Attempting Regeneration from Cultured Cotyledons and Plant Regeneration from Cotyledonary Nodes in Common Bean (*Phaseolus vulgaris* L.)

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Summary: Dry seeds from two cultivars of common bean (*Phaseolus vulgaris* L.) were germinated on sterile cotton and sterile deionized distilled water. Cotyledonary node tissue of seedlings were cultured on Murashige and Skoog(MS)-based media supplemented with different combination of N^6-benzyl-aminopurine (BAP) and indole-3-acetic acid (IAA), and benzyladenine (BA) and a-naphthaleneacetic acid (NAA). The results revealed that the regeneration percent and the average number of buds and shoots per explant were influenced by the type of explants and exogeneously added hormones. Multiple shoot induction on dry bean cotyledonary node that contain 4–5 mm from cotyledons and hypocotyl on a medium containing full concentration of MS inorganic salts supplemented with 0.5mg/l BA and 0.1mg/l NAA was feasible and the method can be applied in transformation experiments.

Introduction

Difficulties in obtaining plants from somatic cells or protoplasts in *Phaseolus* species have up to now hampered the production of transgenic plants by the application of the most common methods available for the introduction of foreign DNA into cells (Genga *et al.* 1990). Large-seeded legumes such as dry bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and pea (*Pisum sativum*) are important to many cropping systems but the lack of regeneration procedures has slowed the improvement of these species via selection in culture and plant transformation (McClean and Grafton 1989). Recently, several regeneration procedures for soybean via embryogenesis and organogenesis have been described. In addition, a recent report describes an embryogenic regeneration procedure for pea.

We report here an attempt to develop a regeneration procedure for dry bean which utilises a lateral segment of cotyledons and cotyledonary node tissue; and to show that the new shoot development appears to be of an adventitious nature.

Material and methods

Bean seeds were obtained from the Department of Genetics and Plant Breeding, University of Horticulture and Food Industry, Budapest, Hungary. Two genotypes, FÖNIX and MAXIDOR from *Phaseolus vulgaris* were used in this study. Dry bean seeds, uniform in shape, were sterilised by submerging in 20% hydrogen peroxide plus some drops Tween 80 for five minutes, they were then rinsed once in 70% ethanol for five minutes, and washed five times with sterile deionized distilled water for five minutes. Uniform germination was achieved only if the seeds which remained unwrinkled during sterilisation was used. One seed was germinated in a 25x80 mm glass tube that contained sterile cotton and sterile deionized distilled water.

After three days,16 and 14 good seedlings were selected respectively from FÖNIX and MAXIDOR, cotyledon parts were prepared from cultured seedlings, the testa, root, hypocotyl and shoot apex were excised from the germinating seeds and each cotyledon was cut into four parts. Each one was placed in a 25x80mm glass tube onto medium containing MS2 major and minor salts (*Murashige*

[1]Abbreviations: MS=Murashige and Skoog(1962); B5=Gamborg et al.(1968); BAP=N^6-benzyl-aminopurine; IAA=indole-3-acetic acid; BA=benzyladenine; NAA=naphthaleneacetic acid.

and *Skoog* 1962) (MS), vitamins according to Gamborg *et al.* (1968) (B5), 2% (w/v) sucrose and 0.7% agar; supplemented with N6-benzyl-aminopurine (BAP) 1.13mg/l and indole-3-acetic acid (IAA) 0.88mg/l. The pH of the medium was adjusted to 5.7 with KOH or HCl before being autoclaved (1.4 $kg.cm^{-2}$ at 121 °C for 20 minutes). Axenic seed explant cultures were performed at 23–27 °C with a 16-h per day photoperiod at 100 $\mu E\ m^{-2}\ s^{-1}$ at the plate surface. In the other part of seedlings, cotyledonary nodes were prepared for culture, cotyledonary node with cotyledons and cotyledonary node without cotyledons. The hypocotyl was removed at the cotyledonary node, the shoot apex was cut. One node was placed in a 55x90 mm glass bottle containing half-strength MS medium, supplemented with 0.75 mg/l benzyladenine (BA) and 0.1mg/l α-naphthaleneacetic acid (NAA).

After one week from germination other explants were prepared from cultured seedlings. Shoot apex and 0.75 mm from cotyledons were removed, and 3–4 explants were placed in a 55x90 mm glass bottle containing half and full strength MS medium supplemented with 0.75 mg/l BA and 0.1 mg/l NAA, and 1.13 mg/l BAP and 0.88mg/l IAA, respectively.

After 3-5 weeks from germination, the efficiency of regeneration from cotyledon parts was compared with that from cotyledonary node with and without cotyledons and other explants by counting the number of buds and shoots. The parts which showed organogenesis from a cotyledonary segment were cut and transferred into a fresh medium, and shoots that attained 0.5^{-1} cm in length were removed and tested for rooting ability.

Results and discussion

Micropropagation of *Phaseolus vulgaris* L. meristems (*Kartha et al.* 1981) and axillary shoots (*Martins* and *Sondahl* 1984) cultured on media containing benzyladenine has been demonstrated; little, if any genetic variability is expected since this new shoot development utilises existing meristems. Tissue culture has long been used to study developmental and physiological processes in legumes, but until recently organogenesis in legumes was rarely reported. Gamborg *et al.* (1974) reported plantlet regeneration from small (2–4 mm^3) macerated cell masses derived from pea shoot tips. Multiple shoots formed on some cell masses grown in culture. They report increased shoot formation with BA treatments, whereas NAA seemed to suppress shoot development and failed to affect root formation. Since one goal of this research was to define a regeneration protocol that may generate culture-induced variation, an alternate approach was sought. Cotyledonary node tissue of soybean prepared from seeds germinated and cultured on BA containing medium exhibited multiple shoot formation from non-meristematic tissue (*Wright et al* 1986, *Wright et al* 1986; and *Cheng et al* 1980). We wanted to determine and compare the usefulness of this explant to other explants for dry bean regeneration.

Two common bean genotypes were used in this study. Different explants were used from each genotype and cultured with different hormones. The results show that the regeneration percent and the average number of buds and shoots per explant were influenced by the type of explants used and by exogeneously added hormones *(Table 1, 2 and 3)*.

Table 1 Efficiency of shoot regeneration in Phaseolus vulgaris cotyledon parts cultured on MS medium supplemented with 1.13mg/l BAP and 0.88mg/l IAA

Cultivar	Number of cotyledonary parts	%
FÖNIX	97	5.15
MAXIDOR	62	9.68

Table 2 Efficiency of shoot regeneration in Phaseolus vulgaris cotyledonary nodes with and without cotyledons cultured on half strength medium supplemented with 0.75 mg/l BA and 0.1mg/l NAA

Cultivar	Cotyledonary node with cotyledons	The average number of buds and shoots per explant	Cotyledonary node without cotyledons	The average number of buds and shoots per explant
FÖNIX	100%	15.25	60%	6.4
MAXIDOR	100%	15	–	–

Four types of explants were compared in this assay. Regenerating buds and shoots were counted on different explants and we found a large variation in regeneration capacity between the four types of explants *(Fig.1. a) and b)*.

The efficiency of shoot regeneration from cotyledonary parts cultured on MS medium supplemented with 1.13mg/l BAP and 0.88mg/l IAA was 2% and 9% in the two

Table 3 The efficiency of shoot regeneration in *Phaseolus vulgaris* from cotyledonary nodes that contains 4–5 mm from the two cotyledons and hypocotyl and giving rise at least three shoots cultured on various media

Cultivar	MS 1.13 mg/l BAP 0.88 mg/l IAA		MS 0.5 mg/l BA 0.1 mg/l NAA		1/2 MS 0.75 mg/l BA 0.1 mg/l NAA	
	%	average number of buds and shoots/explant	%	average number of buds and shoots/explant	%	average number of buds and shoots/explant
FÖNIX	100	16.8	88.9	31.14	100	24.69
MAXIDOR	100	10.0	100	20.25	–	–

genotypes. If the part of cotyledon tissue was transferred and cultured further on the same fresh medium after one month from the initial culture, we found that callus and organogenesis were developed. *(Fig. 2).*

Only 5.15 and 9.68 percent of cotyledonary parts regenerated shoots in case of Fönix and Maxidor respectively. The percentage of cotyledonary nodes with and without cotyledons that regenerated shoots, which were cultured on 1/2 MS medium supplemented with 0.75 mg/l BA and 0.1 mg/l NAA was 100 and 60, respectively, in FÖNIX and 100 in MAXIDOR with cotyledonary nodes with cotyledons only. The maximum number of buds and shoots were 24 and 17 on cotyledonary nodes with and without cotyledons respectively in FÖNIX, and 25 with cotyledonary nodes with cotyledons only in MAXIDOR. The average number of buds and shoots were 15.25 and 6.4 on cotyledonary nodes with and without cotyledons, respectively, in FÖNIX, and 15 on cotyledonary nodes with cotyledons only, in MAXIDOR. The number of regenerating shoots from the fourth explant type, which was cultured on MS supplemented with 1.13mg/l BAP and 0.88 mg/l IAA or 0.5 mg/l BA and 0.1mg/l NAA, and on 1/2 MS supplemented with 0.75 mg/l BA and 0.1mg/l NAA were 38, 55 and 34, respectively, in FÖNIX and 18, 32 and 27 in MAXIDOR. The percentage of the four explant type that regenerated shoots when were cultured on three types of media was 100, 88.9 and 100, respectively, in FÖNIX and 100 and 100 in MAXIDOR. The average number of buds and shoots per one explant was 16.8, 31.14 and 24.69 respectively in FÖNIX and 10.0 and 20.25 in MAXIDOR *(Table 3.)*.

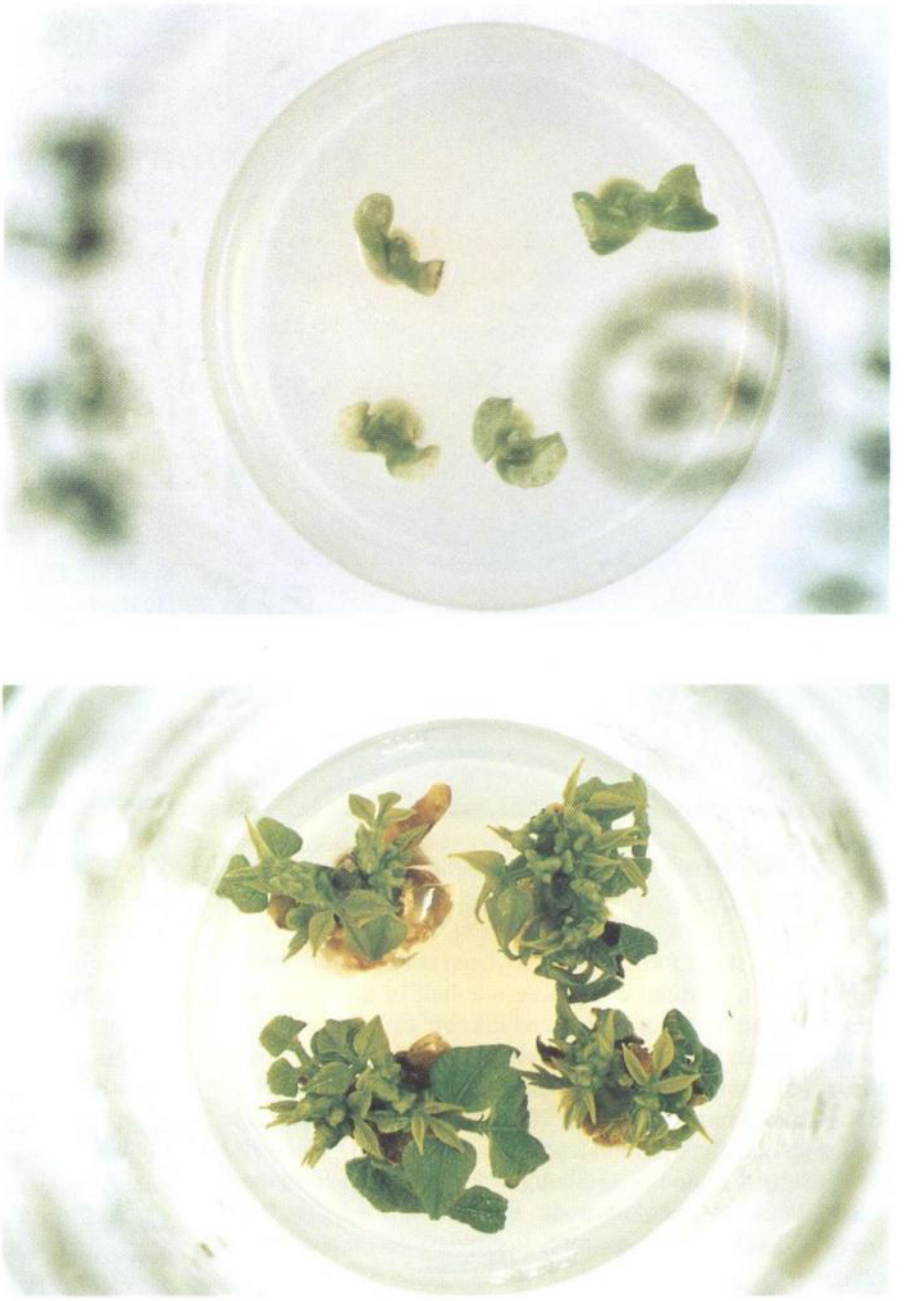

Figure 1a) and b) Shoot formation from cotyledonary nodes of *Phaseolus vulgaris* L., cultured on MS medium supplemented with
0.75 mg/l BA and 0.1 mg/l NAA.

The results show that the type of explant influenced the regeneration process. Malik and Saxena (1991) observed that leaf explants, consisting of the petiole and a portion of lamina, produced shoots if cultured in the presence of BAP. Although regeneration occurred only from petiolar tissue, the presence of an attached portion of lamina was essential for shoot formation. This observation prompted us to investigate the role of different types of explant on subsequent shoot formation. The use of different types of explant required labour-intensive manipulation, including optimisation of many factors such as the size, and physiological age of the explant, and its orientation in the culture vessel. Our results on efficient shoot formation indicate that the type of explant plays an important role in shoot formation in *Phaseolus vulgaris L.* Malik and Saxena (1991), already mentioned, that the presence of the lamina was necessary to induce shoots regeneration from petioles of juvenile leaves. When they were cultured separately, neither the petiole nor the lamina developed shoots. Franklin *et al.* (1991) also observed that cotyledonary-node explants consisting of an intact axillary bud and a part of the cotyledon regenerated a higher number of shoots than explants without them, and we obtained similar results.

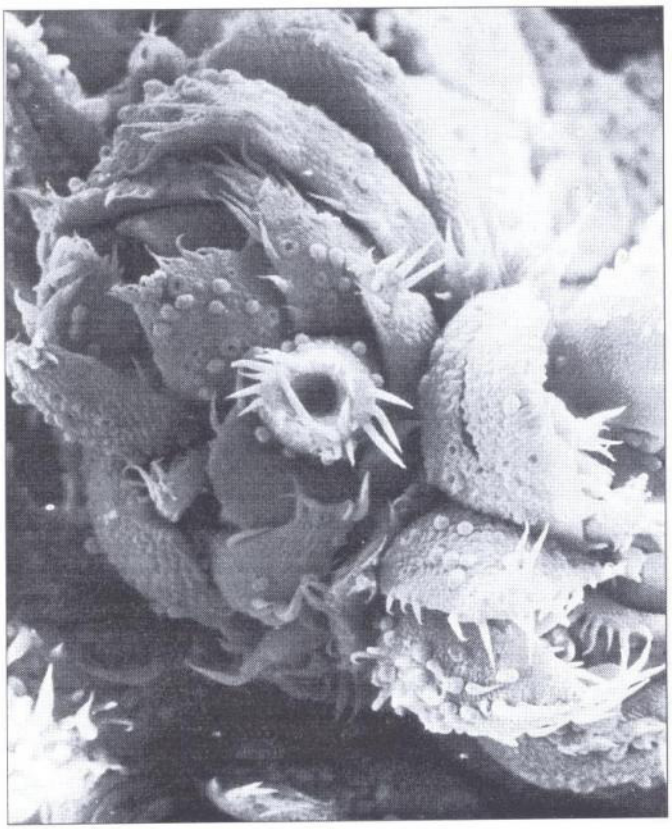

Figure 2 Organogenesis from cotyledonary segments of *Phaseolus vulgaris* L., cultured on MS medium supplemented with 1.13 mg/l BAP and 0.88 mg/l IAA.

Multiple shoot formation on soybean coty!edonary nodes cultured on a medium containing one-half of the published concentration of Murashige and Skoog (1962) inorganic salts has been reported Wright *et al.* (1986). We compared the media containing 0.5, 0.75mg/l BA and 0.1 mg/l NAA, B5 vitamins Gamborg *et al.* (1968) to augmented media with either full or one-half concentration of MS inorganic salts. The results revealed that although multiple shoot formation on dry bean cotyledonary nodes that contains 4-5 mm from two cotyledons and hypocotyl was possible on a medium containing one-half strength MS inorganic salts, but the number of buds and shoots was less than from nodes cultured on a medium containing the full concentration of MS inorganic salts. By these experiments we suggest that this salt concentration may be optimal for the common bean.

Shoots developed at one of two locations on the explant. The most frequent location was in the nodal region where buds began to appear about 7 days after explant preparation. Buds normally appeared in clusters and the clusters were distributed randomly in the nodal region. Bud and shoot development occasionally occurred on the epicotyl. Shortly after elongation, bud and shoot development progressed on the epicotyl. The cellular origin of these shoots has not been determined.

Axillary shoots (0.5 to 1cm) were removed during counts of bud and shoot numbers. Each explant was saved and transferred to full and half-strength MS with B5 vitamins, 20 g sucrose/l, and 7g agar/l without plant growth regulators, in order to induce root formation and plantlet development.

In conclusion, these experiments describe a procedure to regenerate multiple-shoots from dry bean plants, and select the best type from different explants for reproducible high frequency of shoot formation. The optimal treatment for the induction of multiple shoot formation was the culturing of cotyledonary node explants on a MS-based media supplemented with 0.5mg/l BA and 0.1mg/l NAA. The shoots are adventitious, and may be useful in genetic transformation of bean and is being assessed as a source of novel genetic variation.

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