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HIGH-FREQUENCY DIRECT SHOOT REGENERATION AND CONTINUOUS PRODUCTION OF RAPID-CYCLING *BRASSICA OLERACEA IN VITRO*

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Summary

An *in vitro* method was developed for high-frequency shoot regeneration and continuous production of rapid-cycling *B. oleracea* in large numbers. The high regenerative capacity was tissue-dependent. Developmental polarity (apical and basal ends) of the explants appeared to play a regulatory role in shoot morphogenesis in this system. High-frequency shoot regeneration was obtained with N^6-benzyladenine or thidiazuron-supplemented media. Delayed and reduced regenerative ability of cultures in air-tight vessels and the dramatic suppression of shoot regeneration in internodal explants by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid implicate a possible involvement of ethylene in shoot morphogenesis in this species. Rooting of regenerated shoots of *B. oleracea* occurred readily on α-naphthaleneacetic acid-supplemented media. Rooted plantlets were successfully established in soil and developed normal fertile flowers and viable seeds.

Key words: rapid-cycling brassica; plant development; morphogenesis; ethylene; thidiazuron.

Introduction

Rapid-cycling *Brassica* spp. (RCBs), selected for their short lifecycle (30–60 d), petite habit, high female fertility and rapid seed maturation (Williams and Hill, 1986), have been used as model systems in both basic (Rood and Hedden, 1994) and applied research (Leung and Williams, 1983). Their small genome size, just three- to four-fold larger than that of *Arabidopsis* (Arumuganathan and Earle, 1991), availability of different, well-defined genetic stocks and the ability to cross with cultivated *Brassica* spp. make RCBs an ideal experimental system for conventional as well as molecular breeding programs. However, RCBs are little exploited for molecular breeding of brassicas largely due to the lack of an efficient plant regeneration system needed for transgenic plant production. Therefore, development of high-frequency regeneration protocols would be of primary importance in facilitating gene transfer and further molecular studies on RCBs.

Tissue culture techniques for commercial cultivars and conventional breeding lines of *Brassica* spp. are plentiful (Bajaj and Nietsch, 1975; Narasimhulu et al., 1992; Wong et al., 1996). Our attempts to adapt tissue culture procedures of commercial cultivars to RCBs using leaf, petiole and cotyledon explants were, however, not successful. It has been shown that considerable physiological differences exist between RCBs and their long-duration counterparts, and this was reflected in a recent study in which we have observed significant differences in *in vitro* morphogenic responses between RCBs and cultivated *Brassica* spp. (Teo et al., 1997). Previous investigations on regeneration of these plant types have relied on culturing protoplasts (Loudon et al., 1989; Kik and Zaal, 1993; Hansen and Earle, 1994), anthers (Aslam et al., 1990), cotyledons (Teo et al., 1997), or root segments (Berthomieu and Jouanin, 1992). Notably, one of the highest regeneration frequencies reported, about 67%, was from callus derived from leaf mesophyll protoplasts of *Brassica oleracea* (Hansen and Earle, 1994). As the involvement of the callus phase is known to induce somaclonal variation (Larkin and Scowcroft, 1981), development of an efficient direct shoot regeneration method would be of considerable practical value. We here describe a simple, high-frequency direct shoot regeneration procedure for the continuous production of a rapid-cycling line of *B. oleracea*, a species with several vegetable crops, and discuss different factors regulating shoot differentiation in this culture system.

Materials and Methods

Plant materials and in vitro *seed germination*. A rapid-cycling genotype of *Brassica oleracea* (CrGC 3–1 Rbo base population; Crucifer Genetics Cooperative, USA) was used for all experiments. Surface-sterilized seeds were germinated on a basal medium (BM) consisting of MS minerals (Murashige and Skoog, 1962) (Sigma Inc., St. Louis, MO, USA), 2% sucrose and 0.25% Phytagel™ (Sigma Inc.) in Magenta GA7™ vessels (Sigma Inc.) at 24 ± 1°C under a 16-h photoperiod provided by cool, white fluorescent lamps with a photon flux density of 40 $\mu mol\ m^{-2}\ s^{-1}$.

Shoot regeneration experiments. Explants derived from different parts of seedlings or *in vitro* plants were used for shoot regeneration experiments. Regenerated plants used as the source of explants were maintained for not more than 3 mo. in culture. Cotyledons with 1–2-mm long petioles and 1.5–2-mm long hypocotyl or root segments obtained from 3-d-old seedlings were cultured on BM containing N^6-benzyladenine (BA; 5, 10, or 20 μM), 1-phenyl-3-(1,2,3-thiodiazol-5-yl) urea (thidiazuron, TDZ; 1.0, 2.5, or 5.0 μM), and indole-3-acetic acid (IAA; 0.5 or 1.0 μM), either alone or in

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TABLE 1

EFFECT OF N^6-BENZYLADENINE ON PERCENTAGE SHOOT REGENERATION FROM COTYLEDON AND HYPOCOTYL EXPLANTS EXCISED FROM 3-d-OLD RAPID-CYCLING *BRASSICA OLERACEA* SEEDLINGS AS WELL AS YOUNG AND MATURE LEAF EXPLANTS AFTER 21 d OF CULTURE

	BA (μ*M*)			
Explant	0	5	10	20
Cotyledon	53	93	86	75
	(2.6 ± 0.6)	(9.3 ± 0.7)	(8.6 ± 1.9)	(11.5 ± 1.9)
Hypocotyl	100	92	95	87
	(3.3 ± 0.7)	(10.6 ± 2.4)	(16.2 ± 2.5)	(19.6 ± 2.3)
Young leaf	2.3	19.7	6.9	17.2
	(2.0 ± 0)	(5.0 ± 3.5)	(6.0 ± 2.5)	(3.5 ± 1.2)
Mature leaf	1.4	3.5	2.3	8.1
	(4.0 ± 0)	(5.0 ± 1.5)	(3.0 ± 1.0)	(4.1 ± 1.5)
Root	0	0	0	0

Values given in parentheses are the average number of shoots per regenerative explant ± SE. The SEs of the means was obtained from five or six replicates (for cotyledons and hypocotyls), 10–12 replicates each with four to six explants (for cotyledons and hypocotyls) or six to nine explants (for young and mature leaves).

different combinations. Explants were cultured either in Petri dishes or in Magenta GA7™ vessels with media gelled with 0.7% (w/v) Phytagar™ (Life Technologies Inc., Bethesda, MD, USA) or 0.25% (w/v) Phytagel™. All plant growth hormones were from Sigma Inc.

In another experiment, *in vitro*-developed shoots were excised and grown on BM to approximately 7–8 cm in height. These shoots were then cut transversally into apical (about 3 cm) and basal (about 3 cm) halves. Internodal segments (2–3 mm long) and leaves with intact petioles from the apical and basal portions of the shoot were cultured separately on BM containing 5, 10, or 20 μ*M* BA. Leaves from the apical and basal halves of shoots are referred to as young and mature leaves, respectively, hereafter. Leaf explants were cultured with the lower surface touching and the cut petiolar end embedded in the medium, while the internodal segments from apical and basal portions of the shoot (referred to as apical and basal internodal segments, respectively) were placed horizontally on the culture media.

To study the effect of IAA on shoot regeneration, 2–3-mm long internodal segments were cultured on BM enriched with 0.5, 1.0, or 2.0 μ*M* IAA alone or in combination with 5.0 μ*M* BA for 21 d.

Role of ethylene on shoot regeneration was examined by culturing apical internodal segments (2–3 mm) on media supplemented with the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC; 20, 50, 100, or 200 μ*M*), or the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG; 2.5, 5.0, 10.0, or 20.0 μ*M*). Both ACC and AVG were from Sigma Inc. Apical internodal segments were cultured on BM with and without BA (5.0 μ*M*) in Duran™ 100-ml screw-cap bottles to provide a culture environment with negligible headspace gas exchange.

Rooting of in vitro*-developed shoots and establishment of plantlets in pots*. Shoots of about 2 cm long were excised and cultured on BM for about 5 d and then on BM containing 0.5, 1.0, or 2.0 μ*M* α-naphthaleneacetic acid (NAA) and 0.8% (w/v) agar for 21 d. Phytagel™ at 0.25% (w/v) and Phytagar™ at 0.7% (w/v) were also tested as alternative gelling agents for rooting media supplemented with 0.5 μ*M* NAA. Thirty rooted plantlets were transferred to a 1:1 peat moss and vermiculite mixture in pots. These plantlets were acclimatized in trays loosely covered with perforated plastic film for a week. They were then transferred to a plant growth room maintained at 24 ± 1°C with a photon flux density of 150 μmol m^{-2} s^{-1}.

Experimental design and statistical analysis. All experiments were of the completely randomized design and repeated at least once. Each experiment consisted of at least five replicates and each replicate was made up of four or five cotyledons, hypocotyl or root explants, six to nine leaf explants or 9–12 internodal explants. Regeneration efficiency of the system was established based on the percentage of regenerative explants and the average number of shoots or roots produced per regenerative explant. Shoot and root regeneration were evaluated after 4, 7, 14, and 21 d of culture. The standard error was calculated, and data were subjected to ANOVA and Fischer's Protected LSD multiple comparison test (Zar, 1984).

TABLE 2

EFFECT OF IAA AND TDZ ON PERCENTAGE SHOOT REGENERATION OF COTYLEDON EXPLANTS EXCISED FROM 3-d-OLD RAPID-CYCLING *BRASSICA OLERACEA* SEEDLINGS AFTER 21 d OF CULTURE

IAA (μ*M*)	TDZ (μ*M*)			
	0	1.0	2.5	5.0
0	39 a	l00 b	91 bc	97 bc
	(2.4 ± 0.7 p)	(7.1 ± 1.9 qr)	(8.0 ± 2.4 qr)	(6.71 ± 1.2 qr)
0.5	ND	92 bc	94 bc	81 c
		(5.4 ± 2.2 q)	(8.5 ± 3.8 r)	(7.7 ± 4.0 rs)
1.0	ND	89 bc	91 bc	75 bc
		(6.0 ± 1.9 qs)	(7.1 ± 1.9 qr)	(7.2 ± 1.7 qr)

Values given in parentheses are the average number of shoots per regenerative explant ± SE. The SE of the means was obtained from at least five replicates with four to six explants each.

Different letters after each value in the two data sets indicate significant difference by Fischer's Protected LSD ($P < 0.05$). Comparisons of arcsine-transformed percentage data and the average number of shoots are designated by different sets of letters, respectively.

ND, not determined.

Results

Seed germination. All the inoculated seeds were germinated within 24 h and reached about 1.5–2.0 cm in height by the third day of culture. Cotyledons of 3-d-old seedlings were considerably expanded and fully green, and were attached to the main shoot axis by 2-mm long petioles.

BA is needed for high-frequency shoot regeneration. Among the different explants tested, hypocotyl and internodal segments were the most regenerative with over 90% of the explants producing shoots on BA-supplemented media within 21 d of inoculation (Table 1). Interestingly, on BM and BA-enriched media, shoot regeneration was observed in all the different types of explants, except the root tissues. Root explant results are, therefore, not included in Table 1. The presence of BA in the medium markedly increased the number of shoots produced per explant. For instance, hypocotyl segments derived from seedling explants, the most regenerative explant, produced nearly 20 shoots per explant on 20 μ*M* BA-containing medium, as compared with three shoots formed on BM (Table 1). Furthermore, shoot regeneration occurred earlier by nearly 4 d in hypocotyl explants in the presence of BA. Shoot buds first appeared at the cut ends of leaf petioles after 10 d *in vitro*. In BA-supplemented media, the young leaves appeared to be more regenerative than the mature leaves (Table 1). Despite the promotive effect of 5 μ*M* BA, shoot regeneration frequency was generally low and erratic in leaf cultures (Table 1).

TDZ and IAA significantly enhanced shoot regeneration. Multiple shoot buds first appeared at the petiolar cut ends of the cotyledons cultured on media containing TDZ or TDZ and IAA within 4 d of culture initiation. Shoots differentiated subsequently between 7 and 14 d of culture. In all the combinations of TDZ and IAA, the

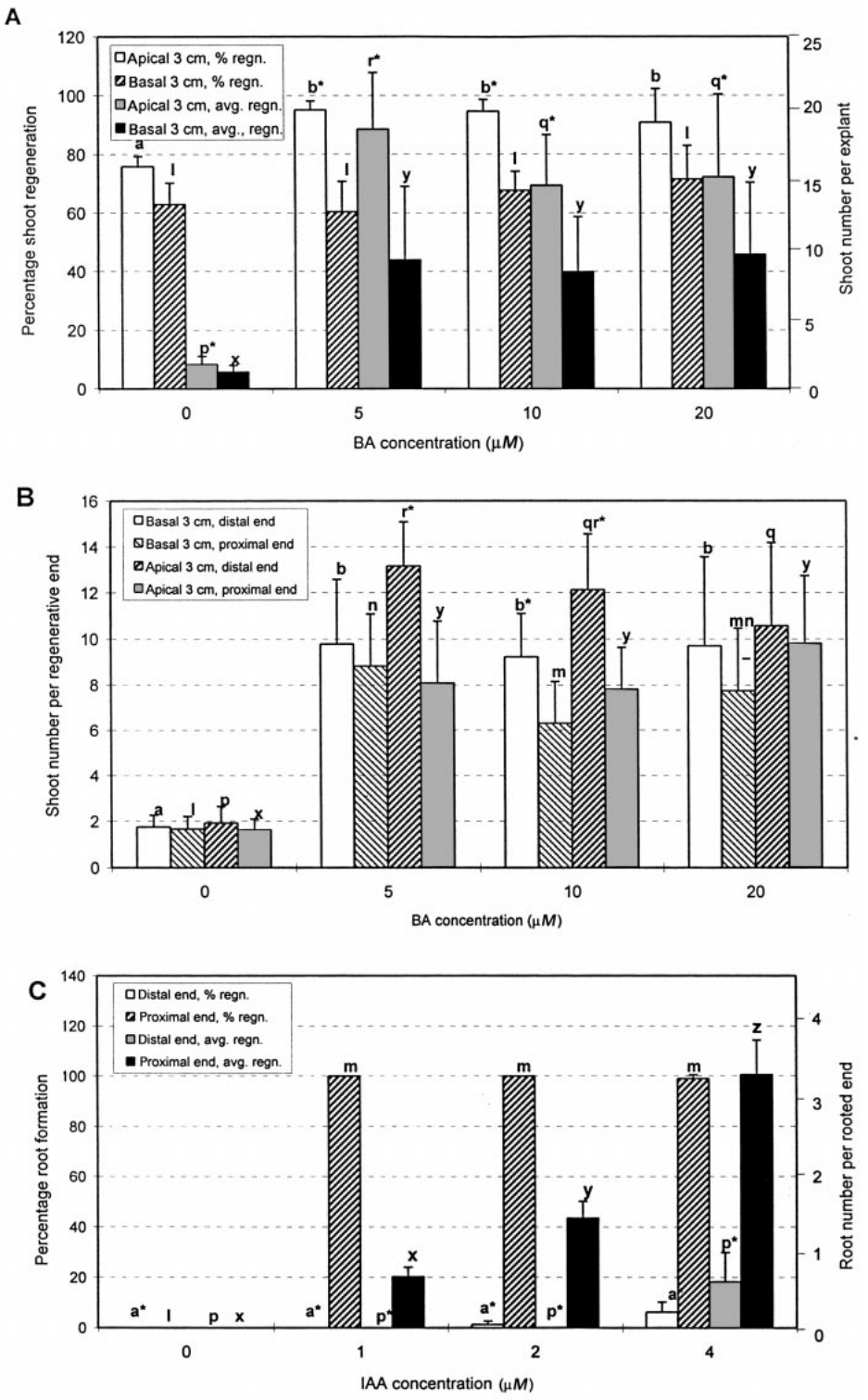

FIG. 1. Developmental polarity during organogenesis from internodal stem explants of rapid-cycling *B. oleracea* after 3 wk of culture on various BA- or IAA-supplemented media. Shoot regeneration frequency and average shoot number per explant were recorded for apical or basal 3 cm stem explants (*A*). Average shoot number per proximal or distal regenerative end were recorded for apical or basal explants after culture on various BA-supplemented (0–20 μ*M*) media (*B*). Rooting frequency and average root number per distal or proximal ends of stem explants were recorded for stem explants grown on various IAA-supplemented (0–4 μ*M*) media (*C*). SE of the means were obtained by arcsine transformation of percentage data, from 10 replicates with 9–12 explants each. Different letters accompanying each bar (of an explant-type) indicate significant difference by Fischer's Protected LSD ($P < 0.05$). '*' Denotes significant difference between the apical and basal stem segments by the same test.

TABLE 3

INFLUENCE OF AVG AND ACC AS WELL AS OF AIRTIGHT CULTURE CONTAINERS ON SHOOT REGENERATION IN STEM EXPLANTS OF *BRASSICA OLERACEA* AFTER 21 d OF CULTURE

Treatment	Concentration (μM)	Percentage shoot regeneration	Mean shoot no. per explant ± SE
AVG	0	76[a]	2.1 ± 0.6
	2.5	41[a]	3.8 ± 2.0
	5.0	59[a]	3.5 ± 1.2
	10.0	60[a]	3.1 ± 1.5
	20.0	72[a]	3.3 ± 1.0
ACC	0	76[p]	1.7 ± 0.6
	20	4[q]	0.1 ± 0.2
	50	6[q]	0.1 ± 0.1
	100	5[q]	0.1 ± 0.2
	200	10[q]	0.2 ± 0.3
GA7 vessels	BM	76	1.7 ± 0.6
	BM + 5 μM BA	95*	18.5 ± 4.0*
Duran airtight bottles	BM	63	1.0 ± 1.0
	BM + 5 μM BA	76*	10.8 ± 5.2*

The SE of means was obtained from 9 or 10 replicates. Different letters following each value within a treatment indicate significant differences by Fisher's Protected LSD ($P < 0.05$).

Comparisons of arcsine-transformed percentage data were performed within each treatment. Similarly, '*' denotes significant difference between cultures in GA7 and in Duran bottles by Fischer's Protected LSD ($P < 0.05$).

percentage of regenerative explants was significantly higher than that of the control (Table 2). On average, seven shoots were produced per regenerative explant in all the TDZ/IAA combinations at the end of 21 d of culture (Table 2). As compared with TDZ, the response to BA treatment of hypocotyl explants was better (Table 1). Since more prolific shoot regeneration was observed with BA than with TDZ, media containing BA were used for further experiments.

Internodal explants are an efficient system for continuous shoot regeneration. Like hypocotyls, internodal segments showed a high regeneration response on BA-containing media. The first appearance of multiple shoot primordia at the cut ends of the internodal segments occurred within a week, but the maximum rate of shoot regeneration was recorded between the second and third week of culture on BA-supplemented media. At the end of 21 d of culture, internodal segments grown on medium containing 5 μM BA produced 15.1 ± 4.6 shoots (about seven-fold more than the control, which produced only 2.1 ± 0.6 shoots per explant). Increasing the concentration of either BA or NAA in combination with BA, however, had little effect on the percentage of morphogenic explants or the number of shoots produced per explant (data not shown).

Polarity of internodal explants influenced organogenesis. Based on the two-factor ANOVA, significant interaction was observed between BA-induced percentage regeneration or average shoot number per explant and positional effects, i.e. whether internodal explants were derived from the apical 3 cm or basal 3 cm of the shoot system (Fig. 1A, B). Nearly 95% of the apical internodal explants cultured on BA-supplemented (5, 10, and 20 μM) media produced about 15–20 shoots by the end of 3 wk of culture (Figs. 1A, 2F, G). In contrast, on average, only three shoots were formed per regenerative internodal explant cultured on BM (Fig. 2E). BA, however, failed to improve the percentage regeneration of basal internodal explants (Fig. 1A) though it considerably increased the number of shoots produced per explant compared with those cultured on BM (Fig. 1B).

A two-factor ANOVA revealed significant interaction between the effects of BA and the developmental polarity (the proximal versus the distal end) of the cut surfaces of apical internodal explants. This interaction, however, was not significant in basal internodal segments. In the presence of BA, no significant difference in percentage shoot regeneration was observed between the distal and proximal ends of apical internodal explants (data not shown). For apical internodal explants, however, the average number of shoots produced at distal ends of explants was found to be higher than that at the proximal ends at 5 and 10 μM BA (Fig. 1B). At these BA concentrations, an average of 12–13 shoots were produced at the distal end of the regenerative explant as compared to eight shoots produced at the proximal end (Fig. 1B).

Polarity effects on IAA-induced rooting of stem explants were also studied. A two-factor ANOVA showed significant interaction between the root-inducing effects of IAA, in terms of percentage root formation and average root number per explant, and the polarity of the cut surface of both internodal explants (data not shown). Root regeneration occurred only in the presence of IAA (1.0, 2.0, and 4.0 μM) (Fig. 1C). For all concentrations of IAA tested, root production and the average number of roots formed per explant at the proximal ends were significantly higher than at the distal ends (Fig. 1C). At the distal cut ends, no root formation occurred in 1.0 μM IAA (Fig. 1C). Frequency of root formation at the proximal ends, in the presence of IAA, was nearly 100% (Fig. 1C).

ACC and accumulation of headspace gas adversely affected organogenesis. Addition of AVG (2.5, 5.0, 10.0, and 20.0 μM) to BM did not significantly influence shoot regeneration of stem explants (Table 3). However, inclusion of ACC (20, 50, 100, and 200 μM) to BM dramatically reduced percentage shoot regeneration of apical explants compared to cultures maintained on BM. The number of explants producing shoots was reduced from 76% in BM to a low range of 4–10% in the presence of 20–200 μM ACC (Table 3). The average number of shoots produced per explant in the presence of ACC was also significantly lower than the control (Table 3).

After 21 d on BM, the percentage of shoot-forming explants and the average number of shoots produced per explant in sealed Duran bottles were not significantly different from those explants cultured in GA7 vessels (Table 3). However, cultures in the airtight bottles showed delayed formation of shoot primordia, since the first appearance of buds occurred only after 2 wk instead of 10 d in GA7 cultures. Further, the number of regenerative explants and the average number of shoots produced per explant cultured on media containing 5 μM BA in sealed Duran bottles were significantly lower compared to those explants cultured in GA7 vessels. Shoot regeneration frequency of stem explants in GA7 vessels was 95% compared to 76% observed in Duran bottle cultures (Table 3). On average, 19 shoots were formed per explant in GA7 cultures compared with 11 shoots recorded for Duran bottles.

NAA-enhanced root formation in regenerated shoots. The first appearance of root primordia occurred after 5 d on both BM- and NAA-supplemented media. The presence of NAA in the culture medium increased the percentage root formation from 34% in BM to nearly 90% in media supplemented with 0.5, 1.0, or 2.0 μM NAA. NAA-enriched media produced three- to four-fold more roots than the media containing IAA. A significant increase in the number

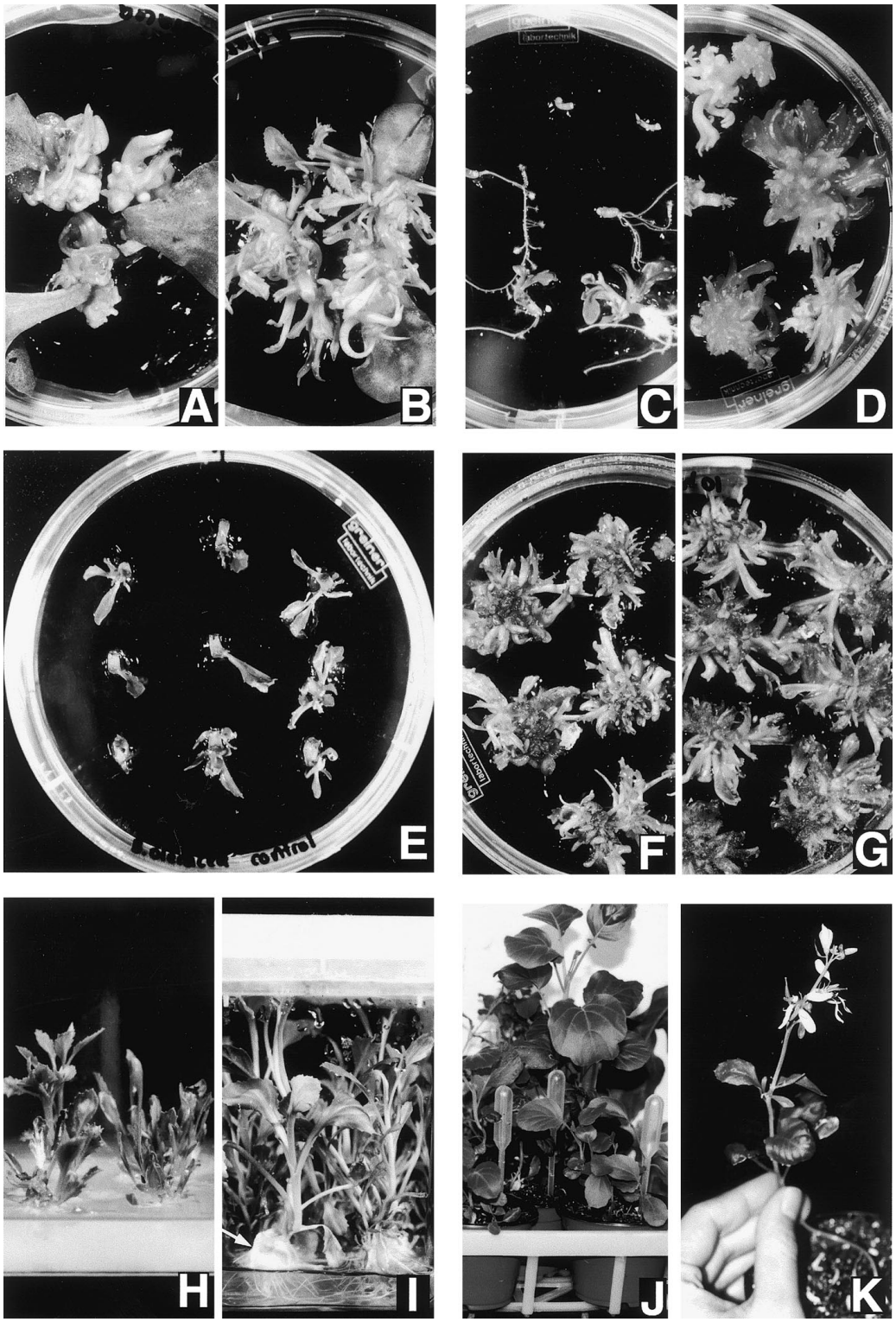

FIG. 2. *A–G*, Shoot regeneration of *B. oleracea* from: cut-petiolar ends of cotyledons after 1 (*A*) and 2 (*B*) wk of culture on 5 μ*M* BA; cut ends of hypocotyl segments after 2 wk of culture on BM (*C*) and BM + 20 μ*M* BA medium (*D*); stem explants cultured on BM (*E*), BM + 5 μ*M* BA (*F*) and BM + 10 μ*M* BA (*G*). Shoot and root formation of *B. oleracea* on BM + 0.5 μ*M* NAA-supplemented medium for 3 wk (*I*) as compared to control on BM after 2 wk (*H*). Establishment of *B. oleracea* plantlets in soil (*J*) and the appearance of normal flowers and seed pods (*K*) 2 wk after transplantation.

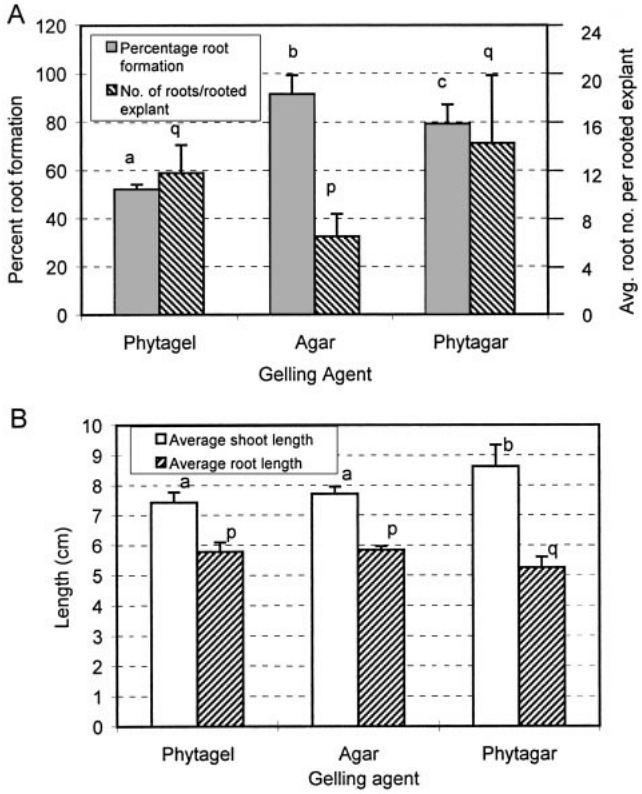

FIG. 3. Effect of gelling agents on rooting frequency or number of roots per rooted explant (*A*) and average root or shoot lengths (*B*) of regenerated shoots of rapid-cycling *B. oleracea* after 3 wk of culture. SE of the means were obtained by arcsine transformation of data collected from 10–12 replicates with four to six explants each. Different letters accompanying each bar indicate significant difference by Fischer's Protected LSD ($P < 0.05$).

of roots formed per explant, about three-fold over the control, was also recorded in NAA-treated cultures. Average root length increased significantly from 4.40 ± 0.3 cm in BM to 6.9 ± 0.4 by the addition of 1 μ*M* NAA in the rooting medium.

The type of solidifying agent used in the media had a significant effect on root regeneration. TC-grade agar was the most effective in promoting root formation (Fig. 3). Almost 90% of the shoots produced roots on medium gelled with TC-grade agar; substituting the agar with Phytagar™ and Phytagel™ reduced the rooting frequency to 79% and 52%, respectively. However, in Phytagar™-gelled medium, each plantlet was capable of producing an average of 14 roots, which was significantly greater than in agar (Fig. 3). There was no significant difference in root lengths for all the three solidifying agents used. Almost 95% of the rooted plantlets transplanted to soil survived (Fig. 2J). They were normal in morphology and produced normal flower buds within 2 wk of transplantation (Fig. 2K). These plants were fertile and formed viable seeds.

DISCUSSION

In this study we have established a highly regenerative system using young internodal explants and simple media that can be employed for mass production of rapid-cycling *B. oleracea*. Among the various explants tested, hypocotyls and young internodal segments from *in vitro* shoots were the most regenerative. We have used internodal explants to develop the regeneration system for the obvious reason that large numbers of explants can be readily harvested from parent shoots maintained *in vitro* without any need for a complete seed to seed cycle. From each *in vitro*-developed shoot (7–8 cm long), the method yields at least eight young, highly regenerative internodal explants (2–3 mm long), capable of producing an average 15 shoots that can be used for the next cycle of shoot production in 6–8 wk duration. Thus, with the current procedure, about 15 000 plantlets can be produced from a single *in vitro* shoot with just two cycles of shoot regeneration. Although there are shoot regeneration procedures (mostly callus-based) established for rapid-cycling *B. oleracea*, a high-frequency, direct and continuous shoot regeneration system has not been described previously.

The apical internodal explants were found to be more regenerative than the basal segments in the presence of BA (Fig. 1). This suggested a position-dependent response to BA in rapid-cycling *B. oleracea*, which is consistent with the previous reports on other long-duration *Brassica* spp. (Stringam, 1977; Yang et al., 1991; Narasimhulu et al., 1992). In *B. carinata* and *B. napus*, the upper segments of the hypocotyl were more regenerative than the lower segments (Yang et al., 1991; Stringam, 1977). The mechanism of this phenomenon is not fully understood (Yang et al., 1991). However, competency for shoot morphogenesis may be attributed to the developmental state of the tissue (Lakshmanan et al., 1997). The effect of developmental age observed in the internodal explants of rapid-cycling *B. oleracea* in this investigation was also evident in rapid-cycling *B. rapa* (Teo et al., 1997). In this context, it is interesting to note that developmental states of morphogenic competence and caulogenic determination are restricted to a narrow window of time and are differentially regulated by different hormones (Lakshmanan et al., 1997). Occurrence of roots at the proximal ends of internodal explants cultured on IAA-supplemented media (Fig. 2H) also indicates the existence of polarity within the internodal explant.

One of the findings in the present study was the spatial regulation of morphogenesis in internodal explants. Most of the shoots were produced in the distal ends, suggesting the existence of a strong morphogenic polarity in the internodal segments. Morphogenic polarity may be associated with polar auxin transport within the main shoot/root axis as well as within isolated stem segments. When the appropriate endogenous hormone thresholds are reached, the terminal cells may regenerate roots (high auxin or auxin/cytokinin ratio) or shoots (low auxin or auxin/cytokinin ratio), respectively. Since polar auxin transport continues even in isolated stem cuttings (Fosket, 1994), and is correlated with the establishment of polar regeneration (Paterson, 1983), this mechanism may account for the higher shoot production at the distal ends of *B. oleracea* stem explants on BA-supplemented media. This may also explain why root regeneration of stem explants almost always occurred at the proximal ends of stem explants cultured on IAA-supplemented media.

Ethylene biosynthesis is mainly regulated by ACC synthase, which converts *S*-adenosylmethionine to ACC, the immediate precursor of ethylene. The presence of ACC dramatically inhibited shoot regeneration of *B. oleracea*, and a delayed and reduced formation of shoot primordia was observed in cultures using airtight containers (Duran bottles) (Table 3). These findings suggest a

possible inhibitory influence of ethylene on shoot morphogenesis in this species. The ethylene inhibitor, AVG, has been found to decrease ethylene production, endogenous ACC synthase activity and the ACC level of *Brassica* explants (Chi et al., 1991; Pua, 1993). In this study, however, addition of AVG did not significantly improve shoot formation (Table 3), indicating that AVG may not be effective in regulating ethylene production in rapid-cycling *B. oleracea*, as it is in some other species (Kumar et al., 1998). Another possibility that cannot be discounted is the low ethylene production or accumulation in these cultures.

In conclusion, we have developed an *in vitro* system for rapid, high-frequency and continuous production of rapid-cycling *B. oleracea* using internodal segments from *in vitro* shoots. As this system produces shoots directly and rapidly without the involvement of a callus phase, it greatly reduces somaclonal variation, making the current methodology more desirable for gene transfer programs. We are currently using this system for generating maize Ac and Ds transposon-tagged lines of rapid-cycling *B. oleracea*.

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