BIOLOGIA PLANTARUM 53 (2): 312-316, 2009

BRIEF COMMUNICATION

High frequency plant regeneration from the cotyledonary node of common bean

W. DANG[1,2] and Z.M. WEI[1]*

Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences[1], *and Graduate School of the Chinese Academy of Sciences*[2], *300 Fenglin Road, 200032, Shanghai, P.R. China*

Abstract

An efficient regeneration system for *Phaseolus vulgaris* was developed from mature seeds germinated on Murashige and Skoog (MS) medium supplemented with thidiazuron or N^6-benzylaminopurine (BA) for 6 d. Using cotyledonary nodes, multiple buds were induced on the MS medium supplemented with 5.0 mg dm^{-3} BA with the induction frequency 71.9 % after 4-week culture. The buds were then transferred onto shoot formation medium containing 1.0 mg dm^{-3} BA, 0.1 mg dm^{-3} gibberellic acid and 2.0 mg dm^{-3} silver nitrate. The addition of $AgNO_3$ enhanced the frequency of the shoot formation from 61.3 to 87.6 %. Root induction medium was half-strength MS medium with 0.75 mg dm^{-3} indolebutyric acid and 0.02 mg dm^{-3} BA. The average root frequency was 84.3 %. The regenerated plantlets with healthy roots grew successfully when transferred to soil. Using this system we obtained over 10 regenerated plantlets from one explant.

Additional key words: bud induction, *Phaseolus vulgaris*, shoot development, silver nitrate, thidiazuron.

Determining an effective and reproducible procedure for regeneration of plants from cells or tissue cultures is essential to improving common bean cultivation through genetic engineering. Previously described bean regeneration procedures include those from shoot apical meristems (Kartha *et al*. 1981, Martins and Sondahl 1984, Rubluo and Kartha 1985, Kalantidis and Griga 1993), cotyledonary and primary leaves node explants (Mohamed *et al*. 1992) devoid of axillary meristems (McClean and Grafton 1989, Mariotti *et al*. 1989) and with axillary buds and a portion of the cotyledons (Franklin *et al*. 1991), explants consisting of the petiole and part of the blade of juvenile leaves (Malik and Saxena 1991), and embryo-derived callus (Zambre *et al*. 1998). Seedling-derived thin cell layers have also been used to regenerate common bean plants (Cruz de Carvalho *et al*. 2000). All of these procedures are problematic because the frequency of regeneration is low, with only four to eight shoots generated from one explant. Malik and Saxena (1992a,b) established a high-frequency induction of direct shoot formation from intact seedlings. But, regeneration using intact seedlings is not suitable for transformation. Induction of somatic embryogenesis in tissue cultures of the common bean has been observed (Saunders *et al*. 1987, Mohamed *et al*. 1993, Nagl *et al*. 1997), however, plantlets have not been successfully regenerated from the embryoids.

Here, we tried to develop an efficient regeneration system *via* organogenesis which might be suitable for genetic transformation of common bean with *Agrobacterium tumefaciens*.

Seeds of *Phaseolus vulgaris* L. cv. Hacaidou No. 1 (from Haerbin Academy of Agricultural Sciences) were surface sterilized with 70 % ethanol for 2 min, followed by 0.1 % $HgCl_2$ for 10 min. Then the seeds were allowed to culture at temperature of 27 °C and a 16-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1}) in Petri dishes containing medium MSB_5 [Murashige and Skoog (1962) basal salts and vitamins after Gamborg *et al*. (1968)], 3 % sucrose and solidified with 0.8 % agar. At this stage, various concentrations of TDZ (0, 0.5, 1.0, 2.0 mg dm^{-3}) or BA (0, 0.5, 1.0, 2.0 mg dm^{-3}) were added to the pre-culture media. After a 6-d culture, epicotyl, hypocotyl and cotyledonary node explants were prepared. Cotyledonary

Received 8 March 2007, *accepted* 25 January 2008.
Abbreviations: BA - N^6-benzylaminopurine; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; TDZ - thidiazuron
* Corresponding author; fax: (+86) 021-54924015; e-mail: zmwei@sippe.ac.cn

nodes were prepared by slicing the embryonic axis into two halves while still attached to the cotyledons, so that the explants contained one cotyledon with a small portion (3 - 4 mm) of split embryonic axis attached to it (including axillary meristem). The epicotyls and hypocotyls were removed 2 mm from the cotyledonary nodes and cut into 1 cm segments. Pre-culture of seeds in the presence of TDZ or BA was also carried out in liquid above-mentioned medium on a gyratory shaker.

The bud induction medium contained MSB_5 and a range of concentrations of BA (0, 0.5, 1.0, 3.0, 5.0, 6.0 mg dm^{-3}). Explants were incubated on the media under conditions mentioned above and after two or four weeks the percentage of explants producing multiple buds and the number of buds were determined.

The multiple buds were then transferred onto shoot formation media containing MSB_5, supplemented with different concentrations of BA (0, 0.5, 1.0, 2.0 mg dm^{-3}) alone or in combination with different concentrations of gibberellic acid (GA_3) (0, 0.05, 0.1, 0.2 mg dm^{-3}). The culture conditions were as above. The ability of filter-sterilized silver nitrate at different concentrations (0, 1.0, 1.5, 2.0, 2.5, 3.0 mg dm^{-3}) to improve the regeneration efficiency and development of regenerated bud shoots was also tested.

The rooting medium contained half-strength MS supplemented with 15 g dm^{-3} sucrose and 8 g dm^{-3} agar. A range of concentrations (0, 0.25, 0.5, 0.75, 1.0 mg dm^{-3}) of indole-3-butyric acid (IBA), was tested for optimization of a good root system. The plantlets with healthy roots were transplanted into a mixture of equal parts (v/v) of sterilized soil and *Vermiculite*. Plants were grown under high relative humidity in a growth room for two weeks, and then they were moved to a greenhouse.

For each treatment, 30 - 50 explants were used and each experiment was repeated three times. Observations were made every week.

No obvious differences were seen in germination frequency of uniform, healthy, viable bean seeds under different cytokinin concentrations. However, the growth and development of the seedlings differed between the various treatments. Seed cultured on MSB_5 germinated normally. Seeds cultured on MSB_5 supplemented with cytokinins germinated abnormally, with enlarged cotyledons, thick and short hypocotyls, and small lateral roots.

The positive effect of TDZ or BA on the regeneration of shoots has been reported in common bean (Malik and Saxena 1992a), soybean, pea, lentil and chickpea (Yoshida 2002, Malik and Saxena 1992b). In general, *in vitro* germination of seeds saved time and resulted in more shoots, compared to the initiation from isolated organs such as hypocotyls and cotyledonary nodes. Our results showed that addition of both BA and TDZ into germination medium enhanced subsequent bud induction (frequency of buds and the number of buds per explant), but at equal concentrations TDZ was more efficient than BA (Table 1). The bud frequency was increased from 12.1 % (MS free of cytokinins) to 71.9 % (MS + 1 mg dm^{-3} TDZ). TDZ may exert its influence by modifying the metabolism of endogenous cytokinins. Germination of seeds in the presence of TDZ or BA was also carried out in liquid medium on a gyratory shaker, but a higher induction percentage in presence of cytokinins was not obtained.

Table 1. The effect of seed germination in MS medium with different cytokinins on subsequent induction of multiple buds from cotyledonary nodes cultured on MSB_5 containing 5.0 mg dm^{-3} BA (after 4-week culture). Mean values of three repeated experiments. Values followed by different letters in a column are significantly different at $P \leq 0.05$ according to LSD multiple range test.

Cytokinin conc. [mg dm^{-3}]		Number of explants inoculated	Multiple buds formation [%]	Number of buds [explant^{-1}]
BA	0	30	12.1f	3.3e
	0.5	48	23.8e	7.3de
	1.0	50	43.8c	12.0bcd
	2.0	52	36.4d	11.3cd
TDZ	0	30	12.1f	3.3e
	0.5	45	47.2c	13.7bc
	1.0	49	71.9a	25.7a
	2.0	48	60.2b	17.0b

BA was an effective inducer on the adventitious shoot buds formation (Vasudevan *et al*. 2007). After 10 d of culture initiation, a ring of compact and light-green meristematic tissue formed at the base of the cotyledon (Fig. 1*A*). At 1 - 6 mg dm^{-3} BA, bean meristems proliferated and produced multiple buds after 10 - 15 d. The concentration of BA markedly affected the ability of cotyledonary nodes to produce multiple buds. The frequency of multiple buds ranged from 15.6 to 71.9 % according to BA concentrations. The number of buds per explant was also dependent on the BA concentration (Table 2).

Table 2. The effect of BA and induction time on induction of multiple buds. Seeds were germinated at 1.0 mg dm^{-3} TDZ. Mean values of three repeated experiments, each with 50 cotyledonary nodes. Values followed by different letters in a column are significantly different at $P \leq 0.05$ according to LSD multiple range test. No buds were induced at 0 or 0.5 mg dm^{-3} BA.

BA [mg dm^{-3}]	Induction frequency [%]		Number of buds [explant^{-1}]	
	2 weeks	4 weeks	2 weeks	4 weeks
1.0	8.0d	15.6c	2.0bc	8.0c
3.0	43.5c	54.5b	5.7b	12.7c
5.0	60.2a	71.9a	12.0a	25.7a
6.0	51.8b	57.0b	14.3a	18.3b

Induction frequency was significantly enhanced, and correspondingly more buds from one explant occurred as the number of culture days increased (Fig. 1*B*). The largest

percentage of nodes was responsive when treated with 5 mg dm^{-3} BA. Furthermore, nodes cultured on 5 mg dm^{-3} BA developed significantly more buds (about 25.0 per explant) than nodes from the other treatments. As the concentration of BA was reduced to 3 or 1 mg dm^{-3}, the number of multiple buds also decreased correspondingly. No multiple buds occurred at 0.5 mg dm^{-3} BA, instead the meristems directly regenerated into single shoots. An increase in the concentration of BA beyond the optimum did not affect the frequency and the number of induction buds significantly, but the buds appeared to be developmentally suppressed and did not grow further.

Different explant types were tested for the induction of multiple buds. Among epicotyls, hypocotyls and cotyledonary nodes, only cotyledonary nodes produced multiple buds. In a previous protocol using cotyledonary nodes as explants (McClean and Grafton 1989), cotyledons and axillary buds were removed, whereas in our protocol the axillary buds and the cotyledons played a major role in the production of buds and shoots. The other two explants expanded and increased rapidly in size during the first two weeks in culture, but thereafter tissues either died or developed small yellow, loose callus that turned brown and eventually died.

In vitro shoot regeneration in the genus *Phaseolus* has been attempted repeatedly (Nagl *et al*. 1997). Common bean is generally recalcitrant to shoot regeneration. The combination of plant growth regulators was usually more effective than one alone on the shoot development (Mallikarjuna and Rajendrudu 2007). In this study, multiple regenerated buds were transferred to fresh medium containing different concentrations of BA alone or in combination with GA_3, and incubated under light. After 10-d culture, the first portion of the shoot appeared (Fig. 1*C*). Over time, more and more shoots commenced and elongated (Fig. 1*D*,*E*). Shoots did not develop to any extent on the medium without growth regulators, suggesting that BA may be essential for shoot development. On medium without BA, the buds turned brown and watery and then died. Some buds also died gradually on medium containing 0.5 mg dm^{-3} BA. BA alone stimulated the formation of shoots at each concentration tested, and was more efficient than GA_3 for shoot formation. It appears that GA_3 plays an important role in normal shoot

Fig. 1. Plant regeneration from cotyledonary node of common bean: *A* - cotyledonary node on induction medium for 10 d; *B* - cotyledonary node on induction medium for 4 weeks; *C* - buds cultured on shoot development medium for 10 d and small shoot commenced; *D*,*E* - healthy shoots formation and elongation on shoot development medium; *F* - Plantlet with healthy roots on rooting medium; *G* - regenerated plant established in soil, growing in the greenhouse; *H* - mature regenerated plant in the greenhouse.

elongation, rather than formation. Of the combinations of BA and GA_3 tested, 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} GA_3 were found to be the most effective and produced the highest frequency of shoot formation (61.3 %).

Production of buds and shoots is a continuous process. In preliminary assays for shoot growth, we observed that with increasing shoot or bud density, shoot elongation and rooting decreased. It was essential to remove the shoots at regular intervals for continued shoot development.

$AgNO_3$ is a potent competitive inhibitor of ethylene (Beyer 1976), which influences cell division and differentiation (Songstad *et al*. 1988). Based on previous results with $AgNO_3$ (Hoyos and Hosfield 1995, Cruz de Carvalho *et al*. 2000), we added a range of concentrations of $AgNO_3$ to the shoot development media. Lower concentrations of $AgNO_3$ did not affect shoot development. When the concentration reached 2.0 mg dm^{-3} $AgNO_3$, the percentage of shoot formation significantly increased. We found that 2.0 mg dm^{-3} $AgNO_3$ in combination with 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} GA_3 is the optimal level for shoot development. The highest percentage was 87.6 %, markedly higher than the frequency observed when no $AgNO_3$ was added. Higher concentration (3.0 mg dm^{-3}) of $AgNO_3$ inhibited the development of shoots, maybe due to the negative effect of the silver ion.

IBA had a positive effect on root induction, and subsequent development of the induced roots proceeded normally. Thick white roots were successfully induced in two weeks (Fig. 1*F*). Shoots cultured on the media without IBA did not develop roots. We found the optimal rooting level was 0.75 mg dm^{-3} of IBA, producing 84.3 % rooting frequency. The survival rate of rooted plantlets transferred to soil and grown in the greenhouse was 95 % (Fig. 1*G*) and these were fertile with normal pod development (Fig. 1*H*).

The protocol described here provides a rapid regeneration system for the common bean. The entire process, from dry seeds to regenerated plantlets, only requires about three months. Using mature dry seeds has several advantages, including ease handling, and availability of seeds year round and in bulk quantities. Our protocol can be incorporated into a gene transfer system of the common bean with *A. tumefaciens*.

References

Beyer, E.M.: A potent inhibitor of ethylene action in plants. - Plant Physiol. **58**: 268-271, 1976.

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

Dillen, W., De Clercq, J., Goossens, A., Van Montagu, M., Angenon, G.: *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. - Theor. appl. Genet. **94**: 151-158, 1997.

Franklin, C.I., Trieu, T.N., Gonzales, R.A., Dixon, R.A.: Plant regeneration from seedling explants of green bean (*Phaseolus vulgaris* L.) via organogenesis. - Plant Cell Tissue Organ Cult. **24**: 199-206, 1991.

Gamborg, O.L., Miller, R.A., Ojiama, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Hoyos, R.A., Hosfield, G.L.: Ethylene inhibitors added to *in vitro* culture media of common bean promotes morphogenesis of cotyledonary node explants. - Annu. Rep. Bean Improvement Coop. **38**: 99-100, 1995.

Kalantidis, K., Griga, M.: Micropropagation of common bean (*Phaseolus vulgaris* L.) by multiple shoot induction. - Plant Prod. (Prague) **39**: 115-128, 1993.

Kartha, K.K., Pahl, K., Leung, N.L., Morginski, L.A.: Plant regeneration from meristems of grain legumes: soybean, cowpea, peanut, chickpea and bean. - Can. J. Bot. **59**: 1671-1679, 1981.

Malik, K.A., Saxena, P.K.: Regeneration in *Phaseolus vulgaris* L.: Promotive role of N^6-benzylaminopurine in cultures from juvenile leaves. - Planta **184**: 148-150, 1991.

Malik, K.A., Saxena, P.K.: Regeneration in *Phaseolus vulgaris* L.: high-frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. - Planta **186**: 384-389, 1992a.

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

Mallikarjuna, K., Rajendrudu, G.: High frequency *in vitro* propagation of *Holarrhena antidysenterica* from nodal buds of mature tree. - Biol. Plant. **51**: 525-529, 2007.

Mariotti, D., Fontana, G.S., Santini, L.: Genetic transformation of grain legumes: *Phaseolus vulgari*s L. and *P. coccineus* L. - J. Genet. Breed. **43**: 77-82, 1989.

Martins, I.S., Sondahl. M.R.: Multiple shoot formation from shoot apex cultures of *Phaseolus vulgaris* L. - J. Plant Physiol. **115**: 205-208, 1984.

McClean, P., Grafton, K.F.: Regeneration of dry bean (*Phaseolus vulgaris* L.) via organogenesis. - Plant Sci. **60**: 117-122, 1989.

Mohamed, F.M., Read, P.E., Coyne, D.P.: Dark preconditioning, CCPU, and thidiazuron promote shoot organogenesis on seedling node explants of common and faba bean. - J. amer. Soc. hort. Sci. **117**: 668-672, 1992.

Mohamed, F.M., Coyne, D.P., Read, P.E.: Enhanced differentiation of somatic embryoids in callus cultures of common bean. - Annu. Rep. Bean Improvement Coop. **36**: 16-17, 1993.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

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

Rubluo, A., Kartha, K.K.: *In vitro* culture of shoot apical meristems of various *Phaseolus* species and cultivars. - J. Plant Physiol. **119**: 425-433, 1985.

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

1987.
Songstad, D., Duncan, D., Widholm, J.: Effect of l-amino-cyclopropane-1-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. - Plant Cell Rep. **7**: 262-265, 1988.
Vasudevan, A., Selvaraj, N., Ganapathi, A., Choi, C.W., Manickavasagam, M., Kasthurirengan, S.: Direct plant regeneration from cucumber embryonal axis. - Biol. Plant. **51**: 521-524, 2007.
Yoshida, T.: Adventitious shoot formation from hypocotyls sections of mature soybean seeds. - Breed. Sci. **52**: 1-8, 2002.
Zambre, M.A., Clercq, J.D., Vranova, E., Van Montagu, M., Angenon, G., Dillen, W.: Plant regeneration from embryo-derived callus in *Phaseolus vulgaris* L. (common bean) and *P. acutifolius* A. Gray (tepary bean). - Plant Cell Rep. **17**: 626-630, 1998.