Evaluation of Different Approaches to Buckwheat (*Fagopyrum esculentum* Moench.) Micropropagation

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Abstract: Plant regeneration by different techniques was evaluated in three buckwheat cultivars: indirect regeneration from cotyledons and hypocotyls, direct regeneration by nodal segment cultivation and induction of multiple shoots from seedling apices. Regenerated shoots were obtained in all procedures. The effects of BA (6-benzylaminopurine), media composition and gelling agent were tested. The regeneration efficiency of shoot apex culture was 2.65–3.33 nodal segments/explant. Cotyledon and hypocotyl segments produced 1.25–2.44 shoots per explant plated. Nodal segment cultivation yielded 4.1–4.8 new nodal segments/explant in 4 weeks. Eighty percent of shoots rooted on the basal medium. Rooting was improved (up to 95.6%) by IBA (3-indolebutyric acid) addition to the culture medium. Regenerated plantlets were transferred to the soil. The most efficient and simple micropropagation of buckwheat was nodal segment cultivation on MS medium solidified by agar with the addition of 1 mg/l BA. This method is advisable for rapid multiplication, *in vitro* conservation or rescue of genetic resources.

Keywords: buckwheat; *in vitro* regeneration; micropropagation; nodal segment; rooting

Buckwheat is known as a moderately productive crop cultivated in countries of the northern hemisphere. The use of buckwheat in human diets is highly recommendable because of the high quality of easily digestible protein and good mineral content (Edwardson 1996). *In vitro* techniques enable rapid multiplication of plants, propagation of pathogen-free material, genetic transformation, long-term conservation and genetic resources rescue.

In vitro regeneration is possible via organogenesis or somatic embryogenesis. The choice of the technique is dependent on the aim of work. Direct organogenesis avoids the callus phase and decreases therefore somaclonal variation, so it is preferred in the case of plant micropropagation. Somatic embryogenesis is mostly advantageous in case of genetic transformation or large-scale production. Regeneration *in vitro* is influenced by many factors such as genotype, type of explant, culture media, growth regulators, gelling agent, physical conditions of culture, etc. An efficient regeneration protocol is needed for the application of *in vitro* techniques and procedures. For the rapid multiplication of species a simple protocol based on axillary and apical meristem shoot proliferation is preferable. The same technique may be used to recover gene resources from a low number of seeds or seedlings and to conserve gene resources *in vitro*. To change plant traits in a desirable way by gene technology, *in vitro* regeneration is needed. Each species usually requires an optimisation of culture conditions.

The regeneration of buckwheat from leaf and stem explants was reported by Rajbhandari *et al.* (1995). The cotyledons isolated from seeds were used as explants by Srejović and Nešković (1981), Miljuš-Djukić *et al.* (1992), Luthar (1996). Shoot induction was also achieved from hypocotyls (Jin

et al. 2002) and cotyledons of seedlings (Woo *et al*. 2000). Nešković *et al*. (1987) cultivated isolated embryos of buckwheat. Regeneration of shoot apices from seedlings was described by Bohanec (1987) and Nešković *et al*. (1990).

In this report, various techniques of buckwheat plant regeneration were compared. It was aimed to develop a simple and efficient micropropagation protocol for buckwheat.

MATERIAL AND METHODS

Three cultivars of buckwheat – Špačinska, Bogatyr and Idel – were provided by the Gene Bank of the Slovak Republic, Piešťany. Their germination ranged between 75% and 95%. Seeds were surface sterilised by rinsing with 70% (w/v) ethanol and immersed for 10 min in 0.1% (w/v) $HgCl_2$ solution, to which 2 drops of the surfactant Tween 20 per 100 ml were added, and washed in sterile distilled water. Afterwards seeds were left to germinate on the basal MS medium (Murashige & Skoog 1962) under a 16/8 h photoperiod. The explants were isolated from 7- or 14-days-old seedlings. Twenty-four to thirty-two explants were plated per variant. Experiments were carried out in two replicates.

All the media were supplemented with sucrose (30 g/l) and agar (8 g/l). The media were adjusted to pH 5.8 and autoclaved at 121°C for 25 min. The cultures were incubated at 25/20°C under 16/8 h photoperiod with light intensity 50 μmol/m^2/s. The subculture interval lasted 4 weeks.

Indirect organogenesis

Cotyledons and hypocotyls were isolated from 7-days-old seedlings of cultivars Špačinska and Bogatyr.

Effect of media composition: Two ways of cultivation were compared. The first one (Rajbhandari *et al*. 1995) – to induce regeneration, explants were grown on MS medium with 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) for 2 weeks, then on MS medium with 4 mg/l BA (6-benzylaminopurine) for another 2 weeks and subsequently on MS medium with 2 mg/l BA and 0.2 mg/l IAA (3-indoleacetic acid). The second way was the cultivation on B5 medium (Gamborg *et al*. 1968) with the addition of 5 mg/l 2,4-D and 0.1 mg/l KIN (kinetin) for 5 days, then the explants were transferred onto B5 medium with 2 mg/l BA and 0.2 mg/l IAA (Srejović & Nešković 1981).

In the additional experiment, we compared regeneration of cotyledons isolated from mature seeds and cotyledons isolated from 7-days-old seedlings by the second way of cultivation.

Nodal segment culture

The explants of all three studied cultivars were prepared by cutting 14-days-old seedlings to individual nodal segments.

Effect of growth regulators: The nodal segments from seedlings were left to regenerate in the presence of BA (0.5 or 1 mg/l) on the MS medium.

Effect of basal media: Three types of basal media without growth regulators were tested – MS, B5 and MSB (MS salts with B5 vitamins).

Effect of gelling agents: To evaluate the effect of different gelling agents, agar (8 g/l), Phytagel (2.5 g/l) and combination of 4 g/l agar and 1.25 g/l Phytagel were applied.

Cultivation of shoot apices

The shoot apices were isolated from 7-days-old seedlings (cultivar Špačinska).

Effect of media composition: The explants were inoculated on the media MS, B5 and MSB (MS salts with B5 vitamins) supplemented with BA (2.2 mg/l) and IAA (0.2 mg/l).

Rooting

To improve rooting of regenerated shoots, half-strength MS medium (1/2 MS) was used and 0.25, 0.5, 1 mg/l IBA (3-indolebutyric acid) was added. Rooted plantlets were rinsed with fungicide [0.15% (w/v) Previcure N, Bayer Crop Science AG, Monheim, Germany], transferred to the soil and acclimatised to *ex vitro* conditions.

In the experiments, we evaluated the regeneration efficiency (number of new nodal segments/explant plated or number of shoots/explant plated – in the case of indirect organogenesis), shoot length (mm) and rooting frequency (%). Data were processed by the analysis of variance. Least significant differences were calculated for the treatment means.

RESULTS

Buckwheat plant regeneration was achieved by all three techniques.

Indirect organogenesis

The first one was the indirect regeneration from cotyledon and hypocotyl segments. The explants were isolated from 7-days-old seedlings. After 2 weeks of cultivation, calli were formed and some of the explants rooted. Better callus formation was observed from hypocotyl explants. Shoot production occurred after 4 weeks. The cultivation on MS or B5 medium yielded 1.25–2.44 shoots per explant.

Explant type: The regeneration was more effective from hypocotyl explants (Figure 1).

Effect of media composition and cultivars: There were no significant differences between the effect of the tested media or the tested cultivars.

In the additional experiment, similar shoot regeneration was obtained by the cultivation of cotyledon segments from seedlings (cultivar Špačinska 1 shoot/explant, cultivar Bogatyr 0.29) and isolated cotyledons from seeds (cultivar Špačinska 0.83, cultivar Bogatyr 0.28).

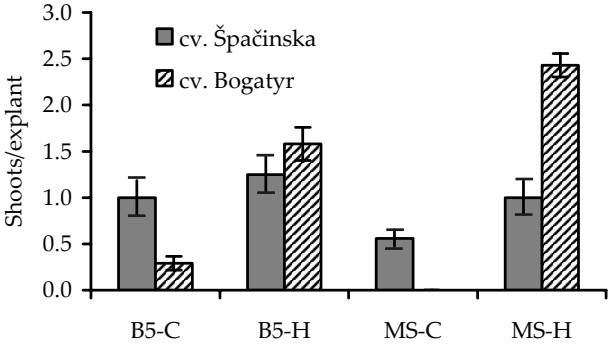


Figure 1. Buckwheat regeneration from cotyledons (C) and hypocotyls (H) of 7-days-old seedlings on MS (with 1 mg/l 2,4-D for 2 weeks, with 4 mg/l BA for 2 weeks, finally with 2 mg/l BA and 0.2 mg/l IAA) and B5 media (with 5 mg/l 2,4-D + 0.1 mg/l KIN for 5 days, then with 2 mg/l BA + 0.2 mg/l IAA) in the cultivars Špačinska and Bogatyr, evaluated after 6 weeks of culture; data represent the mean of 32 explants from 2 replicates

Nodal segment culture

Direct organogenesis occurred from the nodal segments of 14-days-old seedlings.

Cultivars: We found out no cultivar differences.

Effect of growth regulators: The explants were cultivated on MS media with BA. The addition of BA to the culture medium significantly improved the regeneration ability of explants (Table 1). Regeneration efficiency of nodal segments regenerated on MS medium with 1 mg/l BA was increased (3.1-times) after transfer to hormone-free medium (Figure 2).

Effect of basal media: To evaluate the effect of basal media composition on buckwheat regeneration we tested MS, B5 and MSB (MS salts with B5 vitamins) media. The growth of buckwheat nodal segments did not change significantly when using media with various compositions (Table 2).

Effect of gelling agents: The results of the application of different gelling agents (agar, phytagel, their combination) indicated that agar was the most effective one for buckwheat. It was demonstrated by growth and also by rooting responses (Table 3). However, the cultivar Idel showed significantly lower regeneration ability than the other two cultivars.

Cultivation of shoot apices

Another approach to buckwheat micropropagation was the cultivation of apical parts of seedlings. Shoot apices of 7-days-old seedlings were cultured on MS, B5 or MSB medium with BA and IAA.

Table 1. The effect of 6-benzylaminopurine (BA) concentration on regeneration of three buckwheat cultivars from nodal segments; evaluated after 4 weeks of culture; data represent the mean ± SE of 24 explants from 2 replicates per cultivar; different letters designate a significant difference ($P \leq 0.05$)

Medium	Špačinska	Bogatyr	Idel	Mean ± SE	Špačinska	Bogatyr	Idel	Mean ± SE
	No. of shoots/explant				No. of new nodal segments/explant			
MS (control)	1.12	1.03	1.13	1.09 ± 0.132c	3.03	2.89	2.59	2.84 ± 0.203c
MS + 0.5 mg BA	1.77	2.29	1.97	1.86 ± 0.126b	3.88	4.08	3.69	3.88 ± 0.193b
MS + 1mg BA	3.05	2.29	2.91	2.74 ± 0.127a	4.76	4.47	4.14	4.46 ± 0.194a
Mean ± SE	1.98 ± 0.132a	1.72 ± 0.12a	2.00 ± 0.033a		3.89 ± 0.202a	3.81 ± 0.184a	3.47 ± 0.204a	

Table 2. The effect of basal culture media MS, B5, MSB (MS salts with B5 vitamins) without growth regulators on buckwheat shoot regeneration and root formation from nodal segments (cv. Špačinska), evaluated after 4 weeks of culture; data represent the mean ± SE of 24 explants from 2 replicates per basal medium; different letters designate a significant difference ($P \leq 0.05$)

Medium	No. of new nodal segements	Mean length of shoots (mm)	Rooting (%)
B5	4.50 ± 1.469a	70.38 ± 39.75a	77.85
MSB	4.74 ± 1.14a	82.23 ± 32.918a	72.65
MS	4.71 ± 1.05a	97.29 ± 36.875a	92.9

Table 3. The effect of gelling agent on buckwheat propagation from nodal segments of three cultivars on basal MS media without growth regulators (a – agar, ph – phytagel); evaluated after 4 weeks of culture; data represent the mean ± SE of 32 explants from 2 replicates per gelling agent treatment; different letters designate a significant difference ($P \leq 0.05$)

Medium	Špačinska	Bogatyr	Idel	Mean ± SE	Špačinska	Bogatyr	Idel	Mean ± SE	Rooting (%)
	No. of new nodal segments/explant				shoot length (mm)				
MSa	4.51	4.30	3.68	4.16 ± 0.128a	94.17	91.68	62.52	82.79 ± 3.676a	69.7
MSph	3.83	3.69	3.58	3.7 ± 0.137b	73.54	67.60	63.43	68.19 ± 3.925b	65.5
MSa/ph	3.59	3.78	3.25	3.54 ± 0.142b	64.19	87.43	68.26	73.29 ± 4.052ab	58
Mean ± SE	3.98 ± 0.13a	3.92 ± 0.142a	3.50 ± 0.135b		77.3 ± 3.708a	82.24 ± 4.07a	64.73 ± 3.875b		

Proliferated shoots were separated and placed onto the basal medium. Differences between the used media were not statistically significant (data not shown). Regeneration efficiency ranged from 2.65 to 3.35 nodal segments per explant and the best rooting frequency occurred on MS medium (Figure 3).

Rooting

The rooting of buckwheat shoots on the basal MS medium amounted to 78.55%. The cultivation of shoots on a medium with decreased concentration of salts (half-strength MS macronutrients and micronutrients) had no positive effect on rooting

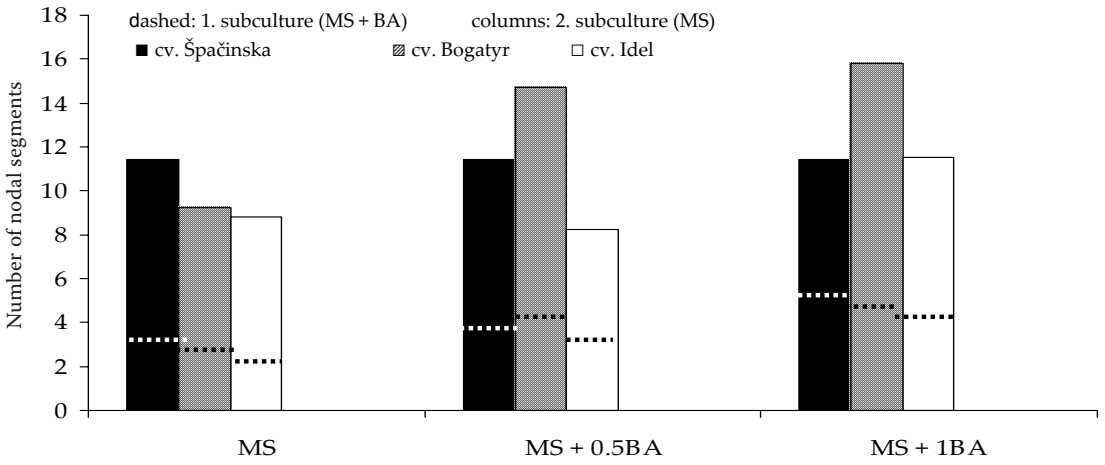


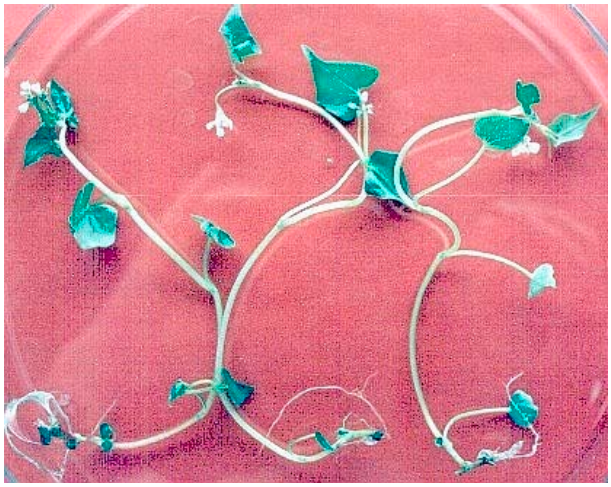
Figure 2. Increasing of buckwheat micropropagation from nodal segments after two subcultures: the first subculture on media with BA (evaluated after 4 weeks), the second one on hormone-free media (evaluated after 8 weeks), in the cultivars Špačinska, Bogatyr and Idel

Table 4. The effect of IBA and half-strenght concentration of salts (1/2 MS) on the rooting of buckwheat shoots regenerated from nodal segments (pooled data for three cultivars), evaluated after 4 weeks of culture; data represent the mean of 32 explants from 2 replicates per medium variant; different letters designate a significant difference ($P \leq 0.05$)

Medium	Rooting (%)
MS (control)	78.55b
MS + 0.2mg IBA	80.35b
MS + 0.5mg IBA	89.05ab
MS + 1mg IBA	95.57a
½ MS	42.5c

(42.5%), however, the shoot treatment with IBA (1 mg/l) significantly increased rooting frequency (Table 4). Rooted plantlets were transferred to the soil. Survival of transferred plants was successful in 41.4%, 57.1% and 62.5% in cv. Bogatyr, Idel and Špačinska, respectively (Figure 4).

(A)



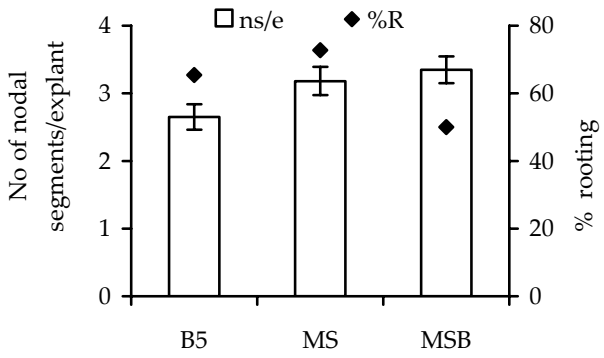


Figure 3. Buckwheat regeneration from apices of 7-days-old seedlings (cultivar Špačinska) on MS, B5 and MSB media (MS salts with B5 vitamins) with 2.2 mg/l BA and 0.2 mg/l IAA, evaluated after 6 weeks of culture; data represent the mean of 24 explants from 2 replicates per basal medium variant

(B)

Figure 4. Regenerated buckwheat plants cv. Špačinska (A) and their acclimation (B)

DISCUSSION

Various techniques of buckwheat plant regeneration are presented in this report: induction from hypocotyls or cotyledons, culture of nodal segments and regeneration of multiple shoots from shoot apices. We have obtained with all three techniques regenerated plants of the tested buckwheat cultivars (Idel, Špačinska and Bogatyr). The shoot regeneration of cultivated hypocotyl segments was 1.25–2.44 shoots/explant. The cotyledons were less responsive (0.28–1.0). There was no difference between seedling cotyledons and cotyledons isolated from seeds. Srejović and Nešković (1981) cultivated isolated cotyledons on B5 medium with 5 mg/l 2,4-D and 0.1 mg/l

KIN. They found that the shoot formation could be induced only when explants were transferred after 3–5 days to another medium with a high cytokinin to auxin ratio. Using such a protocol, Miljuš-Djukić *et al*. (1992) observed 35% and Luthar (1996) 1.14% regeneration from isolated cotyledons. Woo *et al*. (2000) reported somatic embryogenesis of buckwheat in 32% of cotyledon explants. Jin *et al*. (2002) regenerated buckwheat plants from hypocotyl explants on MS medium supplemented with 2 mg/l BA and 1 mg/l KIN. Gradual cultivation on MS medium with 2,4-D and BA or MS medium with BA and IAA was used to regenerate plants from leaf and stem segments by Rajbhandari *et al*. (1995). The authors indicated genotypic differences in buckwheat, in contrast to our observations. We compared regeneration according to Srejović and Nešković (1981) and Rajbhandari *et al*. (1995), and no differences were found in regeneration efficiency.

The culture of nodal segments produced 4.1–4.8 new nodal segments per explant plated. In the second subculture, the efficiency increased to 11.6–16.5 (number of new nodal segments/explant plated). BA was added to the culture medium to improve multiplication (concentration 1 mg/l was superior). We did not observe any differences in growth between basal media composition (MS, B5, MSB). Published papers described MS or B5 media used in equal proportions in buckwheat *in vitro* cultivation. The gelling agent significantly influenced regeneration efficiency. Agar was found to be the best gelling substance for buckwheat. However, Veramendi *et al*. (1997) concluded that Phytagel can allow in potatoes better micropropagation and microtuberization than agar.

Shoot apices of seedlings were cultivated on a medium with 0.2 mg/l IAA and 2 mg/l BA according to Nešković *et al*. (1990). The regeneration efficiency of 2.65–3.35 nodal segments/explant was recorded. Our results are similar to those reported by other authors (Bohanec 1987 – 3.9 nodal segments/explant; Nešković *et al*. 1990 – 4 nodal segments/explant). Several shoots produced flowers, which was also documented by Bohanec (1987).

Regenerated shoots rooted on the basal MS medium at the frequency 78.55%. To improve rooting, IBA (0.2, 0.5, 1 mg/l) was applied. The concentration of 1 mg/l increased rooting frequency to 95.6%. Jin *et al*. (2002) used NAA (1-naphthalene acetic acid) with IBA for root initiation. Other reports described the addition of only IBA to the media to improve rooting (Nešković *et al*. 1987; Srejović & Nešković 1981; Bohanec *et al*. 1993; Miljuš-Djukić *et al*. 1992). 41.4%–62.5% of plants were successfully transferred to the soil. It is in agreement with published results, where acclimation ranged between 30% and 50% (Nešković *et al*. 1990; Luthar 1996).

In consequence, our results indicated that regeneration from nodal segments on MS medium with 1 mg/l BA solidified by agar was the most efficient technique of buckwheat micropropagation and also of *in vitro* maintenance. This method is advisable for rapid multiplication, *in vitro* conservation or gene resources rescue. In contrast, indirect regeneration from cotyledons or hypocotyls may be useful for obtaining higher range of somaclonal variation.

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