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Short communications

Regeneration in *Phaseolus vulgaris* L.

Promotive role of N^6-benzylaminopurine in cultures from juvenile leaves

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Abstract. Plants were regenerated from cultured young leaves of *Phaseolus vulgaris* L. cv. Kinghorn. For inducing shoot regeneration the explant had to consist of the petiole and a portion of the lamina, and N^6-benzylaminopurine (BAP) had to be present in the culture medium. Furthermore, the frequency of shoot regeneration increased more than seven-fold if donor seedlings were raised on a medium containing 5 μM BAP, followed by culture of the leaf explants on a medium containing 20 μM BAP. Regenerated shoots developed roots on basal (hormone-free) medium and the resulting plantlets could be transplanted to soil.

Key words: Cytokinin and shoot regeneration – Petiole – *Phaseolus* (regeneration) – Shoot regeneration

The use of cell culture and genetic engineering is viewed as a logical approach to improve legume crops (Hildebrandt et al. 1986; Mok et al. 1986; Morginski and Kartha 1985). However, most large-seeded legumes have proven to be recalcitrant to regeneration in vitro, soybean being an exception as a number of protocols for regeneration and transformation for this species are currently available (Ranch et al. 1986; Wright et al. 1986; Hinchee et al. 1988). Although plants have been recovered from cultures of apical (Kartha et al. 1981) and axillary (Martins and Sondahl 1984a) meristems of *Phaseolus vulgaris*, the success in inducing organogenesis or somatic embryogenesis has been rather limited. In the first report of regeneration in this species, only two plants were regenerated on a medium containing bean seed extract of an undefined composition (Crocomo et al. 1976). Later, early stages of somatic embryogenesis were observed in callus and cell-suspension cultures (Allavena 1984; Martins and Sondahl 1984b) but these embryoids failed to develop into plants. More recently, McClean and Grafton (1989) reported a direct regeneration of shoots and plants from mechanically disturbed axillary nodal meristems of bean. All axillary buds were excised and if the buds were not visible, the meristem region was scratched prior to culturing the nodal explants on N^6-benzylaminopurine supplemented medium. In this communication, we report a novel procedure for shoot morphogenesis in *Phaseolus vulgaris* L. using explants consisting of the petiole and a portion of the leaf, and recovery of whole plants.

Abbreviations: BAP = N^6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; MS = Murashige and Skoog (1962) medium

The basic culture medium used was that of Murashige and Skoog (1962) (MS) containing its standard macro- and micronutrients, 3% sucrose, vitamins according to Gamborg et al. (1968), and 0.25% gelrite (Scott Laboratories, Carson, Cal., USA). It was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or N^6-benzylaminopurine (BAP) used at 1–20 μM. The pH was adjusted to 5.7 prior to adding gelrite and the media were sterilized by autoclaving at 0.122 MPa for 20 min.

Seeds were placed in a beaker and about 40–50 ml of undiluted sulphuric acid were gently poured into the beaker. After 45–60 s, the acid was decanted and the seeds were rinsed twice with water prior to adding 50 ml of 95% (v/v) ethanol. The alcohol was replaced after 1 min by 50 ml of a commercial (5.6%) solution of sodium hypochlorite, diluted 1:4 (with water) and containing two to five drops of the surfactant "Tween 20" (polyoxyethylenesorbitan monolaurate; Sigma Chemical Co., St. Louis, Mo., USA) and the contents stirred continuously using a magnetic stirrer. After 20 min, the seeds were washed five times with sterile deionized water (250 ml each wash) and only unwrinkled, healthy seeds were used for germination.

Ten seeds were placed in each of the 300 ml Magenta boxes (transparent plastic culture boxes; Magenta Corporation, Chicago, Ill., USA) containing 40 ml of MS medium with or without 5 μM BAP. The cultures were kept in darkness at 24° C and 10-d-old seedlings were used for explant preparation. The first pairs of leaves (2–4 cm long) were harvested and placed in sterile water until used for explantation; care was taken to ensure that no bud or a portion thereof was attached to the petiole. Each leaf was sliced using a sharp razor into three segments, each about 0.5–1.0 cm long, designated as the upper, middle, and basal portions. Ten explants were cultured per Petri dish (100 mm diameter, 15 mm high) containing 25 ml of the culture medium supplemented with various concentrations of 2,4-D or BAP. Subcultures were carried out every two weeks on the same medium. Since the size of the explants on BAP-supplemented medium increased considerably (two- to six-

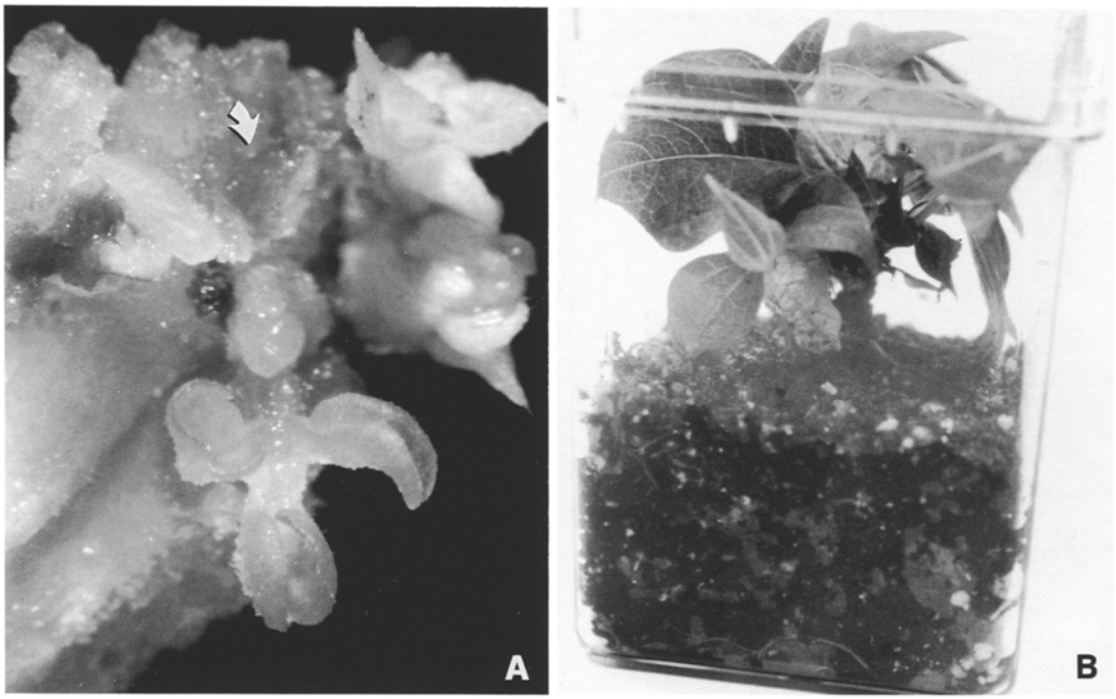

Fig. 1 A, B. Regeneration of plants from juvenile leaf explants of *Phaseolus vulgaris* L. **A** The basal leaf explant cultured on MS medium with 20 μM BAP. Note the initiation of callus on the cut end of the petiole (*arrow*) and development of shoots after four weeks of culture. **B** A 16-week-old plantlet transferred to soil

Table 1. Shoot formation from cultured leaf explants of *Phaseolus vulgaris* L. originating from seedlings grown on MS basal (MS) and on MS medium with 5 μM BAP

Medium	No. of explants	Responding cultures (%)	Shoots per culture
MS	20	8	2
MS + 5 μM BAP	20	60	4

fold) during the culture period, only five explants were subcultured per Petri dish. Shoots developed after three to four weeks of culture. They were separated from the callus and transferred to MS basal medium, i.e. without any hormone (25 ml per Petri dish). After another two weeks, the developing shoots were transferred to Magenta boxes each containing 40 ml of MS basal medium, to induce root formation. Roots started to develop within 12 weeks; after eight to ten weeks plantlets with well formed roots were washed thoroughly with sterile water and transferred to autoclaved soil. All cultures were kept in darkness for the first four weeks and thereafter illuminated under a photoperiod of 16 h (40–60 $\mu mol \cdot m^{-2} \cdot s^{-1}$; Phillips, Scarborough, Ont., Canada. F20-T12 cool-white fluorescent lamps) at 24° C. Twenty explants per treatment were used and the experiments were repeated six times.

Initially, explants from various parts of the seedling such as hypocotyl, cotyledonary nodes, shoot apex and leaves were cultured on MS medium supplemented with 2,4-D or BAP. In all treatments with 2,4-D callus formation occurred within a week at 1–20 μM concentration; with higher concentrations the cultures turned brown and died. Occasionally, in some callus cultures (<1%) of leaf explants origin, organized translucent structures resembling early stages of somatic embryo development were seen but they did not develop any further on the same medium or on a fresh medium lacking 2,4-D. This observation is in agreement with several previous studies (Allavena 1984; Martins and Sondahl 1984b; Saunders et al. 1987) which recorded the presence of smooth green or translucent embryoids or embryo-like structures in bean cell cultures raised on a medium with 2,4-D. Whole plants were not regenerated in any of these experiments.

By contrast, when cultured on a medium supplemented with 10 μM BAP, explants from the upper and middle portions of the leaf showed great increase in size (four- to eight-fold) with little callus formation, whereas basal explants developed callus around the peripheral region of the cut end of the petiole (Fig. 1A, arrow) within two weeks; other areas including the lamina attached to the petiole formed little or no callus. Only 8% of the basal explants formed shoots with an average of two shoots per responding culture. The developing shoots (Fig. 1A) turned green on transfer to light and developed roots on transfer to MS medium without hormones in 10–12 weeks. The plantlets were able to survive the transplant to soil (Fig. 1B). The tissue-culture-derived plants appeared to be morphologically normal and developed flowers and pods with apparently normal seeds.

The efficacy of BAP to induce shoot formation led us to compare the regeneration potential of leaf explants originating from plants raised on MS basal medium with those from seedlings grown on a medium containing BAP (5 μM). The culture of seedlings on the BAP-supplemented medium had a significant promotive effect

on shoot regeneration in explants, raising its frequency to 60% compared to 8% in explants of seedlings raised on MS basal medium. The number of shoots per responding culture also increased to four.

The usefulness of explants excised from seedlings grown on a medium supplemented with a cytokinin was originally described by Wright et al. (1986) for inducing shoot morphogenesis from nodal explants of soybean, and the technique was later extended to bean (McClean and Grafton 1989). In our experiments, the recovery of shoots from leaf explants lacking pre-existing meristems, raised and cultured in the presence of BAP, confirms the morphoregulatory role of cytokinin(s) in the regeneration of *Phaseolus vulgaris* while the induction of morphogenic callus from the petiole demonstrates the potential of this tissue for in-vitro regeneration of common bean. However, regeneration was observed only from those petioles that had a portion of the lamina attached to them. There may be some physiological interaction between the lamina and the petiole which is important for morphogenesis as a variety of cytokinins have been shown to be present in *Phaseolus* leaves (Koshimizu and Iwamura 1986). However, it is also possible that the lamina simply enlarges the size of the explants as the petioles cultured without lamina showed limited increase in size. Presently, experiments are in progress to characterize this regeneration system and to increase the frequency of shoot regeneration further.

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