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In vitro regeneration of shoot buds and plantlets from seedling root segments of *Brassica napus* L.

Dedicated to Dr. Friedrich Constabel on the occasion of his 60th birthday

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Abstract. Root segments obtained from aseptically germinated seedlings of *Brassica napus* cv. Westar were used to optimize conditions for high-frequency shoot bud differentiation. The presence of low kinetin (0.5 μM) and relatively high indole-butyric acid (1.0 μM) levels facilitated optimum shoot bud differentiation. Modified MS medium (MMS) was superior to the other three basal media tested (MS, B5 and White's). Elevated sodium dihydrogen phosphate levels increased the differentiation of shoot buds. Increasing or decreasing the level of sucrose from 3% reduced the frequency of explants forming shoot buds. Addition of glutamine enhanced both the frequency of responding explants, as well as the number of shoots per responding explant. Root segments from 13-day-old seedlings produced the highest response (58%) in the presence of 100 mg l^{-1} glutamine. The position of the segment on the main root, size, and the presence or absence of lateral roots altered the morphogenic response. Sealing of the donor seedling cultures with Parafilm® instead of Stretch' n seal® resulted in a higher production of shoot buds, although root segment cultures were not affected by the type of sealing. Spontaneous rooting occurred on all developed shoots.

Introduction

Almost all the economically important species of the genus *Brassica* have been regenerated from in vitro cultures. In *B. napus*, shoot bud differentiation has been obtained from diverse explants which include leaf discs [4], stem cell layers [12], root protoplasts [26], hypocotyl-derived protoplasts [7], anthers [11] and isolated microspores [20]. Although seedling root explants have been used successfully for obtaining plantlet regeneration in other *Brassica* species, e.g. *B. oleracea* [2, 14] and *B. carinata* [10], very little work has been carried out with *B. napus* root segments, except for a comparative response from two cultivars of this species with that of *B. oleracea*, where 2.6% and 27% of the explants regenerated shoot buds [14].

Root cuttings have been previously used for the propagation of breeding stocks of *B. napus* var. *napobrassica* in vitro [8] and of *B. oleracea* in vivo [9]. Some abnormalities, like 'glossy' plants with reduced or altered leaf wax architecture, multiple branching of the stem, precocious flower formation from the apex, the stem, or the leaves, and abnormal leaves, were reported in the latter study, and it was suggested that these resulted from chromosomal mutations. No such abnormalities or variations in shoots formed from roots have been reported, but it is expected that if these shoots are derived from single cells or cell clusters, they may possess variability to be exploited for agronomic improvement of this crop. Indeed, there is evidence to suggest that plants regenerated from somatic explants directly or after slight callusing can be utilized to obtain somaclonal variants [3, 25]. Furthermore, establishment of fast-growing root cultures which maintain their totipotency over several subcultures [15] can provide an excellent source of test material for mutation breeding and selection experiments. In pursuit of these objectives, we undertook studies on optimization of conditions for the regeneration of shoots from root segments of *Brassica napus* cv. Westar.

Material and methods

Seeds of *Brassica napus* cv. Westar, obtained from Dr. G. Seguin-Swartz of Agriculture Canada, Saskatoon, were sterilized with 0.2% (w/v) mercuric chloride. After 3–4 rinses in sterile distilled water, 15 seeds were sown onto 25 ml hormone-free Murashige & Skoog (MS) medium [19] at pH 5.8 (adjusted prior to autoclaving) and solidified with 0.8% (w/v) Difco Bacto agar in 50 × 19 mm sterile plastic Petri plates. The cultures were initially kept at 5°C in the dark for 24 h to ensure synchrony of seed germination, and then incubated at 25°C with a 16 h photoperiod from Gro-Lux fluorescent tubes at a photon fluence rate of ca. 80 μmol $m^{-2}s^{-1}$. Unless otherwise mentioned, the plates were routinely sealed with Parafilm® (American Can Co.).

Root segments were usually obtained from 14 day-old seedlings, except in one experiment where root segments were taken from 5, 7, 11, 13, 16, 20 and 23-day-old seedlings. In an experiment designed to select the best explant, segments from three distinct regions of the primary root were used. They were S1, which comprised of the proximal 10 mm of the root 5 mm below the hypocotyl/root junction, S2 comprising a 10 mm length midway, and S3 comprising of the distal 10 mm length 5 mm behind the root tip. For all the subsequent experiments the proximal 10 mm root segment (S1) was generally used, after removing pre-existing lateral roots. In a separate experiment where S1 segments were used, all the lateral roots were left intact in order to study their influence on organogenesis.

Unless otherwise mentioned, the organogenic response of root segments was compared on modified MS medium (MMS) [14] containing 0.5 μM kinetin and 1.0 μM indole-butyric acid (IBA). This combination was found to be optimum for shoot bud differentiation compared to concentrations of 0, 0.1, 0.5, 1.0 and 5.0 μM tested in a Latin square design. MMS medium differs from the MS medium in that it comprises of the inorganic constituents of MS and 300 mg l^{-1} NaH_2PO_4 instead of 170 mg l^{-1} KH_2PO_4, as well as the organic constituents present in B5 medium [6]. Other media variables included with MMS were different concentrations of picloram (4-amino-3,5,6-trichloropicolinic acid) or naphthaleneacetic acid (NAA) instead of IBA; N^6-benzyladenine (BA) or 2-isopentenyladenine (2-iP) instead of kinetin, sucrose, glucose, casein hydrolysate (CH), glutamine, and activated charcoal. MS, B5, and modified White's medium [14] were also compared with MMS. The phosphorus content of B5 and MMS was also manipulated by either doubling the concentration of NaH_2PO_4 in the former, or adding 170 mg l^{-1} KH_2PO_4, as in MS, instead of 300 mg l^{-1} NaH_2PO_4 in the latter.

Eight explants were inoculated onto each Petri plate containing 25 ml of the appropriate medium and solidified with 0.8% Difco Bacto agar at pH 5.8 adjusted prior to autoclaving). The plates were routinely sealed with Stretch' n seal®, except in one experiment where Parafilm® was used as the sealant. At least 40 explants were used per treatment and the cultures were incubated under the conditions reported above. Each experiment was repeated at least twice. The cultures were checked weekly for organogenesis and the final observations were made after 40 days. Significant differences amongst means were tested using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test [27]. The data comprising of frequencies were analysed by χ^2 test of homogeneity of proportions, and significant treatment differences selected by post hoc multiple comparisons test [18].

Results

The cultured root segments produced 2–5 new lateral roots within 4–6 days after culture initiation (Fig. 1A). This was followed by greening of the explant and the appearance of nodular structures by 8 days (Fig. 1B). By 10–12 days the first shoots appeared and continued to develop up to 20–25 days (Fig. 1C). All the green nodules did not develop into shoots. Shoots generally appeared either at the proximal cut end or near the middle region and never at the distal cut end where more lateral roots were produced. Whenever two or more shoots appeared, they were always around the same locus. All of the shoots started forming roots on the same medium within

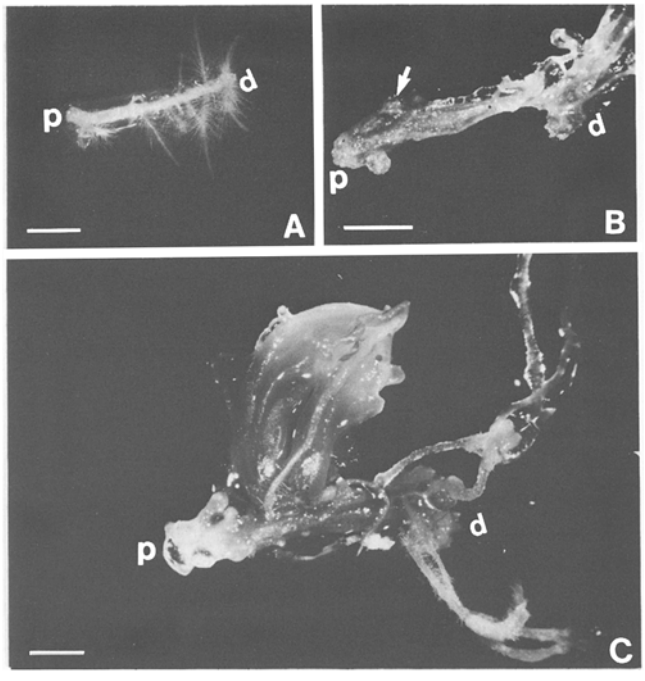

Fig. 1. Regeneration of shoot buds from seedling root segments of *Brassica napus* on MMS medium containing 0.5 μM kinetin and 1.0 μM IBA. (*A*) 4-day-old culture showing the formation of new lateral roots prior to shoot bud differentiation. Note the lateral roots are more numerous at the distal end (d) than at the proximal end (p). Bar = 3 mm. (*B*) 8-day-old culture showing the formation of green nodules (arrow) mainly on the proximal end (p) of the root segment. Bar = 3 mm. (*C*) 20-day-old culture showing a fully developed shoot formed from the middle portion of the explant. Note the presence of numerous lateral roots at the distal end (d). Bar = 2.0 mm.

40 days in culture, and could be directly transplanted to a peat/vermiculite (1:) mixture with 100% success (Fig. 2A). The transplanted shoots flowered normally after 35 days (Fig. 2B) and produced seeds.

Growth regulators

The presence of both cytokinin and auxin was essential for shoot bud differentiation. Shoots formed only on media containing either 0.5 μM or 1.0 μM kinetin in combination with all the IBA levels tested, although the frequency varied; hence data are presented for these two kinetin levels only (Fig. 3). Generally, media containing 0.5 μM kinetin produced higher frequencies of shoots with all five levels of IBA. The optimum combination of phytohormones was found to be 0.5 μM kinetin and 1.0 μM IBA. With this

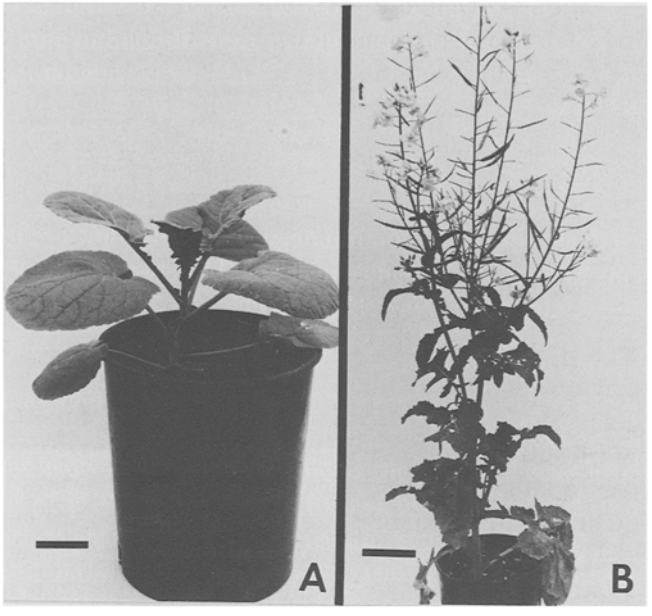

Fig. 2. A normal plantlet derived from a seedling root segment of *Brassica napus*: (*A*) 6 weeks (bar = 4 cm), and (*B*) 12 weeks (bar = 8 cm) after transplantation to peat/vermiculite mixture.

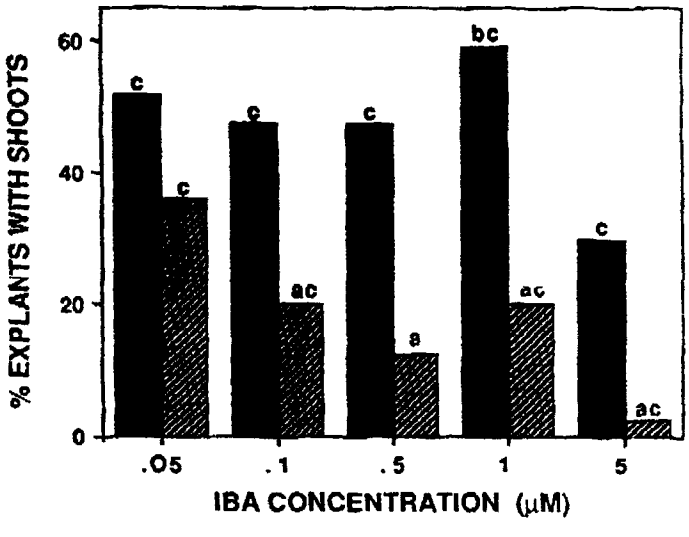

Fig. 3. Effect of kinetin and IBA on shoot bud differentiation from seedling root segments. ■ 0.5 μM kinetin, ■ 1.0 μM kinetin. Basal medium: MMS. Columns with similar letters are not significantly different at $P = 0.05$ (post hoc multiple comparisons test).

combination, although the number of shoots per responding explant was no different than other combinations, a greater proportion of the explants formed shoot buds. Substitution of IBA with picloram or NAA, and of kinetin by BA or 2-iP was inhibitory for shoot bud differentiaion at all equimolar levels tested (data not presented).

Basal media

Four different basal media were tested to examine their effect on the morphogenic potential of the root segments. MS, MMS with the same level of NaH_2PO_4 or KH_2PO_4 as in MS, B5 with the same or double the level of NaH_2PO_4(equal to that present in MMS), and White's were used. All the media proved to be inferior to MMS for shoot bud differentiation from the root segments (Table 1). However, MMS and B5 media were comparable in terms of green nodule formation on the explants. Increasing the level of NaH_2PO_4 twofold in B5 medium increased the frequency of explants with shoots by more than twofold in comparison to normal B5 medium, while reducing it in MMS to that of MS in the form of KH_2PO_4 greatly reduced shoot formation.

Media addenda

MMS medium was variously modified with the addition of different levels of sucrose, glucose, glutamine, CH and charcoal individually to test their

Table 1. Effect of four different media on the morphogenic response of root segments of *Brassica napus*.

Medium[1]	Explants with roots[2] (%)	Explants with green nodules[2] (%)	Explants with shoots[2] (%)	Number of shoots per explant[3]
MS	87ab	22a	9c	0.4 ± 0.02ac
MMS	98b	80b	53b	2.1 ± 0.04b
MMS ($-NaH_2PO_4$) + 170 mg l^{-1} KH_2PO_4	65a	46ac	26cb	0.9 ± 0.04bc
B5	88ab	63bc	10cd	1.2 ± 0.02bc
B5 + 2 × NaH_2PO_4	86ab	74bc	28bd	1.0 ± 0.03bc
White	79ab	23a	3c	0.5 ± 0.02ac

[1] All media contained 0.5 μM kinetin and 1.0 μM IBA with 3% sucrose.

[2] In each column, numbers followed by the same letters are not significantly different at $P = 0.05$ (post hoc multiple comparison test).

[3] Mean ± SE; means followed by the same letters are not significantly different at $P = 0.05$ (Tukey's multiple comparisons test).

Table 2. Effect of sucrose and glutamine on the morphogenic response of root segments of *Brassica napus*.

Constituent[1]	Explants with roots[2] (%)	Explants with green nodules[2] (%)	Explants with shoots[2] (%)	Number of shoots per explant[3]
Sucrose ($g\,l^{-1}$)				
10	65ac	19a	7a	0.4 ± 0.02ac
20	91a	73bc	17a	0.9 ± 0.05bc
30	90a	81bc	49b	1.7 ± 0.06d
40	72ac	85c	21ab	1.0 ± 0.03b
50	40bc	66bc	17ab	0.8 ± 0.05b
60	25b	51ab	3a	0.7 ± 0.03ab
Glutamine ($mg\,l^{-1}$)				
25	79a	75a	29a	0.9 ± 0.06a
50	80ab	70a	39ab	0.8 ± 0.05a
100	93ab	82ab	58b	2.8 ± 0.10d
200	100b	90ab	52ab	1.9 ± 0.08c
300	100b	100b	48ab	1.8 ± 0.06bc
400	90ab	100b	46ab	1.6 ± 0.05b

[1] All media contained MMS with 0.5 μM kinetin and 1.0 μM IBA. In the sucrose experiments, glutamine present at $100\,mg\,l^{-1}$, while in the glutamine tests, sucrose present at $30\,g\,l^{-1}$. Data for sucrose and glutamine experiments analysed separately

[2] In each group, numbers followed by the same letters are not significantly different at $P = 0.05$ (post hoc multiple comparisons test)

[3] Mean ± SE; means in each group followed by the same letters are not significantly different at $P = 0.05$ level (Tukey's multiple comparisons test)

effect on the morphogenic response of cultured root segments. Amongst these, all the levels of glucose tested, viz. 1,2,3,4 and 5% proved to be totally inhibitory for both lateral root and shoot bud differentiation. CH (25,50 and $100\,mg\,l^{-1}$) and charcoal (0.05 and 0.5%) did not affect the morphogenic response (data not presented). Sucrose (3%) already present at that level in MMS was optimal for shoot bud differentiation. Varying sucrose by decreasing the level reduced the frequency of responding explants, while the number of shoots per responding explant was not affected (Table 2). Higher levels of sucrose were also inhibitory for the formation of lateral roots, as well as the formation of green nodules. Glutamine at a concentration of $100\,mg\,l^{-1}$ or more produced a marginally superior response, both quantitatively and qualitatively (Table 2). With glutamine, the shoots appeared 3–4 days earlier. Glutamine was also beneficial in the formation of lateral roots and green nodules on the explant.

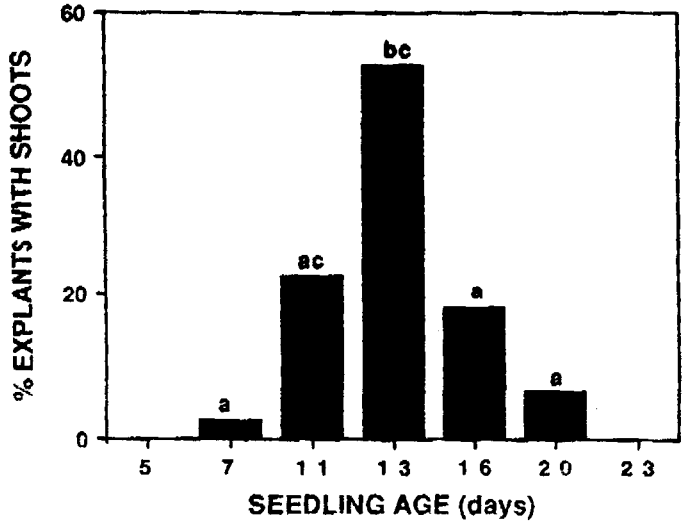

Fig. 4. Effect of age of the explant donor seedlings of *Brassica napus* on shoot bud differentiation from seedling root segments. Culture medium: MMS + 0.5 μM kinetin + 1.0 μM IBA. Columns with similar letters are not significantly different at $P = 0.05$ (post hoc multiple comparisons test).

Seedling age

Seedling age greatly influenced the differentiation of shoot buds from root segments. Explants taken from 5-day-old seedlings failed to respond. Root segments from older seedlings responded positively, reaching a maximum at 13 days (Fig. 4). After this the frequency of responding root segments again declined with increasing seedling age. However, the age of donor plants had no influence on the number of shoots per responding explant.

Explant

Three types of root segments, viz. S1, S2 and S3, were used to compare their morphogenic potentials. Adventitious shoot buds could only be obtained from S1. Segments S2 and S3 failed to produce shoots or S2 regenerated shoot buds only sporadically and in an inconsistent manner (Table 3). The presence of lateral roots inhibited shoot bud differentiation. Only 8–10% of the S1 segments produced shoots compared to about 60% when the S1 segments were devoid of lateral roots. Reducing the size of the S1 segment from 10 mm to 5 mm also reduced shoot bud differentiation, where the frequency of responding explants declined by 40–45% of the controls (Table 3).

Table 3. Effect of the explant origin and size on morphogenic response from root segments of *Brassica napus.*

Explant[1]	Explants with roots[2] (%)	Explants with green nodules[2] (%)	Explants with shoots[2] (%)	Number of shoots per explant[3]
S1	91a	86a	60c	1.8 ± 0.08a
S2	63ac	21b	9ab	1.2 ± 0.12b
S3	23b	20b	0a	
S1 + laterals	40bc	30b	10ab	1.0 ± 0.08b
1/2 S1	65ac	46b	25b	1.0 ± 0.07b

[1] Culture medium: MMS + 0.5 μM kinetin + 1.0 μM IBA + 100 mg l^{-1} glutamine

[2] In each column, numbers followed by the same letters are not significantly different at $P = 0.05$ (post hoc multiple comparisons test)

[3] Mean ± SE; means followed by the same letters are not significantly different at $P = 0.05$ (Tukey's multiple comparisons test)

Sealing of cultures

The type of sealant, viz. Stretch' n seal® or Parafilm®, used for sealing the plates greatly affected the frequency of explants forming shoot buds. Although the type of sealing of the cultured root segments did not affect the response, the sealing of donor seedlings proved to be very crucial (Table 4). The seedling cultures sealed with Parafilm® produced a significantly greater frequency of explants which formed shoot buds. However, the type of sealant used with the seedling cultures or explant cultures did not have any effect on the number of shoot buds per responding explant.

Discussion

The presence of both a cytokinin (low) and an auxin (relatively high) was essential for shoot bud differentiation from the *Brassica napus* root segments. This finding is in agreement with an earlier report [14], where higher levels of kinetin (above 0.5 μM) were inhibitory. This is in contrast to the morphogenic response of the aerial explants, like thin cell layers of *B. napus* stem [12] or leaves and hypocotyls of *B. oleracea* [4,16], where a higher cytokinin:auxin ratio favoured shoot bud differentiation.

The physiological status of an explant is influenced by age of the donor plants. In *Brassica juncea*, 5-day-old seedlings provided the most regenerative cotyledons [22], while in *Pinus radiata*, the cotyledons lose the potential to respond to cytokinin treatment for shoot formation within 3 days after

Table 4. Effect of the type of sealant used for sealing the cultures of explant donor seedlings and seedling root segments of *Brassica napus* on morphogenic response.

Treatment[1]	Explants with roots[2] (%)	Explants with green nodules[2] (%)	Explants with shoots[2] (%)	Number of shoots per explant[3]
Seedlings				
Stretch'n seal®	80.3	72.5ab	20.0a	1.4 ± 0.12
Parafilm®	78.5a	65.6a	56.0b	1.5 ± 0.10
Root explants				
Stretch'n seal®	100b	89.5b	52.0b	1.6 ± 0.08
Parafilm®	95.2ab	92.1b	50.0b	1.7 ± 0.06

[1] The seedling cultures were sealed with Stretch'n seal® or Parafilm® and the explant cultures then sealed with Stretch'n seal®. In the case of root segments, the explant donor seedling cultures were sealed with Parafilm® and the explant cultures then sealed with either Stretch'n seal® or Parafilm®. Culture medium for explants: MMS + 0.5 μM kinetin + 1.0 μM IBA + 100 mg l^{-1} glutamine

[2] In each column, numbers followed by the same letters are not significantly different at $P = 0.05$ (post hoc multiple comparisons test)

[3] Mean ± SE; no significant difference in the response (one-way ANOVA at $P = 0.05$, $F = 1.91$)

germination [1]. As previously observed with root segments of *Brassica oleracea* [14], the morphogenic potential of the root segments of *Brassica napus* also depended on the age of the explant donor seedlings. In both studies, more mature roots proved to be responsive. In contrast, the in vivo root stocks of *Brassica oleracea* formed shoot buds spontaneously irrespective of age of the parent plants [9].

The use of young and meristematic tissues has, in many cases, permitted regenerative cultures, when mature and differentiated explants failed to show such a response [24]. In the present study, as previously observed [14], the proximal segment (S1) was more responsive in terms of formation of adventitious shoot buds. Hence, the lower response (26%) reported to be obtained in *B. carinata* [10] could possibly be due to the fact that they cultured root segments from 7-day-old seedlings and used the middle segment (corresponding to S2). In addition, reducing the size of the 10 mm segments by half was also inhibitory for shoot formation. This may be related to increases in uptake or leakage from the explants or to relatively higher levels of wound-induced compounds in the small explants [17].

The type of sealant used to seal the explant donor seedlings could also modify the physiological status and in turn the regeneration potential of the explants. Sealing the seedling cultures with Stretch' n seal®, which is im-

permeable to gaseous exchange, reduces the potential of their root explants to subsequently differentiate shoot buds in comparison to those sealed with Parafilm®, which is relatively gas permeable. In contrast, the type of sealants used for the explant cultures caused no significant difference in organogenesis. Presumably, therefore, the physiological status of the explant donor seedlings was affected by sealing their cultures with Stretch' n seal®. Thus, the selection of appropriate sealants and closures for the cultures of each plant species and the organ in question may be an important limiting factor determining morphogenic response, as observed also in conifer cultures [13].

In the comparison of different basal media, MMS proved to be most satisfactory, as was reported earlier [14]. MS and White's media were markedly inferior to MMS to B5 although other investigators working with root explants of *Brassica* have used MS medium, without comparing it with other media formulations [1, 10]. It is interesting to note, as indicated earlier, that MMS comprises of organic constituents as present in B5 medium in addition to inorganic constituents of MS and 300 mg l^{-1} NaH_2PO_4 instead of 170 mg l^{-1} KH_2PO_4. As a single component of MMS, NaH_2PO_4 seemed to be very critical for the organogenic response. Although the exact role of phosphorus and its source remains to be worked out, it is known to be actively involved in the energy metabolism of cells, and organogenesis is an energy-dependent process [23].

A reduced nitrogen source has been shown to be essential for organogenesis in several tissue culture systems [5], and root bud formation is known to be stimulated by high nitrogen levels in several species [20]. In the present investigations, although glutamine did not affect shoot formation significantly, it enhanced the process and produced a higher number of shoots per explant. In addition, these were healthier and possessed more vigorous growth.

The beneficial effect of 3% sucrose could not be replaced by glucose, which failed to support any shoot differentiation at any of the tested levels. Even substitution of glucose for some of the sucrose in the medium has been shown to significantly reduce organogenesis [14]. However, for shoot bud differentiation from thin cell layers of *Brassica napus*, sucrose and glucose were equally supportive with the optimal levels at 4% and 3%, respectively [12]. Shoot organogenesis in *B. campestris* leaf discs was also inhibited if the sucrose concentration was greater than 4% [4].

In conclusion, this study has shown that root explants of *Brassica napus* can be effectively used for obtaining direct regeneration of shoot buds (and plantlets) and hence the method is potentially useful for the selection of somaclonal or mutagen-induced variants.

Acknowledgements

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