J. Plant Biotechnology (2005) Vol. 7 (4). pp. 267~272

Journal of Plant Biotechnology

De novo Regeneration of Fertile Common Bean (*Phaseolus vulgaris* L.) Plants

Margareth M. C. Albino[1,2], Giovanni R. Vianna[1], Rosana Falcão[1], Francisco J. L. Aragão[1*]

[1]Embrapa Recursos Genéticos e Biotecnologia, Laboratório de Introdução e Expressão de Genes, PqEB W5 Norte, 70770-900, Brasília, DF, Brazil. [2]Universidade de Brasília, Departamento de Botânica, Instituto de Ciências Biológicas, C.P. 04457, 70919-970, Brasília, DF, Brazil.

Abstract

Common bean (*Phaseolus vulgaris* L.) plants were regenerated via organogenesis from mature embryonic axes, cultured on MS medium supplemented with ildole-3-ecetic acid (IAA) and thidiazuron (TDZ) for one week in the dark. Embryonic axillary regions were excised, longitudinally cut to split the both sides, and cultured for two weeks on MS medium supplemented with IAA and TDZ. The combination 0.5 mg · l^{-1} TDZ/0.5 mg · l^{-1} IAA presented the higher efficiency in shoot regeneration and the combination 0.5 mg · l^{-1} TDZ/0.25 mg · l^{-1} IAA presented the higher efficiency in conversion of shoots to plants. Regenerating explants were transferred to MS medium containing 1 mg · l^{-1} BAP for shoot development. All elongated shoots were rooted *in vitro*, presented normal phenotype and produced viable seeds. Histological analysis confirmed the mode of regeneration as *de novo* shoot organogenesis.

Key words: gene expression, gene transfer, organogenesis, regeneration, shoot formation

Introduction

In the tropical and sub-tropical regions of Latin America, Africa and Asia, the common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for direct consumption. Consequently, there is considerable interest in the introduction of agronomically useful traits into beans by classical and molecular breeding. However, molecular genetic manipulation for applied purposes is only possible with the development of an efficient protocol for transformation. Transformation procedures will be efficient only if an efficient protocol of regeneration has previously been established.

Numerous attempts have been made to regenerate common bean plants from several types of isolated cells and tissues. *In vitro* shoot organogenesis (through multiple shoot induction) from the cotyledonary node, shoot tips and petioles with leaf have been reported (reviewed by Nagl et al. 1997; Svetleva et al. 2003). Cruz de Carvalho et al. (2000) used the transverse thin cell layer method (tTCL) to optimize the frequency of shoot regeneration, without intermediate callus. The induction of multiple shooting in the apical and lateral meristematic region in bean has been demonstrated (Kartha et al. 1981; Martins and Sondahl 1984). Neoformation of shoots in the meristems has been induced by cytokinin (N^6 benzylaminopurine, BAP). The induction of morphogenesis in bean apical regions has been achieved, through the culture of mature embryos in high doses of cytokinins, such as kinetin, zeatin and BAP (McClean and Grafton 1989; Franklin et al. 1991; Malik and Saxena 1992; Mohamed et al. 1992, 1993; Aragão et al. 1996). Compounds that present cytokinin-like effects, such as TDZ (thidiazuron) and CPPU (forclhrofenuron) have also been evaluated (Mohamed et al. 1992).

Although several combinations of hormones, explants and culture conditions have been used to regenerate somatic embryos of *P. vulgaris* (Allavena and Rossetti 1983; Allavena 1984; Martins and Sondahl 1984; Saunders et al. 1987; Nagl et al. 1997), no plantlets have been successfully obtained from the embryoids. In addition, reports on regeneration via callus are limited in *Phaseolus* species. Induction of morphogenic callus has been reported in *P. acutifolius* (Kumar et al. 1988; Dillen et al. 1996), *P. coccineus* (Genga and Allavena 1991), *P. vulgaris* (Zambre et al. 1998) and *P. polyanthus* (Zambre et al. 2001).

* Corresponding author, E-mail: aragao@cenargen.embrapa.br
Received Aug. 17, 2005; Accepted Dec. 22, 2005

The lack of an efficient regeneration protocol of common bean has been obstructed the development of efficient transformation systems. Nevertheless, multiple shoot induction, in association with the biolistic process, allowed recovering transformed common bean plants with agronomic traits (Russel et al. 1993; Aragão et al. 1998; Aragão et al. 2002). However, in our experience, for each 30-40 obtained transgenic lines, one has the potential to be introduced into a breeding program. Consequently, the development of an efficient regeneration protocol should accelerate the development of transgenic bean varieties with improved agricultural characteristics. Here we report the development of efficient regeneration system for common bean.

Materials and Methods

Plant material

Hand harvested mature seeds of the common bean (*Phaseolus vulgaris* L.), cv. Olathe Pinto, were soaked in 70% ethanol, stirred for 1 min and surface sterilized in 1% sodium hypochlorite for 20 min. They were rinsed four times in sterile distilled water and soaked in distilled water for 16-18 h. The embryonic axes were excised from the seeds and the primary leaves and radicles were removed.

Plant regeneration and acclimatization

The embryonic axes were cultured on MS medium supplemented with IAA (0, 0.25, 0.5 and 0.75 mg · L^{-1}) in combination with TDZ (0, 0.25, 0.5 and 0.75 mg · L^{-1}) for one week in the dark. Then, the axillary regions were excised, longitudinally cut to split the both sides, and cultured for two weeks in Petri dishes containing MS medium supplemented with IAA (0, 0.25, 0.5 and 0.75 mg · L^{-1}) in combination with TDZ (0, 0.25, 0.5 and 0.75 mg · L^{-1}). The explants were positioned with the cut region in contact with the medium. The explants were transferred to MS medium containing 1 mg · L^{-1} BAP and were cultured for two weeks for shoot development. The elongated shoots (1 to 2 cm long) were detached from the callus and cultured in baby food jars containing MS medium without growth regulators, and subcultured after every 15 days for rooting. The jars and Petri dishes were arranged in a randomized complete block design. All experiments were repeated three times and each treatment. Each experiment was set up in three or four repetitions (baby food jars or Petri dishes). Unless specified, the explants were cultured under a photoperiod of 16 h (50 μmol · m^{-2} · s^{-1}) at a temperature of 26 ±2℃.

The rooting plantlets were individually transferred to a plastic pot containing 0.2 dm^3 of autoclaved fertilized soil: vermiculite (1:1), covered with a plastic bag sealed with a rubber band and maintained in a greenhouse. After one week, the rubber band was removed. After an additional week, the plastic bag was also removed. As soon as the acclimated plantlets reached approximately 10 cm in length they were transferred to a pot containing 5 dm^3 of fertilized soil and allowed to set seeds. The temperature in the greenhouse was 25±5℃ and the relative humidity above 80%.

Light microscope

Explants from all regeneration stages were fixed with FAA (acetic acid: formaldehyde: ethanol; 1:1:18; v/v/v) for 24 h at 4℃, dehydrated in increasing concentrations of ethanol and embedded in paraplast. Sections were cut at 7 μm, mounted on glass slides and re-hydrated in a descending ethanol series. Slides were stained in Johansen's safranin/fast green schedule as described (Sylvester and Ruzin 1994).

Microparticles bombardment and GUS histochemical assay

The bombardment was carried out as described by Aragão et al. (1996), utilizing a high-pressure helium-driven particle acceleration device (Sanford et al. 1991). The plasmid vector pBI426, which contains the *gus-neo* gene fusion (Datla et al. 1991) driven by a double 35S cauliflower mosaic virus (CaMV) promoter plus a leader sequence from alfalfa mosaic virus (AMV), was utilized. The explants were analyzed for *gus* gene expression according Jefferson et al. (1987).

Results

Plants regeneration

The effect of several combinations of IAA/TDZ on regeneration of the common bean was studied. Embryonic axis cultivated for one week in the dark presented an albino elongated phenotype and were transferred to light. After one week exposed to light, the explants became green (Figure 1a), the axillary regions were excised and transferred to MS medium containing IAA and TDZ. After one week, a green callus formation was observed (Figure 1b). The combination 0.25 mg · L^{-1} TDZ with 0.25, 0.5 or 0.75 mg · L^{-1} IAA presented a higher green calluses formation and growing. The green calluses were transferred to MS medium supplemented with BAP. After two weeks shoots formatting and elongating from the green callus were observed (Figure 1c).

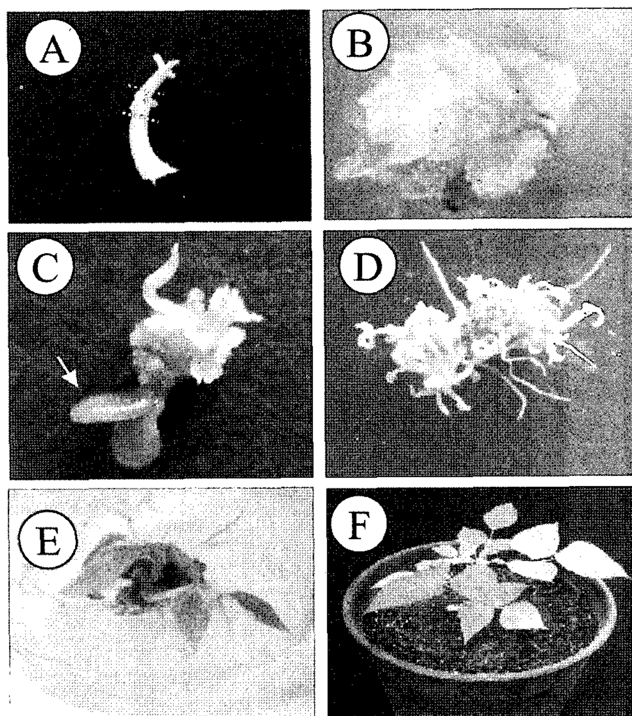

Figure 1. Regeneration of common bean plants. (a) Embryonic axis cultured for two weeks on MS medium supplemented with TDZ and IAA. Punctuated lines shown the explants used in the subsequent culture stages. (b) Green callus formed nine days after cultivation of hypocotyl explants on MS medium supplemented with TDZ and IAA. The arrow indicates the pre-existing axillary bud. (c) First shoots regenerating after cultivation for two weeks on MS medium supplemented with BAP. (d) Elongating shoots after two weeks on MS medium without growth regulators. (e) Rooted plantlet. (f) Acclimatized plant in the green house.

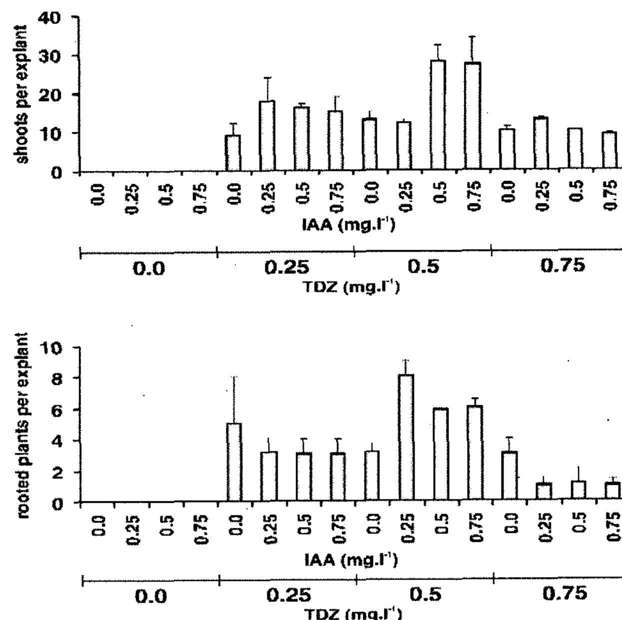

Figure 2. Effect of IAA (0, 0.25, 0.5 and 0.75 mg · L^{-1}) in combination with TDZ on shoots formation and their conversion into mature plants. Error bars represent standard deviation from the mean. (n=12).

Although the combination of 0.5 mg · L^{-1} TDZ/0.5 mg · L^{-1} IAA have presented the higher efficiency in shoot regeneration (27 shoot/explant), the combination 0.5 mg · L^{-1} TDZ/0.25 mg · L^{-1} IAA have shown to render the higher efficiency in conversion of shoot to plantlets (7 plants/explant) (Figure 2). Explants cultivated on MS medium supplement only with IAA were unable to regenerate plantlets (Figure 2). Eighty percent of the plantlets were able to root when transferred to MS medium without growth regulators (Figures 1d and 1e). The establishment of *in vitro* rooted plantlets to the soil under greenhouse conditions was achieved with a frequency of 100%. All regenerated plants presented a normal phenotype (Figure 1f) and produced viable seeds.

Histological analysis of the regenerating explants

The axiliary regions cultured on MS medium containing TDZ and IAA were analyzed using light microscopy. After cultivation for two weeks, explants revealed cell agglomeration in the upper sides (Figure 3a). The explants were cut and transferred to MS medium containing TDZ and IAA. After three weeks, the explants revealed the formation of *de novo* meristematic regions arising from epidermal regions (Figures 3b and 3c). The regenerating explants were then transferred to BAP and primordial shoots initiated to emerge from both epidermal and sub-epidermal layers without vascular connection with the explant (Figure 3d). Buds continued to elongate and differentiate into shoots after explant cultivation on MS medium without growth regulators (Figures 3e and 3f).

Transient gus gene expression

The competence for transformation of both embryonic axis and hypocotyls-derived explants was evaluated using the biolistic process. The initial embryonic axes, axillary regions and green calli were bombarded with the vector pBI426, containing the *gus* gene. The explants revealed a strong *gus* gene expression 24 hours after the bombardment (Figure 4). However, the embryonic axis revealed a greater number of blue spots when compared to axillary regions and green calli (Figure 4a).

Discussion

In this study, fertile plants of *P. vulgaris* were regenerated

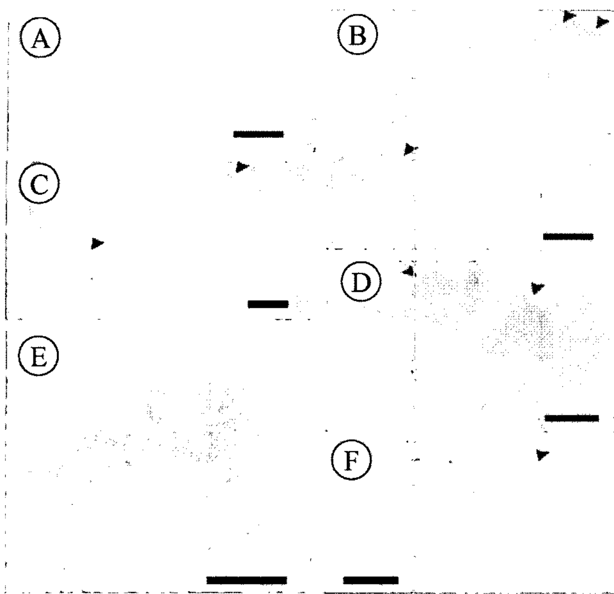

Figure 3. Histological analysis of the regenerating explants. (a) Hypocotyls excised from the embryonic axis cultured for two weeks on MS medium containing TDZ and IAA. (b and c) *de novo* meristematic regions formation (arrows) after cultivation for three weeks on MS medium supplemented with TDZ and IAA. (d to f) Buds neoformation and elongation after cultivations for two weeks on MS medium supplemented with BAP. Bars represent 100 μm (in a, b, c, d and f) and 200 μm in e.

Figure 4. Common bean explants expressing of the *gus* gene 24 h after particle bombardment. (a) Embryonic axes. (b) Axillary buds. (c) Green callus.

via organogenesis. This system was generated in order to be utilized for genetic transformation. The cultivation of axillary buds on MS medium supplemented with TDZ and IAA allowed the achievement of new formation of buds, which were converted in fertile plants. The period of cultivation on a combination of TDZ/IAA was crucial for the achievement of high frequency of green callus formation and further shoots regeneration. Moreover, the cultivation of responsive explants on medium supplemented with BAP was essential for shoot formation, development and surviving. The cytokinin-auxin ratio is known to be critical in shoot organogenesis and rooting (Nagl et al. 1997; Zambre et al. 2001; Sugiyama, 1999; Svetleva et al. 2003). Therefore, the response of the explants to several combinations of TDZ/IAA ratio was studied. Explants cultivated on MS medium supplemented only with IAA were unable to regenerate. *Phaseolus* species have been regenerated combining TDZ and IAA, such as *P. vulgaris*, *P. accutifolius* and *P. polyanthus* (Zambre et al. 1998; Zambre et al. 2001). Nevertheless, for *P. vulgaris* the frequency of regeneration was lower and rooted elongated shoots died upon transference to soil (Zambre et al. 1998). In addition, embryonic axis of *P. vulgaris* cv Xan-159 produced green callus that deteriorated during successive subcultures, and no shoots were recovered (Zambres et al. 1998). Cruz de Carvalho et al. (2000) were able to regenerate *P. vulgaris* cultivating transverse thin cell layers (tTCL) on MS medium supplemented with TDZ, optimizing the frequency of shoot regeneration. The importance to TDZ for shoot organogenesis has also been reported for several other leguminous plants, such as pigeon pea (Eapen et al. 1998; Singh et al. 2003), chickpea (Bajaj and Dhanju 1979; Malik and Saxena 1992), lentil (Malik and Saxena 1992), Guar (Joersbo et al. 1999), peanut (Kanyand et al. 1994; Kanyand et al. 1997; Akasaka et al. 2000), pea (Böhmer et al. 1995) and *Vigna mungo* (Sen and Guha-mukherjee 1998).

The development of shoots, emerging from superficial layers, and absence of vascular connection to axillary buds is generally taken as evidence for a *de novo* shoot formation (Fakhrai et al. 1989; Pickardt 2003). Although pre-existing meristematic tissues are present in the original explants, the histological analysis carried out to determine the origin of initials buds suggested that the generated meristems were not formed from pre-existing axillary meristematic region.

Efforts to achieve an efficient methodology for bean transformation have been obstructed due to the lack of an efficient tissue culture system to regenerate plants from transformed cells. The unique bean transformation system available is based on the gene bombardment of apical meristematic cells from mature embryonic axes (for a review see Nagl et al. 1997 and Svetleva et al. 2003). Nevertheless, we have been able to achieve transgenic bean plants using the biolistic process to introduce genes for virus resistance, herbicide tolerance and improvement of protein quality (Arag ão 2003). However, the genetic engineering of the common bean is still not trivial, due to the lack of an efficient regeneration system. The results obtained with the transient expression of the *gus* gene introduced by the biolistic process indicated that the explants were adequate for transformation. The embryonic axes presented the greatest number of blue spots and were considered more suitable for plant transformation experiments. In addition, explant transformation in the early regeneration stages might facilitate the selection and the achievement of transgenic plants.

The regeneration system described here form the foundation for the development of an efficient genetic transformation system for common bean. It may accelerate the obtain of elite transgenic lines as well as functional gene studies.

Acknowledgements

This work was supported by FINEP. We are grateful to Dr. Josias C. Faria (Embrapa Arroz e Feijão, Brazil) for providing the common bean seeds, Warley Almeida and Luiz Lemos for technical assistance.

References

Akasaka Y, Daimon H, Mii M (2000) Improved plant regeneration from cultured leaf segments in peanut (*Arachis hypogaea* L.) by limited exposure to thidiazuron. Plant Sci 156: 169-175

Allavena A (1984) Beans (*Phaseolus*). In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds), Handbook of Plant Cell Culture, Vol. 2, Macmillan Publishing CO., New York, pp137-168

Allavena A, Rossetti L (1983) Efforts in somatic embryogenesis of *Phaseolus vulgaris* L. Acta Hort 131: 239-246

Aragão FJL (2003) Genetic transformation of common bean via particle bombardment. In Jaiwal PK, Singh RP (eds), Applied Genetics of Leguminosae Biotechnology, Kluwer Academic Publisher, Dordrecht, pp. 35-45

Aragão FJL, Barros LMG, Brasileiro ACM, Ribeiro SG, Smith FD, Sanford JC, Faria JC, Rech EL (1996) Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. Theor Appl Gent 93:142-150

Aragão FJL, Ribeiro SG, Barros LMG, Brasileiro ACM, Maxwell DP, Rech EL, Faria JC (1998) Transgenic beans (Phaseolus vulgaris L.) engineered to express viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. Mol Breed 4: 491-499

Aragão FJL, Vianna GR, Albino MMC, Rech EL (2002) Transgenic dry bean tolerant to the herbicide glufosinate ammonium. Crop Sci 42:1298-1302

Bajaj YPS, Dhanju MS (1979) Regeneration of plants from apical meristem tips of some grain legumes. Curr Sci 48: 906-907

Böhmer P, Meyer B, Jacobsen HJ (1995) Thidiazuron-induced high frequency of shoot induction and plant regeneration in protoplast derived pea callus. Plant Cell Rep 15: 26-29

Cruz de Carvalho MH, Van Le B, Zuily-Fodil Y, Pham Thi AT, Tran Thanh Van K (2000) Efficient whole plant regeneration of common bean (*Phaseolus vulgaris* L.) using thin-cell-layer culture and silver nitrate. Plant Sci 159: 223-232

Datla RS, Hammerlindl JK, Pelcher LE, Crosby WL, Selvaraj G (1991) A bifunctional fusion between beta-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. Gene 101: 239-146

Dillen W, De Clercq J, Van Montagu M, Angenon G (1996) Plant regeneration from callus in a range of *Phaseolus acutifolius* A. Gray genotypes. Plant Sci 118: 81-88

Eapen S, Tivarekar S, George L (1998) Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L. Millsp.). Plant Cell Tiss Org Cult 53: 217-220

Fakhrai F, Evans PK (1989) Morphogenic potential of cultured explants of *Crocus chrysanthus* Herbert. cv. E. P. Bowles. J Exp Bot 40: 809-812

Franklin CI, Trieu TN, Gonzales RA, Dixon RA (1991) Plant regeneration from seedlings explants of green bean (*Phaseolus vulgaris* L.) via organogenesis. Plant Cell Tissue Organ Cult 24:199-206

Genga A, Allavena A (1991) Factors affecting morphogenesis from immature cotyledons of *Phaseolus coccineus* L. Plant Cell Tiss Org Cult 27:189-196

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907

Joersbo M, Brunstedt J, Marcussen J, Okkels, FT (1999) Transformation of the endospermous legume guar (*Cyamopsis tetragonoloba* L.) and analysis of transgene transmission. Mol Breed 5: 521-529

Kanyand M, Dessai AP, Prakash CS (1994) Thidiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea*) plants *in vitro*. Plant Cell Rep 14: 1-5

Kanyand M, Peterson CM, Prakash CS (1997) The differentiation of emergences into adventitious shoots in peanut, *Arachis hypogea* (L.). Plant Sci 126: 87-95

Kartha KK, Pahl K, Leung NL, Mroginski LA (1991) Plant regeneration from meristems of grain legumes: soybean, peanut, chickpea and bean. Can J Bot 59:1671-1679

Kumar AS, Gamborg OL, Nabors MW (1988) Regeneration from long-term cell suspension cultures of tepary bean (*Phaseolus acutifolius*). Plant Cell Rep 7: 322-325

Malik KA, Saxena PK (1992) Thidiazuron induces high frequency shoot regeneration in intact seedling of pea (*Pisum sativum*), chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris*). Aust J Plant Physiol 19: 731-740

Martins IS, Sondahl MR (1984) Multiple shoot formation from shoot apex cultures of *Phaseolus vulgaris*. J Plant Physiol 115:205-208

McClean P, Grafton KF (1989) Regeneration of dry bean (*Phaseolus vulgaris*) vis organogenesis. Plant Sci 60: 117-122

Mohamed MF, Read PE, Coyne DP (1992) Plant regeneration from *in vitro* culture of embryonic axis explant in common bean and terapy bean. J Am Soc Hort Sci 117:332-336

Mohamed MF, Coyne DP, Read PE (1993) Shoot organo-

genesis in callus induced from pedicel explants of common bean (Phaseolus vulgaris). J Am Soc Hort Sci 118:158-162

Nagl W, Ignacimuthu S, Becker J (1997) Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. J Plant Physiol 150: 625-644

Pickardt T (2003) *In vitro* regeneration and genetic transformation of *Vicia* species. In Jaiwal PK, Singh RP (eds), Applied Genetics of Leguminosae Biotechnology, Kluwer Academic Publisher, Dordrecht, pp. 121-131

Russel DR, Wallace KM, Bathe JH, Martinell BJ, McCabe DE (1993) Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration. Plant Cell Rep 12: 165-169

Sanford JC, Devit MJ, Russel JA, Smith FD, Harpending PR, Roy MK, Johnston AS (1991) An improved helium driven biolistic device. Technique 3: 3-16

Sylvester AC, Ruzin SE (1994) Light microscopy: I. Dissection and microtechnique. In Freeling M, Walbot V (eds), The Maize Handbook, Springer-Verlag New York, pp. 83-95

Saunders JW, Hosfield GL, Levi A (1987) Morphogenetic effects of 2,4-dichlorophenoxyacetic acid on pinto bean (*Phaseolus vulgaris* L.) leaf explants *in vitro*. Plant Cell Rep 6: 46-49

Sen J, Guha-mukherjee S (1998) *In vitro* induction of multiple shoots and plant regeneration in *Vigna*. *In Vitro* Cell Dev Biol-Plant 34: 276-280

Singh ND, Lingaraj S, Neere BS, Pawan KJ (2003) The effect of TDZ on organogenesis and somtic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). Plant Sci 164: 341-347

Sugiyama M (1999) Organogenesis *in vitro*. Curr Opi Plant Biol 2: 61-64

Svetleva D, Velcheva M, Bhowmik G (2003) Biotechnology as a useful tool in common bean (*Phaseolus vulgaris* L.) improvement. Euphytica131: 189-200

Zambre MA, De Clercq J, Vranova E, Van Montagu M, Angenon G, Dillen W (1998) Plant regeneration from embryo-derived callus in *Phaseolus vulgaris* L. (common bean) and *P. acutifolius* A Gray (terapy bean). Plant Cell Rep 17: 626-630

Zambre M, Geerts P, Maquet A, Van Montagu M, Dillen W, Angenon G (2001) Regeneration of fertile plants from callus in *Phaseolus polyanthus* Greenman (year bean). Ann Bot 88: 371-377