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Techical Note

In vitro plant regeneration from the petioles of primary leaves of mungbean *Vigna radiata* L.

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Abstract High frequency *in vitro* plant regeneration via multiple shoots is reported from petioles of leaf explants of mungbean (*Vigna radiata* L.) on B5 culture medium supplemented with 6-benzyladenine (BA). The explants comprised of petiole cut at the node bearing the basal half of the lamina. Regeneration frequency varied with age of explant source and concentration of BA. Explants produced shoot-buds and shoots within 2 weeks. The explants harvested from 10-day-old seedlings produced higher number of shoots (8–9) than those from 4-day-old seedlings. Explants consisting of the petiole bearing either the entire lamina or atleast the basal half were more responsive than the explants without lamina. Histological studies confirmed the adventitious nature of shoot-buds. Shoots were rooted on B5 medium supplemented with 0.5 mg l^{-1} indolebutyric acid. The plantlets, hardened and transferred to soil with 90% survival developed normal flowers and viable seeds. This efficient plant regeneration protocol can be used for genetic transformation to produce transgenic mungbean.

Key words: *In vitro* plant regeneration, *Vigna radiata*, petiole, primary leaves.

Mungbean (*Vigna radiata* L.) (2n=22), family Leguminosae (sub family: Fabaceae), is an important grain legume crop and a good source of dietary protein. It is cultivated in Southeast Asia (including India), Africa, South America and Australia. Mungbean is recalcitrant and the lack of an efficient regeneration protocol has limited its improvement via tissue culture and genetic transformation. The importance and applicability of the direct shoot regeneration system in tissue culture and/or genetic manipulations of mungbean and other Asiatic *Vigna* species was discussed earlier by Avenido and Hattori (2001) and Avenido et al. (2001). Techniques making use of multiple shoot formation around pre-existing meristems may provide an ideal system for *in vitro* culture and transformation of grain legumes (Ramsay 1993; Olhoft and Somers 2001). There are a few reports on plant regeneration in mungbean from cotyledon explants (Gulati and Jaiwal 1990), shoot tip explants (Mathew 1987; Gulati and Jaiwal 1992) and cotyledonary node explants (Gulati and Jaiwal 1994; Avenido and Hattori 2001; Avenido et al. 2001). Leaves obtained from *in vitro* grown seedlings eliminate the risk of contamination. The presence of lamina was found to be essential for direct shoot bud formation on the petiolar region of leaves. Direct shoot regeneration is preferred since it reduces the possibility of somaclonal variation (genetic variation) common in plants regenerated from cultured cells or tissues (Misra and Datta 2001; Dayal et. al. 2003; Mujib 2005). We report efficient and high frequency multiple shoot regeneration from petioles (cut at the node) of primary leaf explants that can be used for genetic transformation to produce transgenic mungbean.

Seeds of two cultivars of mungbean (*Vigna radiata* L.) viz: K-851 and LGG-407 obtained from National Seeds Corporation of Hyderabad, India and LAM Agricultural Farm, Guntur, A.P. India respectively were surface-sterilized with 0.1% mercuric chloride for 10 min and rinsed 4–5 times in distilled water. After rinsing, the seeds were allowed to soak for 8 h and germinated (after removing the seed coats aseptically) on B5 basal medium (Gamborg et al. 1968) containing 3% (W/V) sucrose and 0.7% (W/V) phyta-agar. Standard culture conditions of 16 h photoperiod at 25±1°C temperature and 75% humidity with a light intensity of 60 μE m^{-2} S^{-1} were maintained in the growth chamber.

Primary leaf explants comprising the petiole (cut at the node) with lamina (half or whole) or without the lamina were obtained from 4-day-old and 10-day-old aseptically grown seedlings. Preliminary experiments established the fact that explants comprising the petioles cut away from the node responded only with callusing. Hence, only explants cut at the node with lamina (half or whole) and without lamina were used for the study with a view to study the response of explants in relation to presence or absence of lamina.

Preliminary experiments using B5 medium supplemented with cytokinins and auxins indicated that shoot-buds could be induced only with the cytokinin benzyladenine (BA). Hence, different concentrations of

Table 1. Frequency of multiple shoot* development from different types of petiole explants on B5 media in two cultivars of mungbean.

Cultivar	Age of seedling	Explant	* Frequency of multiple shoot development on B5 medium containing different concentrations of BA (mg l^{-1})*			
			0.0	0.2	0.5	1.0
K-851	4-day-old	Petiole with whole lamina	Nil	52.3±1.4	65.3±1.2	84.3±1.4
		Petiole with half lamina	Nil	3.6±0.8	77.6±1.4	87.0±0.5
		Petiole without lamina	Nil	56.0±0.5	63.3±0.8	67.0±1.1
	10-day-old	Petiole with whole lamina	Nil	60.6±1.2	74.0±1.1	81.0±0.5
		Petiole with half lamina	Nil	67.0±1.1	82.0±1.1	95.3±0.8
		Petiole without lamina	Nil	59.0±0.5	65.6±0.6	73.3±0.8
LGG-407	4-day-old	Petiole with whole lamina	Nil	52.0±1.1	64.6±0.8	82.0±1.1
		Petiole with half lamina	Nil	62.6±1.4	74.0±1.1	84.0±0.5
		Petiole without lamina	Nil	52.6±0.8	62.6±1.4	66.3±0.8
	10-day-old	Petiole with whole lamina	Nil	56.0±0.5	66.3±0.8	77.6±1.4
		Petiole with half lamina	Nil	66.0±1.2	75.3±1.2	95.0±0.5
		Petiole without lamina	Nil	57.3±1.4	64.6±0.8	71.0±0.5

* Percentage of the total cultured explants producing multiple-shoots, 2 weeks after inoculation presented as mean±S.E. of the three replicates

BA were used for shoot regeneration studies viz.: 0.2, 0.5 and 1.0 mg l^{-1}. One hundred explants were inoculated on each culture medium with three replicates and incubated in the growth room for shoot regeneration. Elongated shoots were transferred to B5 medium supplemented with indolebutyric acid (IBA) (0.5 mg l^{-1}) for induction of roots. The rooted plantlets were gently rinsed in tap water, transplanted in pots (with 1 : 1 mixture of sand and soil) and gradually acclimatized in a glasshouse. The results pertaining to multiple shoot regeneration (2 weeks after the inoculation of explants) and those pertaining to the rooting and survival (6 weeks after inoculation) were recorded and statistically analyzed.

The present regeneration protocol developed from pre-existing meristem of petiole of primary leaf supports and confirms earlier reports (Gulati and Jaiwal 1992; Gulati and Jaiwal 1994; Avenido and Hattori 2001; Avenido et al. 2001) that the BA could induce highly efficient multiple shoot development in mungbean followed by successful rooting, transplantation and survival. The concentration of BA at 1.0 mg l^{-1} was found to be the most responsive in the production of multiple shoot-buds (Table 1). The explants taken from 10-day-old seedlings produced a higher number (8–9) of shoot-buds and shoots within 2 weeks, compared to those taken from 4-day-old seedlings (5–6). Explants consisting of the petiole and basal half of the lamina or the entire lamina were more responsive than the explants without lamina in case of the explants taken from both 4-day-old and 10-day-old seedlings indicating that the lamina was very essential for induction of multiple shoots. These results support the view of Dayal et al. (2003) in pigeonpea.

The multiple shoot regeneration occurred from the petiole of the primary leaf at the cut end that possessed the axillary bud tissue (Figure 1A). Histological observations point to the development of meristematic regions in the nodal tissue, which later developed into shoot meristems (Figure 1B and C). The multiple shoots are adventitious and appear to develop from sub-epidermal parenchyma cells. They could be sufficiently

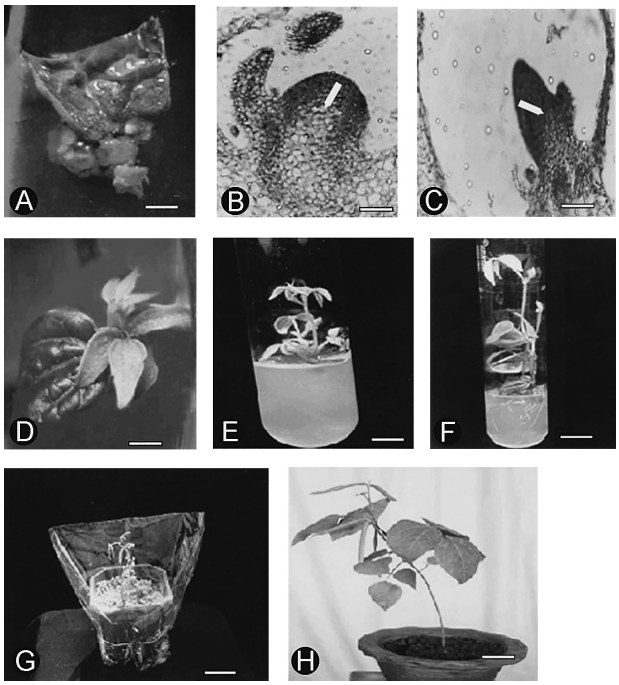

Figure 1(A to H). Studies on *in vitro* plant regeneration from the petioles of primary leaves of mungbean. (A) Multiple shoot development from the petiole (cut at the node) of primary leaf explant, on the 8th day after inoculation (bar=100 mm). (B, C) Histological study of developing adventitious shoot meristems (appear to develop from sub-epidermal parenchyma cells), stained with 0.1% safranin (bar=50 mm). (D) Regenerated shoot on the 14th day of culture (bar=40 mm). (E) Elongated shoots on the 26th day of culture (bar=40 mm). (F) Regenerated plantlet with well developed roots on the 40th day of culture, ready for transplantation (bar=40 mm). (G) Acclimatization of regenerated plantlet, 6 d after transplantation (bar=40 mm). (H) Healthy transplanted plantlet in soil on the 65th day after initiation of culture (bar=40 mm).

elongated in the same culture medium in 2 weeks after which they were ready to be rooted (Figure 1D and E).

Profuse roots (2–3 cm long) were induced on the rooting medium in 2–3 weeks after which the plantlets were ready for transfer to pots (Figure 1F). Rooted plantlets were transferred to pots containing a 1 : 1 mixture of sand and soil and acclimatized for 1 week prior to transfer to the glasshouse with 90% survival (Figure 1G and H). The results indicate that the explants obtained from 10-day-old seedlings were more responsive to plant regeneration with higher shoot regeneration, rooting and survival potential compared to those obtained from the 4-day-old seedlings in both the cultivars of mungbean.

Tissue culture may lead to abnormal plants leading to somaclonal variations but multiplication from existing meristems appears to prevent them. Hence, direct plant regeneration from meristems is the most ideal for genetic transformation. Previous reports on multiple shoot regeneration of mungbean have utilized shoot apices (Gulati and Jaiwal 1992) or cotyledonary node explants (Gulati and Jaiwal 1994; Avenido and Hattori 2001; Avenido et al. 2001). The regenerated plantlets produced presently from leaf explants were healthy and produced normal flowers and pods and set viable seeds. They were similar to plants germinated from seeds and did not reveal any variation.

Earlier reports on direct multiple shoot regeneration from various explants (Gulati and Jaiwal 1992; Gulati and Jaiwal 1994; Avenido and Hattori 2001; Avenido et al. 2001) have claimed a moderate regeneration frequency and a lesser number of shoots per explant compared to the present report. Previous studies on plant regeneration from various explants of mungbean obtained through callus also reported very low frequency of regeneration with low survival (Mathews 1987; Patel et al. 1991; Mendoza et al. 1992; Gulati and Jaiwal 1994). Plant regeneration from somatic embryos has also been reported albeit with a lower frequency (Devi et al. 2004). The highly regenerable petiole explants consisting of axillary meristem cells reported here may provide suitable target tissue for *Agrobacterium*-mediated as well as microprojectile method of genetic transformation for production of transgenic mungbean.

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