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In vitro induction of multiple shoots and plant regeneration from shoot tips of mung bean (*Vigna radiata* (L.) Wilczek)

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Abstract

Conditions for plant regeneration from excised shoot tips of *Vigna radiata* were studied. Complete plants were regenerated directly without an intervening callus phase from shoot tips on basal medium (MS salts + B_5 vitamins). Regeneration frequency varied with genotype, explant size and growth regulator combinations in the medium. Addition of cytokinins induced a variable amount of callus at the base of the shoot tip, followed by multiple shoot formation. Benzyladenine (BA), kinetin and zeatin at 5×10^{-6} M each induced multiple shoots in 100% of the explants but the highest number of regenerants per explant (9) was produced with BA. The efficacy of BA for shoot multiplication was not improved when it was supplemented with naphthaleneacetic acid (NAA) or indoleacetic acid (IAA). NAA or adenine sulphate, when applied alone, induced complete plantlets. The growth regulator requirement of explants for the induction of multiple shoots varied with explant size. The shoot tip explants maintained proliferation ability on subculture. None of the treatments was effective in inducing shoot bud differentiation from callus. Regenerated shoots were rooted on MS basal medium and MS supplemented with either IAA or indolebutyric acid. The rooted plants were transferred to the field; 60% subsequently survived and grew.

Abbreviations: BM – basal medium [MS (Murashige & Skoog 1962) salts + B_5 (Gamborg et al. 1968) vitamins], BA-6-benzyladenine, AdS-adenine sulphate, IAA-indole-3-acetic acid, NAA-1-naphthaleneacetic acid, IBA-indolebutyric acid

Introduction

Grain legumes are the main source of dietary protein in the developing countries, especially where animal protein is insufficient or is taboo. Seeds of leguminous crops in general, grain-legumes in particular, carry viruses internally (Kartha et al. 1981). Spread of virus within a growing crop usually results in extensive losses in seed quality and yield. Meristem culture has been successfully used in the elimination of viral pathogens, including seed-borne viral infections in forage and grain-legumes (Kartha et al. 1979, 1981; Kartha 1982), in mass propagation, in germplasm preservation (Kartha et al. 1979, 1980; Rubluo & Kartha 1985) and in the international exchange of genetic material (Kartha 1982). There have been only a few attempts to regenerate mung bean plants via tissue culture (Mathews 1987; Gulati & Jaiwal 1990). Recovery of single plants from mung bean meristems on basal medium was reported by Goel et al. (1983) and Mathews (1987). However, only single (Bajaj & Dhanju 1979) or few (0–3) shoots (Singh et al. 1985) were produced on basal medium supplemented with one cytokinin and one auxin. Previous studies have been restricted to one or two cultivars (Bajaj & Dhanju 1979; Goel et al. 1983; Mathews 1987). Moreover, none of the earlier workers studied the various factors that influence plant differentiation in detail. In the present study, six cultivars have been investigated and culture conditions for efficient plant regeneration from shoot tips have been worked out in one.

Materials and methods

Seeds of six cultivars (listed in Table 5) of *V. radiata* were obtained from the Directorate of Pulse Research, ICAR, Kalyanpur, Kanpur and Division of Genetics, IARI, New Delhi. Of these, only cv. K-851 was used for detailed studies.

To raise aseptic seedlings, the seeds were rinsed in 70% alcohol for 1 min, disinfested in 0.2% (w/v) aqueous $HgCl_2$ solution for 10 min, thoroughly rinsed in sterile distilled water and planted on BM containing 3% (w/v) sucrose and 0.7% (w/v) agar (Hi-media, Bombay) in test tubes (25 mm × 150 mm) plugged with non-absorbent cotton wrapped in one layer of cheese cloth. All media were adjusted to pH 5.8 with 0.1 N NaOH or 0.1 N HCl before the addition of agar and were autoclaved at 121°C for 15 min. The seeds were germinated in a 16-h photoperiod (80 $\mu mol\ m^{-2}\ s^{-1}$) at 25 ± 2°C with 60% humidity.

Shoot tips either measuring 0.5–0.6 mm or 5–6 mm of 7-day-old seedlings served as explants. All leaves except a pair of leaf primordia were removed from the explants under a stereo microscope in a laminar flow cabinet. These dissected shoot tips having a pair of leaf primordia were cultured on BM and BM supplemented with either 5×10^{-7} or 5×10^{-6} or 5×10^{-5} M BA. The shoot tips were planted with the cut end slightly embedded in the medium. Callus which developed at the embedded end of the cultured explants was isolated and cut into small pieces of approximately 50 mg (4 mm × 4 mm) and transferred to the same or different media for plant regeneration.

To study the effect of different cytokinins on shoot regeneration, shoot tip explants (5–6 mm) were cultured on BM supplemented with either kinetin or zeatin or AdS at an equimolar concentration of 5×10^{-6} M. To assess the influence of auxins (NAA and IAA) either alone or in combination with BA on shoot production, explants (5–6 mm) were cultured on BM supplemented with 10^{-6} M of NAA or IAA alone or in combination with 5×10^{-6} M BA.

The effect of genotype on shoot multiplication was tested by culturing the shoot tips (5–6 mm) of cvs K-851, ML-1, ML-323, ML-337, SML-32 and Pusa baisakhi on BM containing 5×10^{-6} M BA.

Rooting

Well-developed shoots (30–40 mm) from proliferating shoot cultures were excised and rooted on MS without auxin or supplemented with 10^{-6} M IAA or NAA or IBA.

All cultures were maintained under the same experimental conditions as for germination of seeds. For each treatment 24 cultures were raised and each experiment was repeated at least twice. Visual observations of the cultures were taken every week and the effect of treatments was quantified on the basis of percentage of cultures showing response and the number of regenerants per culture. The data pertaining to number of shoots per culture were subjected to analysis of variance and significant treatment differences selected by Newman-Keuls multiple range test (Bruning & Kintz 1977).

Transplantation

Plantlets with well-developed roots were removed from the culture tubes and, after washing their roots in running tap water, were transferred to pots containing sterile vermiculite. A glass beaker was inverted over each plant to ensure high humidity during the first few days after transfer. Subsequently, the plants were transferred to field conditions.

Results

Isolated shoot tips cultured on BM started expanding after 7 days with no or insignificant callus formation at the cut end and gradually developed into solitary shoots, 4 cm long, within 14 days. In a further 15–20 days, the shoots subsequently developed roots from the basal end to form 8–10 cm long plantlets with large leaves. The regeneration of complete plants was 100% on the basal medium (Table 1).

Addition of various concentrations of BA to BM induced a variable amount of callus at the base of the explants, followed by multiple adventitious shoot differentiation from explants within 10–15 days of culture (Table 1). The shoots remained stunted and did not produce roots even after prolonged incubation on BA-containing media. At 5×10^{-6} M BA, 100% of explants formed shoots and number of shoots per explant was maximal (Table 1). The length of shoots showed an inverse relationship with the concentration of BA. Growth of the basal callus increased with an increase in BA concentration and was greatest at 5×10^{-5} M (Table 1).

The shoot buds regenerated on BA medium were transferred to BM for elongation. Within 15 days, the shoots elongated to 3–4 cm without root formation. Well-developed shoots ≥3 cm in height were excised and transferred to rooting medium, while the initial explants were subcultured on their respective shoot proliferation medium, i.e. BM containing different concentrations of BA. On subculture, the frequency of explants that showed regeneration decreased but the number of shoots per regenerating explant increased. Both these parameters were highest at 5×10^{-6} M BA. On media containing BA (5×10^{-6} M, 9 shoots per explant could be regenerated after one subculture (Table 1). The green callus isolated from shoot tip culture grew profusely but failed to differentiate shoots on basal medium or any of the BA treatments.

Effect of different cytokinins

Since maximum frequency of regeneration and the number of shoots per culture occurred with 5×10^{-6} M BA, the effect of three cytokinins (kinetin, zeatin and BA) and AdS was compared at this concentration (Table 2). All cytokinins induced variable amounts of callus at the base of the shoot tip. However, further morphogenic

Table 2. Effect of different cytokinins and adenine sulfate (5×10^{-6} M) on shoot-tip (5–6 mm) cultures of *Vigna radiata* cv. K-851.[1,2]

Medium	Cultures regenerating (%)	Mean shoots per explant[3]	Amount of basal callus (%)
BM	100	1.0 ± 0.0	0
BM + BA	100	8.8 ± 0.6[a]	38 ± 4.4
BM + kinetin	100	2.2 ± 0.2[b]	18 ± 1.1
BM + AdS	100	2.9 ± 0.1[b]	6 ± 1.3
BM + zeatin	100	2.2 ± 0.2[b]	21 ± 2.3

Values are mean ±S.E.
[1] Data based on 24 explants per treatment
[2] Data scored after 1st subculture (12 weeks of culture)
[3] Means followed by the same letter are not significantly different according to (Newman-Keuls multiple range test ($p = 0.05$).

Table 1. Morphogenic response of shoot tips (5–6 mm) of *Vigna radiata* cv. K-851 on BM and BM supplemented with different concentrations of BA in primary cultures and subcultures[1].

BA concentration (M)	Primary cultures[2]				In 1st subcultures[3]		Total shoots per explant after 1st subculture[4]	Amount of basal callus (%)
	Cultures regenerating (%)	Mean shoots per explant	Cultures forming roots (%)	Shoot length (cm)	Cultures regenerating (%)	Mean shoots per explant		
0	100	1.0 ± 0.0	100	9.2 ± 0.3	90	1.0 ± 0.0	1.0 ± 0.0	0.0
5×10^{-7}	100	1.7 ± 0.2	0	2.7 ± 0.4	62	1.8 ± 0.5	1.9 ± 0.3[a]	25 ± 0.8
5×10^{-6}	100	4.2 ± 0.2	0	1.6 ± 0.1	92	5.0 ± 0.4	8.8 ± 0.6[b]	38 ± 4.4
5×10^{-5}	58	2.4 ± 0.3	0	0.6 ± 0.1	58	2.8 ± 0.3	5.3 ± 0.6[c]	52 ± 5.2

Values are mean ±S.E.
[1] Data based on 24 explants per treatment
[2] Data scored after 6 weeks of culture
[3] Data scored after 12 weeks of culture
[4] Means followed by same letter are not significantly different according to Newman-Keuls multiple range test ($p = 0.05$).

response of the explants varied with the cytokinin and AdS. BA and kinetin favoured multiple shoot development, while zeatin not only induced multiple shoots but also rooting in 20% of the explants. AdS induced multiple shoots that subsequently developed roots. All cytokinins and AdS induced shoots in all of the explants but the highest number of regenerants per explant was obtained with BA.

Interaction of BA and auxins

Shoot tips cultured on media supplemented with 10^{-6} M NAA or IAA produced plantlets and/or multiple shoots in 100% and 83% cultures, respectively (Table 3). Both of the auxins, in combination with BA, not only induced multiple shoots but also roots developed at the base of the shoots. The efficacy of BA for shoot multiplication was decreased when it was supplemented with auxins. Profuse callus production from the base of shoot tips occurred with NAA, alone and in combination with BA (Table 3).

Size of explant

Shoot tips of 0.5–0.6 mm and 5–6 mm size responded differently to different concentrations of BA (Tables 1 and 4). The smaller explants (0.5–0.6 mm) exhibited a decline in the number and length of regenerated shoots with increase in the concentration of BA. Callus growth at the base

Table 3. Effect of auxins, IAA or NAA (10^{-6} M) alone or in combination with BA (5×10^{-6} M) on shoot regeneration from shoot tips (5–6 mm) of *Vigna radiata* cv. K-851[1].

Media	Primary cultures[2]		In 1st subcultures[3]		Total shoots per explant after 1st subculture[4]	Amount of basal callus (%)
	Cultures regenerating (%)	Mean shoots per explant	Cultures regenerating (%)	Mean shoots per explant		
BM	100	1.0 ± 0.0	90	1.0 ± 0.0	1.0 ± 0.0	0.0
BM + NAA	100	1.0 ± 0.0	75	2.0 ± 0.2	2.6 ± 0.3[a]	66 ± 3.8
BM + IAA	83	1.0 ± 0.0	75	1.8 ± 0.2	2.0 ± 0.4[a]	35 ± 4.2
BM + BA	100	4.2 ± 0.2	92	5.0 ± 0.4	8.8 ± 0.6[b]	38 ± 3.4
BM + BA + NAA	92	1.8 ± 0.3	83	3.0 ± 0.3	4.5 ± 0.6[c]	57 ± 3.1
BM + BA + IAA	92	2.5 ± 0.3	92	4.3 ± 0.4	6.8 ± 0.6[d]	35 ± 2.7

Values are mean ±S.E.
[1] Data based on 24 explants per treatment
[2] Data scored after 6 weeks of culture
[3] Data scored after 12 weeks of culture
[4] Means followed by the same letter are not significantly different according to Newman–Keuls multiple range test ($p = 0.05$)

Table 4. Morphogenic response of small shoot tips (0.5 – 0.6 mm) of *Vigna radiata* cv. K-851 cultured on BM and BM supplemented with different concentrations of BA in primary cultures and subcultures[1].

BA concentration (M)	Primary cultures[2]			In 1st subcultures[3]		Total shoots per explant after 1st subculture[4]	Amount of basal callus (%)
	Cultures regenerating (%)	Mean shoots per explant	Shoot length (cm)	Cultures regenerating (%)	Mean shoots per explant		
0.0	100	1.0 ± 0.0	5.5 ± 0.8	90	1.0 ± 0.0	1.0 ± 0.0	15 ± 3.7
5×10^{-7}	90	2.3 ± 0.3	1.8 ± 0.5	100	1.9 ± 0.2	4.0 ± 0.5[a]	67 ± 4.8
5×10^{-6}	64	2.0 ± 0.2	0.5 ± 0.1	54	2.2 ± 0.3	3.8 ± 0.5[a]	75 ± 6.7
5×10^{-5}	0	0	0	0	0	0	100 ± 0.0

Values are mean ±S.E.
[1] Data based on 24 explants per treatment
[2] Data scored after 6 weeks of culture
[3] Data scored after 12 weeks of culture
[4] Means followed by the same letter are not significantly different according to Newman-Keuls multiple range test ($p = 0.05$)

of the meristem exhibited a direct relationship with BA concentration. At 5×10^{-5} M, shoot tips failed to grow into shoots and only callus formation occurred. The maximum frequency of differentiation as well as the number of regenerants per explant occurred with 5×10^{-7} M BA (Table 4). For regeneration from large shoot tips (5–6 mm) 5×10^{-6} M BA was found to be optimum (Table 1).

Effect of genotype

Multiple shoot formation occurred in all the cultivars tested, but the frequency of regeneration and the number of shoots per explant varied with the cultivar (Table 5). Cultivars K-851, SML-32 and Pusa baisakhi produced multiple shoots without roots while ML-1, ML-323 and ML-337 produced multiple shoots and also roots in 15.6%, 16% and 9% of the cultures, respectively. Cultivar K-851 produced the greatest number (8.3) of shoots per explant.

Rooting and transplantation

Well-developed shoots of those cultivars that were not rooted on BA medium were excised and cultured on MS without auxin or containing 10^{-6} M IAA or NAA or IBA. Roots emerged from the cut end of all the shoots within 15 days. Fifty rooted shoots of each cultivar were transferred to the pots and later established in the field where 60% of them survived and resumed growth.

Discussion

In *Vigna*, plant regeneration from shoot meristems has been reported in *V. unguiculata* (Kartha et al. 1981), *V. mungo* (Bajaj & Dhanju 1979; Goel et al. 1983; Hoque et al. 1984) and *V. radiata* (Bajaj & Dhanju 1979; Goel et al. 1983; Singh et al. 1985; Mathews 1987). In the present study, shoot tips grew directly into complete plants without an intervening callus phase on basal medium. Similar results were obtained in *V. unguiculata* (Kartha et al. 1981) and *V. radiata* (Goel et al. 1983; Mathews 1987). The basal media used by previous workers for *V. radiata* (Goel et al. 1983; Mathews 1987) were different from the one used in present study and gave less regeneration, i.e. 58% of the total explants (Mathews 1987), than the present work (100%). Since plant cells do not generally grow in the absence of growth regulators, regeneration noted here is attributable to hormones synthesized by primordial leaves (Jaiwal & Bhambie 1990).

In all the previous studies dealing with shoot tip cultures of *V. radiata*, an auxin and a cytokinin were used in combination for differentiation. Earlier researchers have reported either the formation of callus (Mathews 1987) or callus and roots (Goel et al. 1983) or callus and shoots

Table 5. Regenerative response of shoot tips (5–6 mm) obtained from different cultivars of *Vigna radiata*[1,2].

Cultivars	Cultures regenerating (%)	Mean shoots per explant[3]	Cultures forming roots (%)	Amount of basal callus (%)
K-851	100	8.3 ± 0.7[a]	0.0	50 ± 4.7
ML-1	100	6.2 ± 0.5[b]	15.6	37 ± 2.9
ML-323	100	5.9 ± 0.4[bc]	16.0	37 ± 2.9
ML-337	100	6.6 ± 0.7[b]	9.0	50 ± 2.5
SML-32	90	3.5 ± 0.6[c]	0.0	39 ± 3.5
Pusa baisakhi	87	4.8 ± 0.8[bc]	0.0	50 ± 4.4

Culture medium: BM + BA (5×10^{-6} M)
Values are mean ±S.E.
[1] Data based on 24 explants per cultivar
[2] Data scored after 1st subculture (12 weeks of culture)
[3] Means followed by the same letter are not significantly different according to Newman-Keuls multiple range test ($p = 0.05$)

(Singh et al. 1985) or plantlets (Bajaj & Dhanju 1979). The frequency of regeneration was 60–70% (Bajaj & Dhanju 1979) and only 3 shoots per explant were produced (Singh et al. 1985). In contrast to these reports, in the present study a cytokinin (BA) alone induced 9 shoots in 100% of the cultures.

Explant size plays an important role in shoot formation in legumes (Hammatt et al. 1986). A direct correlation between the size of meristem and the percent regeneration of plant of *V. radiata* was observed by Bajaj & Dhanju (1979). In the previous studies (Bajaj & Dhanju 1979; Goel et al. 1983; Singh et al. 1985; Mathews 1987), the explant size was smaller than used in the present work (0.2–2 mm vs 0.5–6 mm). However, in the present study, the morphogenic response of different sizes of explants was similar on BM. But, the growth regulator requirements for explants were different for optimal shoot multiplication. Explants of size 0.5–0.6 mm showed optimal response at 5×10^{-7} M BA and those of 5–6 mm was at 5×10^{-6} M BA. Moreover, the former produced only callus whereas the latter produced 6 shoots at 5×10^{-5} M BA. These differences in growth regulator requirement for shoot multiplication may be due to differences in the levels of endogenous hormones in the explants.

Regeneration in tissue culture is a genetically controlled trait (Bhojwani et al. 1984; Templeton-Somers & Collins 1986). The present results show that the frequency of shoot regeneration and the number of shoots per explant vary among cultivars. These differences are attributed to genotypic differences equivalent to those reported in the regenerating capacity of other grain legumes (Malmberg 1979; Rubluo et al. 1982; Rubluo & Kartha 1985). The influence of genotype in eliciting various morphogenic responses was also noted for meristems of several grass species (Dale 1977) and *Trifolium* (Bhojwani 1981).

Formation of multiple shoots from shoot tip cultures of *V. radiata* could be of practical application for raising hybrid seedlings of difficult crosses and mutagenesis *in vitro*. Regeneration of multiple plantlets from shoot-tip explants on simple medium might be used for the production of virus-free plants and for the storage and maintenance of germplasm.

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