/l agar	2.07	9.27	Present results
Shoot tip	4 weeks	MS	10 μM TOP	8 g/l agar	3.41	14.42	

^a: mean of three cultivars (Špačinska, Bogaty, Idel)

^b: MSB: MS salts and B5 vitamins

explant of a subculture is a shoot tip and shoot multiplication occurs by repeated formation of axillary branching; and (2) single or multiple node culture, where the explant is a segment of *in vitro* shoots having one or more nodes and further multiplication can occur by cutting each shoot into single- or several-node pieces (George and Debergh 2008).

Cytokinins are N⁶-substituted adenines with growth regulatory activity in plants and they are one of the most important factors during shoot development and shoot multiplication (Howell *et al.* 2003). There are two main classes: isoprenoid and aromatic cytokinins, which differ from each other in their biochemistry, receptors, biological activity and metabolism (Werbrouck *et al.* 1996; Strnad *et al.* 1997).

The aim of the present work was to evaluate the effects of different types of cytokinins in the shoot multiplication stage of micropropagation of buckwheat. The effects of different aromatic cytokinins, namely: 6-benzylaminopurine (BA), which has a benzylamino side chain and its N⁹-ribosides (BAR); kinetin (KIN) with a furfurylamino side chain; meta-topolin (TOP), which is a hydroxylated analogue of BA; 6-(3,3-dimethylallyl-amino)purine (2iP), which is an isoprenoid cytokinin, were studied. Beside the role of cytokinins, the effect of explant type used for a subculture on the rate of shoot multiplication and on the growth type of developing new *in vitro* shoots was examined.

MATERIALS AND METHODS

Mature seeds of *Fagopyrum esculentum* Moench cv. 'Hajnalka' were dehulled and surface sterilized by soaking them in a 1.2 g l⁻¹ sodium dichloroisocyanurate (Fitosept[®] tablet, Biomark, Hungary) solution for 30 min, then in 25% calcium hypochlorite solution for 30 min, passed through a 70% ethanol solution for 3 min and finally washed three times with sterile distilled water. Afterwards seeds were germinated aseptically on wet filter paper (MN 619). After 5 days shoot tips of seedlings were excised and cultured on MS medium containing MS salts and vitamins (Murashige and Skoog 1962) to induce shoot proliferation. The medium was supplemented with 100 mg l⁻¹ *myo*-inositol, 3% sucrose, 0.8% agar-agar BIOLAB bacteriological agar, 10 µM BA and 1 µM indole-3-acetic acid (IAA). The cultures were grown at 22°C with a 16-h photoperiod at PPFD of 105 µmol s⁻¹ m⁻² and subcultured at 4-week-intervals for shoot multiplication experiments.

Shoot multiplication experiments were conducted on media with different cytokinin contents. Five types of cytokinins were tested: BA, BAR, TOP, KIN and 2-iP in the following concentrations: 1, 5, 10, 15 and 20 µM. Other components of the media were the same as those used for initial shoot proliferation. The effects of the following cytokinin: auxin (using 1 µM IAA) ratios were also tested: 1: 1, 5: 1, 10: 1, 15: 1 and 20: 1 for each type of cytokinin. In shoot multiplication experiments the multiplication of two explant types were tested. In the one series of experiments, the subcultures were initiated by 15-mm-long shoot tips of *in vitro* plantlets and in the other series the subcultures were initiated by nodal segments of *in vitro* plantlets.

The experiments were carried out in 400 ml Kilner jars; in each jar four explants were placed on 20 ml of medium and they were grown at the same light and temperature conditions as shoot multiplication for four weeks. Each treatment consisted of 15 jars, i.e. 60 explants in total. The experiments were repeated in triplicate.

At the end of the experiments multiplication rate (number of new shoots per explant), shoot length (mm) and number of nodes of newly developed shoots were measured, number of new nodal segments per explant were counted (multiplication rate × the number of nodes of newly developed shoots per respective explant); moreover, the type of multiplication (shoot rosettes (SR) or long shoots with several nodes (NS) or mixed type, based on George and Debergh 2008) (Fig. 1) was observed. The measured data were analysed statistically by one- and two-way-ANOVA followed by Tukey's test or *t*-probe by using SPSS 13.0 for Windows software.



Fig. 1 Growth type. *SR growth type*: shoot rosettes when shoot multiplication occurs by axillary branching; *NS growth type*: shoot multiplication occurs by a long shoot with several nodes; *mixed type*: both shoot rosettes and long shoots with several nodes develop on initial explants.

RESULTS

Statistical analysis of observed data proved both the effect of initial explants and cytokinin content (type and concentration) of the medium to be significant and there were significant interactions between them ($P < 0.01$).

Multiplication rate and the number of new nodal segments per explant

Concerning the main effects of the type of cytokinins, the multiplication rate (number of new shoots per explant) was highest when BA was used as the cytokinin, independent of the type of initial explant. In the case of the other cytokinins their effects were also dependent on the type of initial explant. When the experiments were initiated by shoot tips, the multiplication rate with BAR (2.99) or TOP (3.04) was statistically the same as with BA (3.05). Using nodal segments for the subculture, 2-iP initiated significantly more new shoots per explant (1.37) after BA (2.12) treatment and KIN resulted in the lowest multiplication rate (1.15). Increasing the concentration of cytokinin in the medium caused an increase in the multiplication rate but this effect was dependent on the type of cytokinin and on the initial explants, as well. When averaging different cytokinins, the multiplication rate was increased most (2.89) by 10 µM when initial explants were shoot tips and by 15-20 µM (1.59-1.66) when experiments initially used nodal segments. The multiplication rate could be increased by using shoot tips as initial explants. The average number of new shoots per explant in each treatment is presented in **Table 2**.

Concerning the main effects of treatments (concentrations or types of cytokinins) it can be concluded that the number of new nodal segments per explant was the highest (12.46) at 10 µM cytokinin or using TOP (12.82) when initial explants were shoot tips, and at 15-20 µM (5.99-6.05) cytokinin or using BA (8.11) when initial explants were nodal segments. Because of the tight significant ($P < 0.001$) interaction between the effect of the types and concentrations of cytokinins, the main effects could be modified in particular cases (**Table 2**). More new nodal segments per explant could be obtained when experiments were initialized with shoot tips compared to nodal segments (**Table 2**).

Growth type

Fig. 2 presents the effects of the type and concentration of applied cytokinins on the growth type observed in the subculture. When experiments were initialized with shoot tips, BA (58%) and BAR (56.5%) promoted SR-type of growth, while KIN (56.7%) and 2-iP (48.7%) significantly stimulated NS-type of growth, however TOP caused a mixed type of growth at 41.4%. High concentrations (> 10 µM) sti-

Table 2 Effects of cytokinins (their types and concentrations) and types of initial explant on the number of newly developed shoots and on the number of new nodal segments.⁺

Cytokinin		№ of new shoots/explant		№ of new nodal segments/explant	
Type	µM	Initial explants:		Initial explants:	
		Shoot tips	Nodal segments	Shoot tips	Nodal segments
BA	1	2.37 a, B	1.91 ^{ns} a, C	9.76 a, B	7.40* a, B
	5	3.25 bc, B	2.07* ab, B	12.78 b, B	9.27* a, B
	10	3.17 bc, B	2.16* ab, B	13.21 b, B	8.70* a, B
	15	2.90 b, B	1.98* a, B	10.12 ab, AB	7.47* a, B
	20	3.58 c, B	2.48* b, C	13.38 b, C	7.73* a, B
TOP	1	2.18 a, B	1.30* a, B	10.17 a, B	4.43* a, A
	5	3.05 b, B	1.20* a, A	13.26 b, B	4.93* a, A
	10	3.41 b, B	1.12* a, A	14.42 b, B	4.39* a, A
	15	3.49 b, C	1.27* a, A	14.33 b, C	5.40* a, AB
	20	3.09 b, B	1.37* a, A	11.95 ab, BC	5.03* a, AB
BAR	1	2.27 a, B	0.88* a, A	10.40 a, B	3.75* a, A
	5	3.08 b, B	1.20* ab, A	12.11 ab, B	4.60* a, A
	10	3.42 b, B	1.12* a, A	12.96 b, B	4.62* a, A
	15	3.03 b, BC	1.57* bc, AB	11.33 ab, B	5.76* ab, AB
	20	4.14 b, B	1.93* c, B	10.58 ab, AB	7.86 ^{ns} b, B
KIN	1	1.50 ab, A	0.88* a, A	8.31 ab, AB	3.78* a, A
	5	1.30 a, A	1.00* a, A	7.18 a, A	4.04* a, A
	10	1.78 bc, A	1.20* ab, A	7.51 ab, A	3.97* a, A
	15	1.96 cd, A	1.48* b, AB	8.58 ab, A	4.61* a, A
	20	2.28 d, A	1.20* ab, A	10.13 b, AB	3.85* a, A
2-iP	1	1.40 a, A	1.06* a, AB	6.35 a, A	3.71* a, A
	5	1.68 a, A	1.19* a, A	8.83 ab, A	3.93* a, A
	10	2.27 b, A	1.43* ab, A	12.83 b, B	4.93* ab, A
	15	1.73 a, A	1.75 ^{ns} b, AB	9.50 ab, AB	6.47 ^{ns} b, AB
	20	1.80 a, A	1.43 ^{ns} ab, A	8.03 a, A	5.80 ^{ns} ab, AB

⁺: Different small letters in the columns mean the significant differences ($P < 0.01$) between the different concentrations of cytokinins in the same cytokinin type; different block capitals in the columns indicate differences between different cytokinin types in the same concentration of cytokinin, based on Tukey's test. *^{ns} in the rows mean the significance of differences between the two types of initial explants: * means significant differences ($P < 0.01$), ^{ns} means no significance, based on *t*-probe.

Table 3 Effects of cytokinins (their types and concentrations) and types of initial explant on the mean length and on the number of nodes of newly developed shoots.⁺

Cytokinin		Mean length of new shoots (mm)		Mean № of nodes per new shoots	
Type	µM	Initial explants:		Initial explants:	
		Shoot tips	Nodal segments	Shoot tips	Nodal segments
BA	1	50.15 c, AB	48.58 ^{ns} d, B	4.13 c, A	3.83 ^{ns} b, A
	5	34.15 b, AB	39.57 ^{ns} c, B	3.93 bc, A	4.55* c, C
	10	32.06 b, B	28.10 ^{ns} b, A	4.14 c, A	3.91 ^{ns} b, AB
	15	24.12 a, A	23.82 ^{ns} ab, AB	3.49 a, A	3.66 ^{ns} b, B
	20	23.29 a, A	19.62* a, A	3.74 b, B	3.28* a, A
TOP	1	51.15 b, AB	26.03* a, A	4.66 c, A	3.41* a, A
	5	40.33 a, B	27.36* a, A	4.35 bc, A	3.83* ab, AB
	10	43.87 ab, C	26.32* a, A	4.22 ab, A	3.93 ^{ns} ab, AB
	15	44.53 ab, C	28.70* a, B	4.07 ab, B	4.23 ^{ns} b, C
	20	37.97 a, B	25.55* a, AB	3.87 a, B	3.68 ^{ns} ab, AB
BAR	1	61.07 d, B	23.61* a, A	4.59 c, A	4.18 ^{ns} a, A
	5	32.56 c, A	19.74* a, A	3.92 b, A	3.83 ^{ns} a, AB
	10	24.65 a, A	17.32* a, A	3.78 b, A	4.14 ^{ns} a, B
	15	26.56 b, A	19.84* a, A	3.89 b, AB	3.68 ^{ns} a, B
	20	20.73 a, A	19.81 ^{ns} a, A	3.36 a, A	4.06* a, B
KIN	1	47.29 bc, AB	57.08 ^{ns} c, B	5.54 b, B	4.26* b, A
	5	51.58 c, C	41.61 ^{ns} b, B	5.53 b, B	3.97* b, BC
	10	37.39 ab, B	26.75* a, A	4.23 a, A	3.28* a, A
	15	36.07 a, B	20.40* a, A	4.38 a, B	3.07* a, A
	20	39.36 ab, B	23.06* a, A	4.44 a, C	3.24* a, A
2-iP	1	37.25 a, A	28.31 ^{ns} a, A	4.54 a, A	3.58 ^{ns} a, A
	5	49.01 ab, C	28.37* a, A	5.22 ab, B	3.28* a, A
	10	51.57 b, D	26.34* a, A	5.65 b, B	3.51* a, AB
	15	53.93 b, D	29.83* a, B	5.48 b, C	3.73* a, B
	20	37.82 a, B	30.80 ^{ns} a, B	4.46 a, C	3.92 ^{ns} a, B

⁺: Different small letters in the columns mean the significant differences ($P < 0.01$) between the different concentrations of cytokinins in the same cytokinin type; different block capitals in the columns indicate differences between different cytokinin types in the same concentration of cytokinin, based on Tukey's test. *^{ns} in the rows mean the significance of differences between the two types of initial explants: * means significant differences ($P < 0.01$), ^{ns} means no significance, based on *t*-probe.

mulated SR-type of growth (41-49.6%) and lower concentrations (1-5 µM) promoted NS-type of growth (59-41%). Using nodal segments for subculture, the effect of the different cytokinin-concentrations was similar to each other. However, the effects of cytokinin type on the growth type were different: the type of SR growth was most stimulated

by BAR (73.6%) and the type of NS growth was most stimulated by TOP (70.7%).

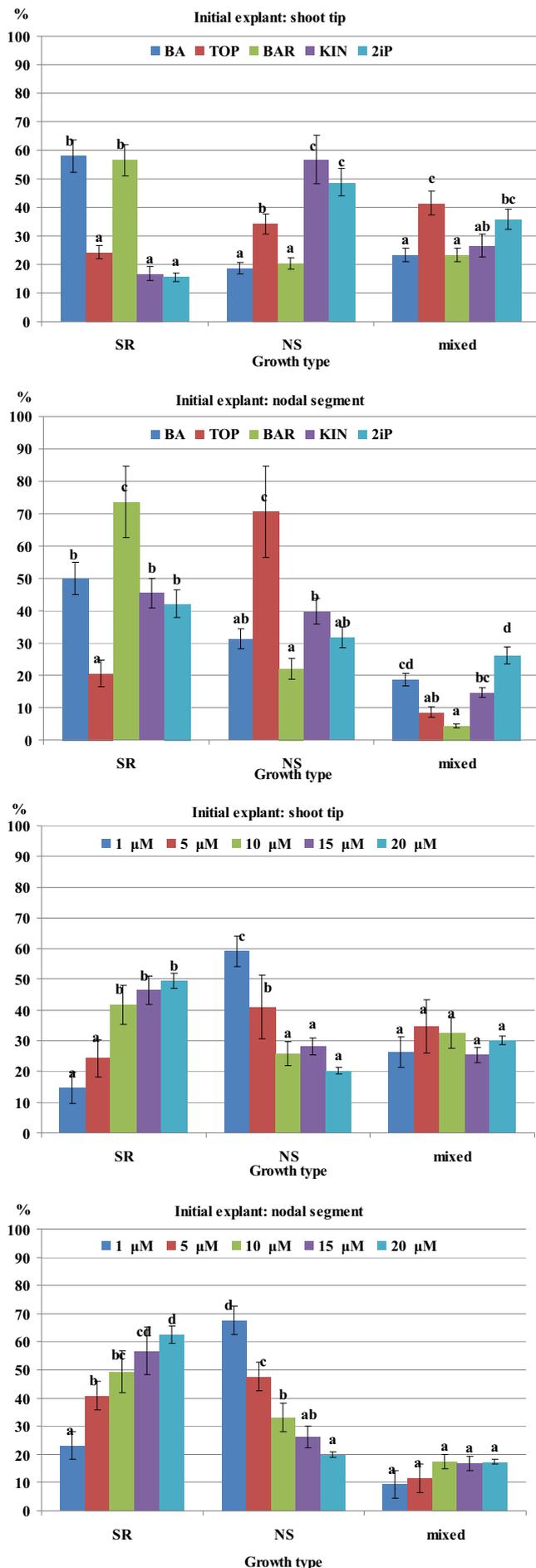


Fig. 2 Effect of initial explant and cytokinin type and concentration on the type of growth.

Shoot length and the number of nodes per newly developed shoot

An increase in the cytokinin concentration resulted in a significant decrease in shoot length in both types of initial explants ($P < 0.001$); the mean shoot length decreased from 52.44 mm to 30.20 mm using shoot tip explants and from 37.65 mm to 23.14 mm using one-node-explants. This was the greatest and clearest effect. When subcultures were initialized using shoot tips, this decreasing tendency was true for all of the tested cytokinins, excepting 2-iP, for which 10-15 μM induced the longest shoots. If initial explants were nodal segments, there were no significant differences between the effects of concentrations in the same cytokinin types, excepting for BA, when the same was true with regards to the main effects of concentrations. Using shoot tips for establishment of a subculture, the mean length of shoots was either significantly higher or not significantly different compared to experiments initialized with nodal segments (Table 3).

The main effects of the type of cytokinins on shoot length also depended on the type of initial explants. When the experiments were initialized with shoot tips, the mean length of shoots was highest (48.23 mm) using 2-iP. However, when the experiments were initialized with nodal segments, the mean length of shoots was the highest when BA (31.89) or KIN (31.56) were used.

Examining the main effects of either the type or the concentration of cytokinin on the number of nodes per newly developed shoots, it can be concluded that it depended significantly ($P < 0.001$) on the type of initial explants used. When shoot tips were used for the subculture, an increase in the concentration of cytokinins decreased the number of nodes per shoot from 4.56 to 3.87, and using 2-iP and KIN resulted in the highest number of nodes per shoot (5.23 and 4.71, respectively). When applying nodal segments for the subculture, the highest number of nodes per shoot was observed at 5 μM cytokinin (3.97) or when BAR (3.96) was used as the cytokinin. The main effects of cytokinin types were considerably modified with particular concentrations – due to the tight interaction between the concentration and the type of cytokinins – as presented in Table 3.

DISCUSSION

The culture of nodal segments resulted in the highest number of new nodal segments per explant (7.40-9.27) after 4-week-long culture if BA was used in the medium. Multiplication could be improved by using shoot tips for subculture and high concentration (from 10 μM) of BA (10.12-13.38 new nodes per explant) or 10 μM BAR (12.96 new nodes per explant) or 2-iP at 10 μM (12.83 new nodes per explant). However, the best results (13.26-14.42 new nodes per explant) were obtained by replacing BA with TOP at a concentration range from 5 to 15 μM in the medium (Table 2). Application of these TOP concentrations allows high multiplication rate (3.05 to 3.49 new shoots per explant, Table 2) and shoots with shoot length of 40.33-44.53 mm (Table 3, Fig. 3) suitable for rooting directly after shoot multiplication. From these results about 14^{11} (4.05×10^{12}) shoots from a single shoot tip can be multiplied yearly, therefore this procedure is suggested for the shoot multiplication of *Fagopyrum esculentum* cv. 'Hajnalka'. A similarly high multiplication efficiency (11.6-16.5 new nodal segments per explant) of common buckwheat was achieved earlier only in the case when a two-step method was applied and nodal segments regenerated on MS medium with 1 mg/l (4.44 μM) BA being transferred to PGR-free medium (Kločová and Gubišová 2008) (Table 1). The high efficiency in our experiment may be due to the fact that the side-effects of BA could be avoided by the use of TOP. The harmful effects of BA may be caused by the N^7 - and N^9 -glucosylation or alanine conjugation resulting in biologically inactive but chemically very stable derivatives and slow release of BA



Fig. 3 Effects of different concentrations of TOP on the shoot multiplication when shoot tips were used for the subculture.

from the derivatives (Werbrouck *et al.* 1995). Using hydroxylated BA analogues, such as TOP, could be an alternative way to avoid the side effects of BA as described earlier in other plant species (Werbrouck *et al.* 1996; Strnad *et al.* 1997; Dobránszki *et al.* 2005). Moreover, in the experiments of Kločová and Gubišová (2008) these harmful effects presumably may be avoided by the transfer to hormone-free medium. Other kinds of published alternatives showed lower efficiency; Romchatngoen *et al.* (1998) calculated 20^8 (2.56×10^{10}) new shoots per year on half-strength MS medium supplemented with 20 μ M BA and 0.15 g/l Gellan gum but generally the number of new nodes was around 4 (Bohanec 1987; Nešković *et al.* 1990; Kločová and Gubišová 2008) (Table 1).

For efficient micropropagation the correct choice of the explant type is important because they have different capacity for regeneration and their reactions to PGRs could be distinct (Gaham and George 2008). In the present experiments the efficiency of shoot multiplication was improved by using shoot tips for a subculture similar to the earlier results of Romchatngoen *et al.* (1998). In contrast, in the experiments of Kločová and Gubišová (2008) regeneration from nodal segments was the most efficient technique of buckwheat micropropagation. Moreover, a significant interaction was observed between the effects of explant type used in the subculture and the cytokinin applied. Results are in agreement with earlier results in which the uptake of different cytokinins from the medium, their transport and metabolism in the plants are different and they can interact with the endogenous cytokinins of an explant distinctly (Strnad *et al.* 1997; Werbrouck *et al.* 1996; van Staden *et al.* 2008).

In conclusion, these results indicate that shoot multiplication from shoot tip explants on MS medium with 10 μ M TOP was the most efficient technique and could be the part of an efficient micropropagation protocol for *F. esculentum* cv. 'Hajnalka'.

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