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In vitro plant regeneration from immature embryo axis and cotyledons of common bean (*Phaseolus vulgaris* L.)

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Summary: *Phaseolus vulgaris* L. is the most important economic species within the genus *Phaseolus*. It is grown in all parts of the world. Genetic improvement by conventional breeding has met considerable success, although production of hybrids between species within the genus has been limited due to sexual incompatibility. Recent advances in tissue culture have offered the opportunity to produce cultivars, which could not be obtained by conventional breeding methods. The use of tissue culture and genetic engineering is viewed as a logical approach to improve bean production. Gene transfer techniques will have a great impact on legumes. Although the concept of cell totipotency is widely proved, *in vitro* morphogenesis has not yet been achieved for a large number of cultivated beans. Regeneration protocols are strongly influenced by the genotype. In tissue and cell culture of beans, the factors controlling shoot morphogenesis and somatic embryogenesis are still unknown. The reported data suggest a possible way for future research.

Key words: *Phaseolus vulgaris*, bean, regeneration, immature cotyledons, embryonic axis

Abbreviations: MS = *Murashige & Skoog* (1962), ABA = Abscisic acid, 2iP = 2-isopentenyl adenine, NAA = -naphthaleneacetic acid

Introduction

Dry common beans are the major source of protein and the most important food legume in the developing world. Most of the bean production is confined to *P. vulgaris*. Its production has not been improved significantly. Consequently, there is considerable interest in the introduction of useful traits (*Mergenthaler & Bisztray*, 2000; *Singh* 2001) into common bean by breeding and genetic engineering. Classical breeding has met limited success. The other alternative approach is transferring desirable genes from other sources. The main hindrance to transformation is that it is recalcitrant to *in vitro* morphogenesis. Genetic transformation of bean has been difficult and challenging till now. The use of cell culture and genetic engineering is viewed as a logical approach to improve legume crops (*Mok* et al. 1986). Genetic transformation is one of the interesting areas of biotechnology for plant breeders because characteristics can be added with minimal alteration of the target plant's genome. Therefore, the direct shoot morphogenesis from the primary tissues is more desirable for transformation process than going through an intermediate callus phase (*Stiekema* et al. 1988). Gene transfer techniques will have a great impact on legumes.

In *Phaseolus*, few experiments have been carried out concerning *in vitro* culture of the young immature embryos. Researchers try to define technique and media suitable for the culture of various *P. vulgaris* genotypes. For the transformation of *P. vulgaris* it is essential to define an *in vitro* culture media that takes into account the *in vivo* properties of bean and allows succesfull plant regeneration. Efficient, repeatable, and rapid *in vitro* regeneration systems are a prerequisite for using recent advances in biotechnology to improve crop plants.

Direct shoot organogenesis from cotyledonary node tissues of *P. vulgaris* was reported by *McClean & Grafton* (1989). Shoot and bud formations at the cotyledonary node tissues from *in vitro* cultures of developing cotyledonary explants were described by *Mohamed* et al. (1991) and plant regeneration from embryonic axis explants of common and tepary beans was reported (*Mohamed* et al. 1992). *Eissa Ahmed* et al. (1998) investigated direct shoot organogenesis from different seedling explants of common bean. Also, shoot bud formation from cotyledonary and primary node tissues, intact seedlings and from *in vitro* cultures of developing explants and intact seedlings, was reported, and its potential applications discussed (*Eissa Ahmed* et al. 1999a, 1999b, 2001, 2002).

Plant regeneration from induced multiple buds via direct shoot bud organogenesis from embryonic axis explants in *Phaseolus* spp. (*Mohamed* et al.1991b, Aragão et al. 1996, *Zambre* et al. 1998) and from immature cotyledons and isolated embryos in soybean (*Lippmann & Lippmann* 1984, *Parrott* et al. 1989, *Lippmann & Lippmann* 1993) has been reported. Organogenesis and plant regeneration from immature cotyledons has been achieved successfully in *P. coccineus* L. (*Allavena & Rossetti* 1986, *Angelini & Allavena* 1989). Regeneration-competent callus of *P. vulgaris* and *P. acutifolius* was obtained from mature embryo explants on a medium containing TDZ and IAA (*Zambre* et al. 1998). For the *P. vulgaris* genotype Xan-159, regeneration was achieved from

cotyledon explants, but not from embryonic axis. Both explants could be used for the *P. acutifolius* genotype NI 574 but embryonic axes gave the best results. In case of faba bean lines, embryo axes and shoot tips were cultured on MS medium containing 1, 2 or 4mg BA and/or TDZ/l (*Khalafalla & Hattori* 2000). Explants cultured on medium with TDZ+BA produced a higher number of shoots compared with either cytokinin alone. The embryo axes promoted the formation of more adventitious shoots than shoot tips. The line "740", cultured with 2mg TDZ+2mg BA gave the highest number of shoots per explant from both embryo axes and shoot tips (10.2 and 7.7, respectively). Explants cultured on MS or (MS+B5 vitamins) media produced more shoots than on B5 or half-strength MS or B5 media. The results of (*Santalla* et al. 1998) indicated that *in vitro* culture response and regeneration ability from seedling explants containing a cotyledon and a small portion of the split embryonic axis varied significantly between species and amongst genotypes. *P. coccineus* produced more shoots per explant than *P. vulgaris*.

If regenerative, the explant meristematic tissues could be used directly for foreign gene insertion to produce transgenic plants (*McCabe* et al. 1988). Shoot bud organogenesis from embryonic explants also could be used to increase the efficiency of *in vitro* production of F_1 hybrids in certain interspecific crosses that fail to germinate using the regular embryo rescue procedures (*Chen* et al. 1990). Based on the introduction of genes into meristematic cells by partcle bombardment, *Aragão* (2002) developed an efficient and reproducible system to achieve routinely transgenic plants of several species, such as dry bean, soyabean and cotton. The apical region of embryonic axes has been an obvious target for the development of systems.

The primary objective of this study was to develop a protocol for regeneration of bean plants from tissue cultures of embryonic axis and immature cotyledons explants. Our aim was to work out an efficient system for the regeneration of bean as a base of transformation. The regenerating capability of different bean genotypes was examined.

Material and method

Plant material

Seeds of *P. vulgaris* – lines Almere (A1), S, St_{52}, STR, ($A1xR_{12}$) and R_{12} were evaluated in this study. Seeds were obtained from the breeder (Prof. *I. Velich*). All the experiments were carried out and kept at the Department of Genetics and Plant Breeding, Faculty of Horticultural Sciences, Corvinus University Budapest (formerly: Szent István University), Hungary.

Method

Six lines of common bean were selected based on their wide genetic diversity and use in the bean genetic and breeding program in Hungary. The plants of each line were grown in the greenhouse. Pods were taken after two weeks after pollination. Pods were harvested early in the morning when plants were at full turgor. Pods of each genotype were collected from greenhouse grown plants. After surface sterilization, with 1% NaOCl for 25 minutes, the embryos were aseptically removed. The embryonic axes were excised from the immature embryos. Immature cotyledons, embryonic axes and immature seeds cultured in 55x90 mm glass bottles containing *Murashige Skoog* (1962) (MS) basal medium (BM) supplemented with 3.3 mg/l Abscisic acid (ABA) or 10mg/l 2-isopentenyl adenine (2iP) and 0.05 mg/l α-naphthaleneacetic acid (NAA). The pH of the media was adjusted to 5.7 with KOH or NaOH before autoclaving for 15 min at 121 °C. Cultures were maintained in a growth chamber at 24 to 25 °C with a 16/8 h light/dark cycle at white light. Percentage of explants developing shoots and plants was recorded after 7 weeks on the primary culture.

Results and discussion

The immature cotyledon explants from seeds taken from two weeks old pods developed callus on ABA and (2iP+NAA) containing media, while the older cotyledons became green and underwent morphogenesis very rarely and formed roots only, or also produced shoots as well. Some of the very young cotyledons bleached and died. Callus formation from bean immature cotyledons was occurred in all six genotypes tested as shown in *Table 1*. The highest percentage of plant rageneration from bean immature cotyledons was 16.67% in genotype STR cultured on MS medium containing (10 mg/l 2iP+0.05 mg/l NAA) and 10% in genotypes A1 and $A1xR_{12}$ cultured on MS media containing 3.3 mg/l ABA (*Table 1*).

These significant genotype effects suggest that genetic factors are important in the response to *in vitro* tissue culture. This *in vitro* culture system carries a high potential for regeneration and transformation of *P. vulgaris*, which could also be incorporated into somatic cell approaches to improve this species. Similar findings were obtained by *Allavena & Rossetti* (1986) who reported that leaves, callus and shoots rose from the immature cotyledons of *P. coccineus* cultured on the medium containing IAA and BA after 24 h treatment on medium containing ABA. I case of *P. coccineus*, *Angelini & Allavena* (1989) also found that the highest percentage of regeneration (37.5%) was observed when the cotyledons were cultured on MS medium containing 10 mg/l 2iP+0.05 mg/l NAA. In addition, *Parrott* et al. (1989) conclude similarly, that the genotype has a large effect on the ability of immature soybean cotyledons to undergo auxin-stimulated somatic embryogenesis. Moreover, among 33 soybean lines, they tested, all those showing good regeneration were found to have in their pedigrees one or both of the highly regenerative ancestral lines. A method for the induction of somatic embryos in soybean tissue cultures was investigated by *Lippmann & Lippmann* (1984). Cotyledons from immature embryos were utilized as explant source. Supplementing the

Table 1 Morphogenesis and plant regeneration from immature cotyledons of 6 lines of *P. vulgaris* cultured on MS medium containing 3.3mg/l ABA or (10 mg/l 2iP+0.05 mg/l NAA)

Genotypes	MS+3.3mg/l ABA	MS+(10mg/l 2iP+0.05mg/l NAA)	Nb of tested immature cotyledons	Callus%	Regeneration Shoots%	Shoots and roots%	Nb of regenerated plantlets%
A1	+	–	50	60	0	10	10
S	+	–	58	27.56	3.44	0	3.44
A1xR$_{12}$	+	–	50	30	0	10	10
R$_{12}$	–	+	50	60	0	0	0
St$_{52}$	–	+	40	100	0	10	10
A1xR$_{12}$	–	+	70	17.14	0	0	0
STR	–	+	36	100	0	16.67	16.67

Table 2 Morphogenesis and plant regeneration from embryo axes of 3 lines of *P. vulgaris* cultured on MS medium containing 3.3mg/l ABA or (10mg/l 2iP+0.05mg/l NAA)

Genotypes	MS+3.3mg/l ABA	MS+(10mg/l 2iP+0.05mg/l NAA)	Nb of tested immature cotyledons	Callus%	Regeneration Shoots%	Shoots and roots%	Nb of regenerated plantlets%
S	+	–	90	0	88.89	11.11	100
A1xR$_{12}$	+	–	60	45	0	85	85
S	–	+	70	0	0	100	100
ST	–	+	36	100	0	33.33	33.33
A1xR$_{12}$	–	+	60	0	0	100	100

Table 3 Morphogenesis and plant regeneration from immature seeds of 5 lines of *P. vulgaris* cultured on MS medium containing 3.3mg/l ABA or (10mg/l 2iP+0.05mg/l NAA)

Genotypes	MS+3.3mg/l ABA	MS+(10mg/l 2iP+0.05mg/l NAA)	Nb of tested immature cotyledons	Callus%	Regeneration Shoots%	Shoots and roots%	Nb of regenerated plantlets%
R12	+	–	58	10.34	0	24.13	24.13
S	+	–	30	0	0	0	0
A1xR12	+	–	132	80.14	0	17.64	17.64
St52	–	+	60	16.67	0	0	0
A1xR12	–	+	88	29.54	0	9.10	9.10
ST	–	+	45	33.33	0	0	0

culture medium with auxins (2,4-D, MCPA, 2,4,5-T, NAA, IAA, IBA) caused formation of meristematic tissue on cotyledon explants. The extent of meristematic tissue formed depended on the kind and concentration of auxin in the culture medium. Embryoid formation rates were influenced by the developmental stage of the embryos serving as explant source and auxin concentration. Also, *Lippmann* & *Lippmann* (1993) have shown that a culture medium containing only 25mM KNO3 as the nitrogen source supported embryo growth in soybean. The tested plant growth regulators, IAA, BAP and GA3, stimulated growth and plant development when added to the medium at a low concentration (0.1µM). The optimal temperature for *in vitro* growth of cotyledon stage embryos was 27 °C.

Our results indicated that the greatest percentage of regenerating plants for all genotypes were obtained using embryo axes explants. Three genotypes were tested [S, (A1xR$_{12}$) and ST)]. Plant regeneration from embryo axes occurred in all genotypes, cultured on MS medium containing 3.3 mg/l ABA or (10 mg/l 2iP+0.05 mg/l NAA). The highest percentage of regeneration was 100% with genotypes (A1xR$_{12}$) and S, when embryo axes were cultured on MS medium containing(10mg/l 2iP+0.05 mg/l NAA), or with genotype S, explatns when cultured on MS medium containing 3.3mg/l ABA only (*Table 2*).

These results are new compared to others in the literature. While *Mohamed* et al. (1992) reported that 15–90% of embryonic axes explants of common bean regenerated multiple shoots on medium containing –10µM BA, media with NAA alone or with 20 µM BA gave only callus. The shoot initials were found only on the meristematic regions and tips of embryonic axes. *Benedicic* et al. (1997) studied the growth and development of *P. vulgaris* L. cv. Zorin meristems *in vitro*. Special attention was paid to the influence of different types of cytokinins and their concentrations on bud induction, shoot growth and callus formation. Although the buds developed on all media used, the regeneration of plants achieved only when meristems were isolated on basal medium containing 1 and 5 µmol/l BA or 6-gamma, gamma-dimethylallylamino-purine and with 20 µmol/l BA and 1.4 µmol/l GA3.

Similar results were obtained by *Ignacimuthu Franklin* (1998), who demonstrated that seed-derived cotyledon and embryonal axis explants of *Vigna mungo* L-Hepper were capable of producing multiple shoots similarly when cultured

on MS medium containing B5 vitamins, BAP and NAA. Regeneration of plants was obtained via organogenesis from mature embryonal axes explants of pigeonpea (*Franklin* et al., 2000) as well. However, shoots were produced from embryonal axes after 20 days of dark incubation on MS medium containing 8.86 µM BAP and 1.07 µM NAA. When the explants were cultured under light-dark (16–8 h) conditions, shoots were initiated only after 65 days of culture initiation.

Data in *Table 3* show that most immature seeds tested produced callus only. The higher number of regenerated plantlets per immature seed was observed for R_{12} and ($A1xR_{12}$). The regeneration process was more efficient in R_{12} and ($A1xR_{12}$) genotypes (*Table 3*).

These results are comparable to *Mohamed* et al. (1991). They used the basal one-third of the cotyledons, including the proximal notch as the explant. They olso detected the effect of the age of the explant. They found that while the cotyledonary explants from 10–12 day-old pods developed callus only, and from the 21 day-old pods only formed roots or also shoots (on 30% of the explants). All genotyps initiated shoots and roots from the 28 day-old pods (15–50%). The effect of the age of the explant could be in connection with changes in enzyme activities and concentration of other factors during bean ontogenesis, as described by *Sárdi et al.* (1999).

Here, we describe a novel procedure to obtain *in vitro* regeneration from immature cotyledons, embryonic axes and seeds in *P. vulgaris*. Success was achieved with 6 genotypes [A1, S, ($A1xR_{12}$), St_{52}, STR, ST]. Immature cotyledon explants gave the best results for 5 genotypes [A1, S, ($A1xR_{12}$), St_{52}, STR]. For embryonic axes explants 2 genotypes [S and ($A1xR_{12}$)] gave sufficient results. For immature seeds genotypes R_{12} and ($A1xR_{12}$) gave the best results. A protocol had been developed for regeneration of common bean from immature cotyledons and embryo axes. The regenerating capability of the bean depends on the genotype. The aim is to develop a highly efficient *Agrobacterium* and particle bombardment-based transformation methods for *P. vulgaris*. Therefore, a procedure for regeneration of shoots from immature cotyledons and embryos in *P. vulgaris* should be of great help to attain genetic transformation of this important species.

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