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RAPID MULTIPLE SHOOT PRODUCTION FROM COTYLEDONARY NODE EXPLANTS OF PEA (*PISUM SATIVUM* L.)

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SUMMARY

Multiple shoots were produced from cotyledonary node explants of pea (*Pisum sativum* L.) cultured on MS medium containing 1 mg l^{-1} BAP. The number of buds formed could be increased by scraping the nodes before culture or by increasing the cytokinin concentration. However, cytokinin levels over 5 mg l^{-1} increasingly produced shoots that were vitrified and difficult to root. With all the genotypes tested, developing buds were visible as soon as 5 days after culture and elongated shoots could be removed after 21 days. Histological studies indicated that the buds and shoots were formed from superficial layers of tissue. The efficiency of this regeneration system compared favorably with previously published methods. The rapid, genotype-independent, high frequency system described here may be of use in the production of transgenic pea plants.

Key words: regeneration; pea; cotyledonary nodes; genotype independent.

INTRODUCTION

The use of many methods for plant improvement using recent advances in biotechnology depends on the establishment of an efficient *in vitro* regeneration system for the species concerned. Such a system must allow for the rapid, repeatable production of shoots that can be rooted and grown to mature plants. In addition, such a system should apply to as broad a range of genotypes as possible so that cultivars in current use can be immediately manipulated.

Plant regeneration in pea (*Pisum sativum* L.) has been reported previously from protoplasts (10,15), as well as from explants via organogenesis (2,6,11,13) and embryogenesis (9). Unfortunately, these regeneration methods are slow, apply only to certain genotypes and generally produce a low frequency of regenerants. This paper describes an improved method of pea regeneration using cotyledonary node tissue. Shoot production is quick, genotype-independent and occurs at high frequency. In addition, the shoots and buds appear to form from superficial layers of the tissue which might facilitate transformation of regenerable tissue by infection with *Agrobacterium*, or bombardment with DNA-coated particles.

MATERIALS AND METHODS

Accessions of *P. sativum* L. from widely diverse provenances (Europe, North America, Afghanistan, India) were used (PI244253, PI269776, PI269814, PI212916, JM9 and cv Afghanistan). These genotypes ranged from commercial varieties to totally unimproved types and exhibited considerable morphological differences (e.g. in plant height and number of tillers, as well as in seed size, color and shape). Seeds were surface-sterilized by immersion in 70% ethanol for 2 min followed by 20 min in 20% Javex (1.2% sodium hypochlorite). After 4 rinses in sterile distilled H_2O, the seeds were germinated in the dark at 22° C in unsealed glass petri dishes containing 2 sterile filter paper discs and 10 ml sterile distilled H_2O. In one experiment the H_2O was replaced by filter-sterilized solutions of 1 or 10 mg l^{-1} benzylaminopurine (BAP).

After 3 to 5 days germination, cotyledonary nodes were excised by cutting off the cotyledons to within 1 mm of the node and trimming the epicotyl and root to 1-2 mm. In some experiments, a longitudinal cut was made through the epicotyl and the resulting 2 buds were cultured separately. Axillary buds (up to 2 mm long) were removed by scraping with a scalpel blade.

Nodes were cultured in petri dishes (100 × 15 mm) containing a medium consisting of MS salts (14), B5 vitamins (3), 3% sucrose, 0.7% agar (Sigma) and varying amounts of BAP or kinetin (pH 5.7) as described in Results and Discussion. The cultures were maintained at 20° C at a photosynthetically active radiance of 60 to 100 $\mu E\ m^{-2}\ s^{-1}$ with a 16 h photoperiod. When developing shoots were greater than 10 mm long they were removed and rooted on 1/2 strength B5 medium with 0.186 mg l^{-1} napthaleneacetic acid (NAA) (8). Rooted shoots were washed in water, planted in autoclaved vermiculite in

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propagating trays with transparent lids, and watered with 1/4 strength Hoagland's solution no. 1 (4). The transparent lids of the containers were removed after 2 to 3 weeks when the plants were established. The plants were transferred to 12 cm pots containing Terra-Lite Redi-Earth (W. R. Grace and Co., Canada) and watered with full strength Hoagland's solution no. 1.

Plumules and immature leaflets were cultured as described by Hussey and Gunn (6) and Mroginski and Kartha (13) respectively. Briefly, these procedures were as follows. Plumules were excised from asceptically germinated seed. These were placed on a nutrient medium consisting of MS salts (14) with 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} thiamine, 1.0 mg l^{-1} pyridoxine, 5.0 mg l^{-1} nicotinic acid, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 6 mg l^{-1} indole-butyric acid (IBA), pH 5.9. Callus formed at the base of the plumules and shoot regeneration occurred from this callus after transfer to the same medium but with IBA reduced to 0.25 mg l^{-1}. In the second method, immature yellowish-white leaflets from the second and third apical leaves were dissected from three-day old asceptically germinated seedlings and placed on the basic nutrient medium used for cotyledonary nodes but with 2.25 mg l^{-1} BAP and 0.0186 mg l^{-1} NAA. All cultures were maintained under the same environmental conditions as above.

For histological studies, cotyledonary nodes at different stages of culture were fixed in formalin/acetic acid/50% ethanol (5:5:90), dehydrated in an ethanol/tertiary butyl alcohol series and embedded in paraffin wax. Sections were cut at 10–15 μm and stained with toluidine blue.

Results and Discussion

Multiple shoots developed from excised cotyledonary nodes from young pea seedlings. If intact explants were placed on MS medium without plant growth regulators, a single shoot developed from one side of the node, suppressing the outgrowth of the bud on the other side. However, culture of the intact nodes on a medium containing 1 mg l^{-1} BAP (MS1), induced the development of multiple buds from both sides in 100% of the explants in all genotypes (Table 1).

TABLE 1

MEAN NUMBER OF BUDS (± STANDARD ERROR) FORMED PER NODE ON COTYLEDONARY NODE EXPLANTS OF SIX GENOTYPES OF PEA AFTER 21 DAYS ON MS MEDIUM WITH 1 mg l^{-1} BAP

	Mean Number of Buds Formed Per Node[a]		
Genotype	Intact Nodes	Scraped Nodes (Axillary Buds Removed)	% of Nodes Forming Buds
cv. Afg	5.8 ± 0.6	8.6 ± 0.9	100
PI244253	3.0 ± 0.4	7.4 ± 0.7	100
PI269776	4.6 ± 0.6	5.6 ± 0.7	100
PI269814	7.7 ± 0.7	9.3 ± 1.2	100
PI212916	3.3 ± 0.2	9.6 ± 1.1	100
JM9	4.2 ± 0.4	9.1 ± 0.8	100

[a]Number of explants per genotype = 20.

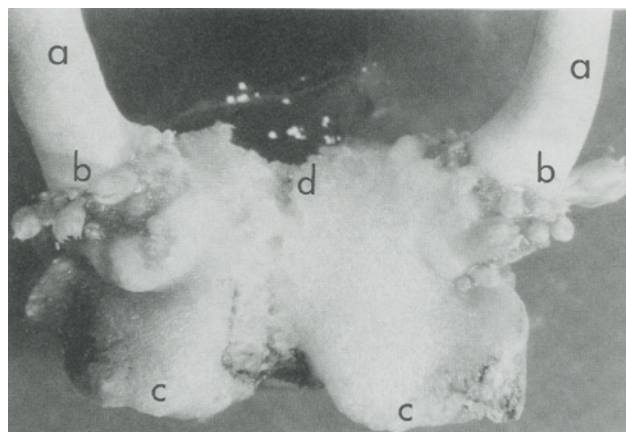

FIG. 1. Cotyledonary node explant with 1 large shoot developing from each of the 2 scraped axillary bud sites and multiple buds forming in a ring around the base of these shoots (×17). *a*) large shoot forming from scraped axillary bud region, *b*) multiple buds produced at the base of the larger shoots, *c*) site of excision of cotyledons, *d*) region from which epicotyl was excised.

The influence of the existing axillary buds was further reduced by the removal of obvious ones from the node explants and disrupting surrounding tissue by scraping with a scalpel blade. Such scraping produced substantial disruption of the meristem although its total removal could not be proven. In each genotype, 100% of nodes still formed multiple buds and the number formed per node was increased significantly (Table 1). The inclusion of BAP in the germination medium had no effect on the production of buds or shoots.

The pattern of bud production and development was studied by analyzing node sections fixed at various culture stages. One shoot initially developed from the scraped axillary bud region cultured on MS1. After 5 days culture, buds formed in a ring around the base of this shoot (Fig. 1). The origin of this first shoot was not clear, but its rapid appearance suggests that it may have been derived from the disrupted axillary bud meristem(s). Histological analysis of explants fixed 9 days after culture revealed that multiple buds were formed from a meristematic zone around the base of the larger shoot (Fig. 2). Three weeks after culture initiation, shoots greater than 1 cm long could be removed. By placing the remaining tissue on fresh MS1 medium, new buds were induced and new shoots were formed. With this method of continual shoot removal, approximately 5 shoots per explant could be produced in a comparatively short period (2 months) and, over the longer term, shoot-producing cultures could be maintained for more than 6 months producing at least 10 to 20 shoots from a single explant. The excised shoots formed roots at high frequency (> 90%) within 2 to 4 weeks after transfer to rooting medium. Most rooted shoots also survived

transfer to soil and the mature plants appeared morphologically normal and set seed.

In initial experiments 1 mg l^{-1} BAP was used, since this concentration was known to produce multiple shoot formation in excised apical meristems (unpublished results, J. A. J.). To determine the optimum concentration for multiple shoot production from cotyledonary node explants, scraped nodes were cultured on media containing various concentrations of BAP. The number of buds formed increased with increasing BAP concentration (Table 2); however, the buds and shoots formed on cultures growing on medium containing 10 mg l^{-1} tended to appear abnormal and vitrified. When transferred to rooting medium, these abnormal shoots formed callus rather than roots. There were no major differences apparent in the response to BAP levels in the three genotypes tested (Table 2). The use of kinetin at various concentrations, as a replacement for BAP, produced no increase in the number of buds formed over the control (0 mg l^{-1} BAP) until very high levels (50 or 100 mg l^{-1}) were reached (Table 2). Again, at these levels abnormal buds and shoots were formed that were difficult to root. The best compromise between quality and number of buds produced was determined to be a cytokinin level of 1 or 5 mg l^{-1} BAP.

TABLE 2

NUMBER OF BUDS FORMED PER NODE (± STANDARD ERROR) ON SCRAPED COTYLEDONARY NODE EXPLANTS OF 3 GENOTYPES OF PEA AFTER 45 DAYS ON MS MEDIUM WITH VARIOUS CONCENTRATIONS OF BAP OR KINETIN (KIN)

Cytokinin Concentration (mg l^{-1})	Mean Number of Buds Formed[a]		
	JM9	PI244253	PI212916
0 BAP	4.0 ± 0.3	2.0 ± 0.3	4.9 ± 0.7
0.5 BAP	11.9 ± 1.6	7.1 ± 1.2	8.7 ± 1.6
1.0 BAP	13.2 ± 1.5	6.7 ± 0.8	13.5 ± 2.5
5.0 BAP	23.1 ± 2.1	17.2 ± 3.9	16.8 ± 2.1
10.0 BAP	33.4 ± 4.7	20.4 ± 2.0	26.5 ± 2.5
0.5 KIN	3.7 ± 0.3	2.3 ± 0.4	3.1 ± 0.6
1.0 KIN	3.5 ± 0.3	2.3 ± 0.2	3.6 ± 0.4
5.0 KIN	4.5 ± 0.3	2.7 ± 0.4	3.6 ± 0.3
10.0 KIN	5.2 ± 0.4	2.3 ± 0.3	4.0 ± 0.5
50.0 KIN	N.D.[b]	14.8 ± 1.9	N.D.
100.0 KIN	N.D.	28.3 ± 2.2	N.D.

[a]Number of explants per genotype-treatment combination = 12.
[b]Not determined.

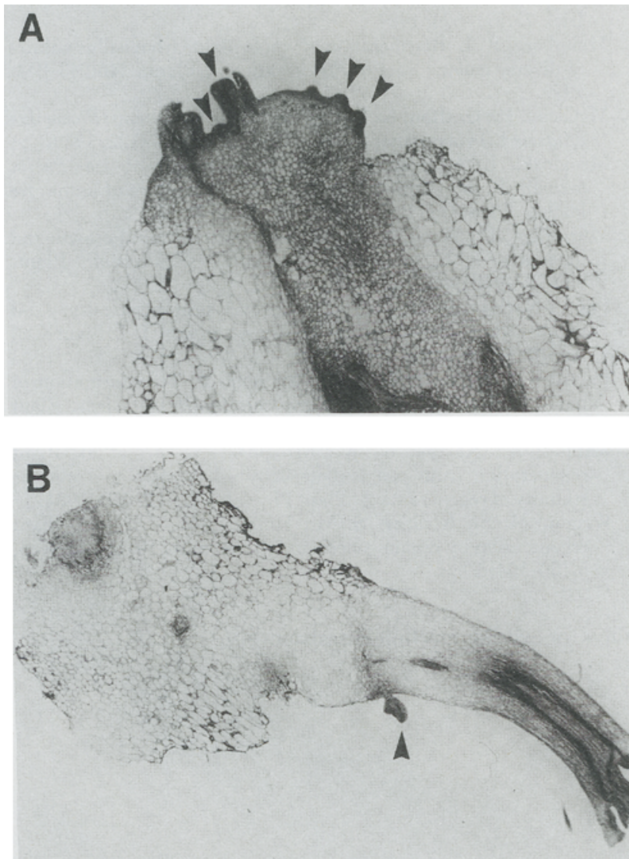

FIG. 2. Sections of cotyledonary node explants 9 days after culture: *A*. Shoot buds (*arrows*) forming from superficial meristematic tissue (×21). *B*. Large shoot developing from the scraped axillary bud region of a cotyledonary node explant with a shoot bud (*arrow*) forming near its base (×17).

Similar concentrations of BAP were found to stimulate regeneration from soybean and dry bean cotyledonary node explants (1,12,18). However, unlike our results with pea (albeit under different germination conditions), in these other species it was essential to include cytokinin in the germination medium as well as the culture medium.

To compare the efficiency of our method with those previously published for pea shoot regeneration (6,13), we took seedlings of 2 genotypes and cultured cotyledonary nodes, as well as plumules and immature leaflets, following exactly the previously described methods (6,13). Buds took longer to appear on the plumule and immature leaflet cultures (30 to 60 days) than for cotyledonary node cultures (5–14 days) and the time to the stage at which the shoots could be excised for rooting was also longer (45–90 days as opposed to 21–28 days). Each cotyledonary node explant produced at least one shoot and further buds would develop into shoots on its removal. Although the plumule and immature leaflet explants were cultured for 8 weeks, as opposed to 3 weeks for cotyledonary nodes, a higher percentage of cotyledonary node explants formed buds and shoots in both genotypes with a higher number per explant (Table 3). The times and numbers for the previously reported regeneration methods are in agreement with published information (6,13). Both the plumule and immature leaflet methods have been reported to be highly genotype specific, with 3 out of 5 genotypes showing no regeneration from plumule-derived cultures (6) and 5 out of 9 genotypes showing less than 25% of explants forming shoots from immature leaflet explants (17). We chose 6 genotypes which vary widely in geographic origin and morphology; all showed regeneration from cotyledonary nodes indicating that this method

TABLE 3

MEAN NUMBER OF BUDS AND SHOOTS PRODUCED BY CULTURES OF 2 PEA GENOTYPES USING DIFFERENT EXPLANT SOURCES

Explant Source	Genotype	No. of Explants Cultured	% of Explants Forming Buds or Shoots	Mean No. of Buds and Shoots Per Original Explant
Plumule[a]	PI244253	18	28	0.3
	c.v. Afg	21	24	0.2
Immature leaflet[a]	PI244253	36	58	0.6
	cv. Afg	36	0	0
Cotyledonary node[b]	PI244253	20	100	7.4
	cv. Afg	20	100	8.6

[a]No. of buds and shoots counted after 8 weeks.
[b]No. of buds and shoots counted after 3 weeks.

should produce desired results from a wide range of important pea varieties.

Pea (*Pisum sativum* L.) is susceptible to *Agrobacterium* and the integration and expression of inserted foreign genes have been demonstrated in callus tissue (5,7,16). However, a remaining obstacle to the production of transgenic pea plants has been reported to be the selection of easily regenerable tissue that is susceptible to this *Agrobacterium*-mediated transformation (16). Further experimentation will determine whether the method of *in vitro* pea regeneration described here can be used to overcome this obstacle.

Conclusions

We have described a rapid, reliable, high frequency method for producing multiple shoots from pea cotyledonary node explants. These shoots are derived from superficial layers of the explants which in turn have been found to form galls when infected with oncogenic strains of *Agrobacterium tumefaciens* (unpublished data, J.A.J.). Therefore, with the use of suitable disarmed *Agrobacterium* strains and cocultivation procedures, or with the use of microprojectile bombardment, this system of plant regeneration might be valuable in the development of a method for the production of transgenic pea plants.

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