Somatic embryogenesis and regeneration of *Vigna radiata*

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Abstract

An efficient regeneration protocol *via* somatic embryogenesis was optimized for mung bean [*Vigna radiata* (L.) Wilczek; cv. Vamban 1]. Primary leaf explants were used for embryogenic callus induction in MMS medium (Murashige and Skoog salts with B5 vitamins) containing 2.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 150 mg dm^{-3} glutamine and 3 % sucrose. Fast growing, highly embryogenic cell suspensions were established from 21-d-old calli in MMS medium supplemented with 0.5 mg dm^{-3} 2,4-D and 50 mg dm^{-3} proline (Pro), and maximum recovery of globular (39.0 %), heart-shaped (26.3 %) and torpedo-stage (21.0 %) somatic embryos were observed in this medium. Mature cotyledonary-stage somatic embryos were cultured for 5 d in half strength B5 liquid medium containing 0.05 mg dm^{-3} 2,4-D, 20 mg dm^{-3} Pro, 5 μM abscisic acid, 1000 mg dm^{-3} KNO_3, 50 mg dm^{-3} polyethylene glycol (PEG 6000) and 30 g dm^{-3} D-mannitol. Mature somatic embryos were germinated after dessication for 3 d and complete development of plantlets accomplished in MMS medium containing 30 g dm^{-3} maltose, 0.5 mg dm^{-3} benzyladenine and 500 mg dm^{-3} KNO_3. Profuse lateral roots, and regeneration frequency (up to 60 %) were observed in half-strength MMS medium containing 0.5 mg dm^{-3} indolebutyric acid (IBA). The regenerated plants were grown to fruiting and were morphologically normal and fertile.

Additional key words: cell suspension cultures, mung bean, plant growth regulators, primary leaf explant.

Introduction

Mung bean [*Vigna radiata* (L.) Wilczek] is an important food grain legume but efforts to improve this crop by conventional breeding methods met with limited success (Jaiwal *et al.* 2001). Mung bean plants have been regenerated *in vitro* from the cotyledons (Mathews 1987, Gulati and Jaiwal 1990, Mendoza *et al.* 1993, Chandra and Pal 1995, Tivarekar and Eapen 2001, Amutha *et al.* 2003), cotyledonary nodes (Amutha *et al.* 2006, Mundhara and Rashid 2006), shoot tips (Mathews 1987, Gulati and Jaiwal 1992, Betal and Sen Raychaudhuri 2001), hypocotyls (Jaiwal *et al.* 2001, Amutha *et al.* 2003, Tazeen and Mirza 2004, Amutha *et al.* 2006) and leaflets (Mathews 1987, Patel *et al.* 1991, Selvi *et al.* 1995, Jaiwal *et al.* 2001, Tazeen and Mirza 2004). However, the plant regeneration *via* somatic embryogenesis is limited (Girija *et al.* 2000, Devi *et al.* 2004) due to problems with maturation and conversion of somatic embryos to plantlets (Eapen and George 1990, Patel *et al.* 1991). Chen *et al.* (1990) reported the production of a large quantity of somatic embryos from the cotyledons of morphologically abnormal embryos derived from crosses between *V. glabrescens* and *V. radiata.* Girija *et al.* (2000) reported successful germination of the somatic embryos, produced in cell suspension cultures, in half strength MS basal medium without mentioning further plantlet development in soil. Recently, Devi *et al.* (2004) reported plant regeneration *via* somatic embryos of two mung bean cultivars (ML-267 and PUSA-16) after making synthetic seeds for clonal propagation. Here we report a simple regeneration protocol *via* somatic embryogenesis which could be extended for genetic transformation of mung bean.

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Abbreviations: ABA - abscisic acid; BA - 6-benzyladenine; B5 - Gamborg medium; CH - casein hydrolysate; CIM - embryogenic callus induction medium; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; Gln - L-glutamic acid-5-amide; EIM - somatic embryo induction medium; EMM - somatic embryo maturation medium; KIN - kinetin; ME - malt extract; MMS - MS salts with B5 vitamins; MS - Murashige and Skoog medium; NAA - α-naphthalene acetic acid; PEM - proembryogenic mass; PEG - polyethylene glycol; Pro - L-proline; Put - putrescine; RM - rooting medium; TDZ - thidiazuron; YE - yeast extract; ZE - zeatin.
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Materials and methods

Mung bean [*Vigna radiata* (L.) Wilczek; cv. Vamban 1] seeds were obtained from the National Pulses Research Centre, Vamban, India. They were surface-sterilized with *Tween 20* (10 min), 70 % ethanol (5 min) and 0.1 % mercuric chloride (10 min), rinsed 5 times in sterile distilled water after each step and then, aseptically germinated in 50 cm^3 sterile half-strength MMS medium [Murashige and Skoog (1962) salts with B5 (Gamborg *et al.* 1968) vitamins solidified with 0.4 % *Phytagel,* pH 5.8] for 10 d. They were maintained at 25 ± 2 ºC, 60 % relative humidity and 16-h photoperiod (irradiance of 36 μmol m^{-2} s^{-1}).

Primary leaf segments (1 cm^2) dissected longitudinally through the midrib were used as explants. These were cultured on MS medium with 2,4-dichlorophenoxyacetic acid (2,4-D; 1, 1.5 and 2 mg dm^{-3}) separately. The MMS medium supplemented with α-naphthalene acetic acid (NAA; 1, 1.5 or 2 mg dm^{-3}) or 2,4-D (1, 1.5 and 2 mg dm^{-3}). The MMS medium containing 2 mg dm^{-3} 2,4-D producing more callus than other media was tried with two cytokinins, 6-benzyladenine (BA) and kinetin (KIN), each at three different concentrations (1, 1.5 or 2 mg dm^{-3}) (Table 1). The effects of organic additives, yeast extract (YE; 100 mg dm^{-3}), malt extract (ME; 100 mg dm^{-3}) and casein hydrolysate (CH; 100 and 150 mg dm^{-3}) were examined in MMS medium containing 2,4-D (2 mg dm^{-3}). Similarly, the effect of L-glutamic acid-5-amide (Gln) on the frequency of callus induction was ascertained by adding it at 100, 150 and 200 mg dm^{-3} to MMS medium containing 2,4-D (1.5 or 2 mg dm^{-3}). As there was induction of embryogenic calli in the presence of 2 mg dm^{-3} 2,4-D and 150 mg dm^{-3} Gln, two concentrations of CH (100 and 150 mg dm^{-3}) were tried in order to observe its additive effect. In another set of experiment, putrescine at three different levels (Put; 50, 100 and 150 mg dm^{-3}) in place of CH was tried (Table 2). All media (pH 5.8) were supplemented with 3 % sucrose and solified with 0.4 % *Phytagel*. Subsequent subculturing of embryogenic calli was done on the same callusing medium.

Three-week-old, rapidly proliferating friable, yellowish, embryogenic calli (2 g) were produced from three different semi-solid MMS media with 3 % sucrose: *1*) MMS + 2 mg dm^{-3} 2,4-D, *2*) MMS + 1.5 mg dm^{-3} 2,4-D + 150 mg dm^{-3} Gln and 3) MMS + 2 mg dm^{-3} 2,4-D + 150 mg dm^{-3} Gln). The initial suspension culture medium was composed of liquid MMS containing 0.25 and 0.50 mg dm^{-3} 2,4-D either alone or in combination with *a*) CH (100 and 150 mg dm^{-3}, *b*) Gln (100 and 150 mg dm^{-3} alone or with CH and *c*) Pro (50, 100 and 150 mg dm^{-3}) at six combinations (Table 3). Further batch of MMS media contained 2,4-D (0.25 and 0.50 mg dm^{-3}), Pro (50 mg dm^{-3}) and Gln (100 and 150 mg dm^{-3}) at four different combinations. The last batch of MMS media contained 2,4-D (0.5 mg dm^{-3}) and Put (50, 100 and 150 mg dm^{-3}) (Table 3). The cultures were shaken at 90 rpm, 25 ± 2 ºC, 60 % RH and 16-h photoperiod (36 μmol $m^{-2}s^{-1}$). Every 5th day, half of the culture was removed and replenished with an equal volume of the respective fresh liquid MMS medium. Established suspensions were maintained by adding 5 cm^3 of the culture to 25 cm^3 of respective fresh liquid medium, at 5-d intervals. Induction of somatic embryos was followed by regular observation (Table 4). A week after suspension initiation, samples were drawn from 10 flasks per treatment.

Heart- and torpedo-stage embryos from cell suspensions were aseptically separated and individually subcultured in 15 dm^3 of MMS either full or half strength, B5 full or half strength, containing 2,4-D (0.05 and 0.10 mg dm^{-3}), 5 μM ABA, 20 mg dm^{-3} Pro and either with 3 % sucrose or mannitol (Table 5). Maturation experiments were carried out in EMM 19 and 20 (half B5 + 0.05 mg dm^{-3} 2,4-D + 5 μM ABA + 20 mg dm^{-3} Pro + 50 mg dm^{-3} PEG 6000 + 1000 mg dm^{-3} KNO_3) supplemented with 3 % mannitol or 3 % sorbitol. Cultures were maintained for 5 d in the same medium with no subculturing. Somatic embryo maturation was followed for each experiment, separately. The mature somatic embryos were subjected to desiccation in B5 liquid medium containing 3 % sucrose for 3 d. Mature cotyledon-stage somatic embryos were desiccated for 5 min on sterile filter paper, prior to germination.

The mature cotyledon-stage somatic embryos were separately germinated on MS, MMS media, and B5 (both half, and full strength) containing plant growth regulators: KIN (0.5 or 1.0 mg dm^{-3}), GA_3 (0.5 or 1.0 mg dm^{-3}), BA (0.5 or 1.0 mg dm^{-3}), KNO_3 (500 mg dm^{-3}), TDZ (0.05 or 0.10 mg dm^{-3}) and maltose (3 %) (Table 6). The resulting plantlets were grown in the respective media for 2 weeks at conditions mentioned above with regular sub-culturing every week.

The plantlets with primary leaves were used to optimize rooting in semi-solid media. Shoots taller than 5 cm were found ideal for the induction of adventitious roots. The well developed shoots were cultured on semi-solid, half-strength MMS medium containing IBA (0.5 and 1.0 mg dm^{-3}) and NAA (0.5 and 1.0 mg dm^{-3}) either alone or in combinations (Table 7). Half-strength MMS medium devoid of growth regulators served as control.

Rooted plantlets were carefully removed, roots washed in sterile distilled water and grown initially in small paper cups later transferred to plastic pots containing potting mixture [sand mixed with autoclaved garden soil (10 % organic matter), 3:2]. Initially, the plantlets were maintained under the same environment, then transferred to mist chamber for secondary hardening.

The hardened plants were, subsequently, transferred to big plastic pots containing enriched soil (sand, red soil and organic manure).

The percentage of callus induction, somatic embryo induction and plant regeneration were calculated following Zale *et al.* (2004). Each experiment was replicated thrice in a completely randomized block design. Data were processed using analysis of variance (*ANOVA*) of the *IRRISAT* statistical package. Mean values were separated by Duncan's (1955) multiple range test (DMRT) at 5 % probability level. Arcsin $(p\%/100)^{½}$ transformation of the variable was performed before analysis and converted back to percentage.

Results

The explants formed callus within a week on different media containing different plant growth regulators (Tables 1,2). The percentage of embryogenic calli induction varied from 16.50 to 88.40 % with different media composition. The highest frequency (88.40 %) of compact friable embryogenic calli was observed in MMS medium supplemented with 2.0 mg dm^{-3} 2,4-D and 150 mg dm^{-3} Gln. Compact friable embryogenic calli, with small vacuoles and dense cytoplasm in the cells were produced in the course of 21 d.

Table 1. Effect of auxins and cytokinins on callus induction from 10-d-old primary leaf explants of mung bean. CIM 1 to CIM 3 (MS); CIM 4 to CIM 15 (MMS); total culture period 21 d; b - brownish, c - compact, e - embryogenic, f - friable, g - greenish, n - nodular, p - powdery. Means ± SE, $n = 3$.

Code	Additives [mg dm^{-3}] 2,4-D	NAA	BA	KIN	Callus [%]	Callus state
CIM 1	1.0				39.50 ± 0.58	b, n
CIM 2	1.5				42.80 ± 0.93	n, e
CIM 3	2.0				52.10 ± 0.62	b, e
CIM 4	1.0				45.60 ± 0.57	p, c, e
CIM 5	1.5				47.50 ± 0.71	p, c, e
CIM 6	2.0				61.50 ± 0.87	p, e
CIM 7	1.0	1.0			32.50 ± 0.76	n
CIM 8	1.5	1.5			38.10 ± 0.91	n, b
CIM 9	2.0	2.0			41.30 ± 0.61	n, b
CIM 10	2.0		1.0		51.70 ± 0.58	g, n
CIM 11	2.0		1.5		48.50 ± 0.67	g, f, n
CIM 12	2.0		2.0		45.70 ± 0.86	g, f, n
CIM 13	2.0			1.0	22.10 ± 0.61	n
CIM 14	2.0			1.5	21.60 ± 0.44	g, n
CIM 15	2.0			2.0	18.10 ± 0.76	g, n

Primary leaf-derived, friable, 21-d-old compact embryogenic calli from CIM 6, 22 and 23 sub-cultured in MMS liquid medium containing 0.5 mg dm^{-3} 2,4-D and 50 mg dm^{-3} Pro (EIM 17) showed the highest frequency of developing embryos (Table 3). In this medium, maximal recovery of globular (39.0 %), heart (26.3 %) and torpedo-stage (21.0 %) somatic embryos was observed in parallel when the callus from CIM 23 was used (Table 4).

Half strength B5 medium with 5 μM ABA, 20 mg dm^{-3} Pro, 0.05 mg dm^{-3} 2,4-D and 3 % mannitol showed greater maturation (31.4 %) compared to 24.1 % observed in the same medium with 3 % sucrose (Table 5). 2,4-D at 0.05 mg dm^{-3} resulted in the maximum maturation irrespective of the maturation media and their composition and further increase in the concentration of 2,4-D reduced the maturation frequency. An enhanced somatic embryo maturation (46.5 %) was observed by the addition of 1000 mg dm^{-3} KNO_3, 50 mg dm^{-3} PEG 6000 and 3 % mannitol to the above medium. Lower maturation (38.6 %) was observed in the same combination when sorbitol (3 %) replaced mannitol (Table 5). At the end of maturation treatment, most of the embryos changed colour from deep yellow to creamy-white indicating the physiological maturity, non-mature somatic embryos appeared colourless.

Mature, creamy-white, cotyledonary-stage somatic embryos were subjected to germination in MS, MMS and

Table 2. Effect of 2,4-D and plant growth additives on callus induction from 10-d-old primary leaf explants of mung bean. CIM 16 to CIM 30 (MMS), total culture period 21 d; b - brownish, c - compact, e - embryogenic, f - friable, g - greenish, n - nodular, p - powdery, s - slow proliferation. Means ± SE, $n = 3$.

Code	Additives [mg dm^{-3}] 2,4-D	YE	ME	CH	Gln	Put	Callus [%]	Callus state
CIM 16	2.0	100					27.80 ± 0.61	p
CIM 17	2.0		100				22.00 ± 0.76	n, b
CIM 18	2.0			100			48.70 ± 1.01	c, e
CIM 19	2.0			150			52.60 ± 0.93	n, e
CIM 20	1.5				100		72.10 ± 0.87	e, s
CIM 21	2.0				100		79.40 ± 0.58	e, s
CIM 22	1.5				150		82.30 ± 0.61	f, e
CIM 23	2.0				150		88.40 ± 0.53	f, e
CIM 24	1.5				200		78.20 ± 0.44	f, e
CIM 25	2.0				200		75.60 ± 0.76	c, n, e
CIM 26	2.0			100	150		69.10 ± 0.87	c, n, e
CIM 27	2.0			150	150		74.90 ± 0.60	c, e
CIM 28	2.0				150	50	22.00 ± 1.01	c, n
CIM 29	2.0				150	100	18.00 ± 0.87	n
CIM 30	2.0				150	150	16.50 ±0.93	n

B5 media supplemented with different cytokinins, osmotica, *Vermiculite* and KNO_3 (data not shown). Most of the mature somatic embryos, after initial recallusing phase, induced plenty of roots within 5 d of transfer to different germination media. Rapid greening followed by recallusing was observed in these media containing different levels of growth regulators (KIN, GA_3 and TDZ). There was no visible response to KIN and GA_3. The differences observed were varying degree of recallusing, greening, and rooting and primary leaf emergence.

Table 3. Different media compostions used for embryo induction in mung bean.

Code	Additives [mg dm^{-3}]			
	2,4-D	CH	Gln	Pro
EIM 1				
EIM 2	0.25			
EIM 3	0.50			
EIM 4	0.25	100		
EIM 5	0.50	100		
EIM 6	0.25	150		
EIM 7	0.50	150		
EIM 8	0.25		100	
EIM 9	0.50		100	
EIM 10	0.25		150	
EIM 11	0.50		150	
EIM 12	0.25	100	100	
EIM 13	0.50	100	100	
EIM 14	0.25	150	150	
EIM 15	0.50	150	150	
EIM 16	0.25			50
EIM 17	0.50			50
EIM 18	0.25			100
EIM 19	0.50			100
EIM 20	0.25			150
EIM 21	0.50			150
EIM 22	0.25		100	50
EIM 23	0.50		100	50
EIM 24	0.25		150	50
EIM 25	0.50		150	50
EIM 26	0.50			
EIM 27	0.50			
EIM 28	0.50			

The germination response and development of complete plantlets markedly differed when mature cotyledonary-stage somatic embryos were cultured in different germination media containing 3 % maltose and various concentrations of BA, TDZ and KNO_3 (Table 6). Upon gradual removal of residual auxin, mature cotyledon-stage somatic embryos exhibited abnormal rooting in MS and MMS basal medium supplemented with 3 % maltose; browning and recallusing were also observed. Upon germination, somatic embryos sprouted cotyledons, hypocotyls and root poles synchronously. Hypocotyls extension was observed in MMS or half MMS medium containing different concentrations of BA + 500 mg dm^{-3} KNO_3. The regeneration frequency was in the range 0 - 60 % in different germination media tried (Table 6).

Inclusion of 500 mg dm^{-3} KNO_3 and 0.5 mg dm^{-3} BA in half-strength MMS medium induced both the hypocotyl extension and plantlet development with regeneration frequency up to 60 %. It, however, fell to 20 % when somatic embryos were cultured in MMS medium containing a higher dose of BA (1 mg dm^{-3}). The germination media supplemented with 0.05 mg dm^{-3} TDZ alone or with 0.5 mg dm^{-3} BA resulted in browning and rapid recallusing.

An initial, spontaneous rooting of embryo was noticed in the germination medium containing maltose (3 %), 0.05 mg dm^{-3} BA and 500 mg dm^{-3} KNO_3, 5 - 7 d after transfer; however, further growth of root was slow. Germinated somatic embryos were transferred 7 d later to half-strength MMS rooting medium containing different levels of IBA or NAA either alone or together in order to increase the lateral roots from primary root (Table 7). The maximal number (7) of roots per shoot with an average root length (5.5 cm) was observed when grown on half MMS + 1.0 mg dm^{-3} IBA. The rooting efficiency was also greatest in this combination (Table 7).

The plantlets at four-leaf-stage were transferred to mist chamber for further emergence of leaflets and elongation of the stem for next 3 weeks. The plantlets were hardened under greenhouse and subsequently grown until maturity for further phenotypic observation. The average regeneration in three different experiments was 60 %. The harvested seeds from regenerated plants did not show any visible variation.

Discussion

Somatic embryogenesis from primary leaf explants has been reported in *Vigna radiata,* an important recalcitrant grain legume. In the present study, embryogenic calli were induced from 10-d-old primary leaf segments of mung bean cultured in MMS medium containing 2.0 mg dm^{-3} 2,4-D and 150 mg dm^{-3} Gln. Leaf explants from young seedlings were found ideal for somatic embryogenesis in mung bean (Devi *et al.* 2004), cowpea (Kulothungan *et al.* 1995, Prem Anand *et al.* 2001, Ramakrishnan *et al.* 2005) and pigeon pea (Anbazhagan and Ganapathi, 1999). Among the two auxins tested, 2,4-D induced higher frequency of embryogenic calli than NAA. These results differ in pea, in which NAA induced the embryogenic callus in MS medium (Ozcan *et al.* 1993). The importance of 2,4-D for callus induction was studied in mung bean (Girija *et al.* 2000, Amutha

Table 4. Effect of media compositions (Table 3) on induction of somatic embryos [%] of mung bean in different stage of development. Total culture period 21 d. Means ± SE, $n = 9$.

Code	CIM 6			CIM 22			CIM 23		
	globular	heart	torpedo	globular	heart	torpedo	globular	heart	torpedo
EIM 1	0	0	0	8.40±1.33	4.30±0.33	2.30±0.67	9.40±0.67	6.30±0.33	4.60±0.88
EIM 2	7.60±1.20	4.60±0.44	2.50±0.50	8.60±1.76	6.70±0.88	2.00±0.44	10.30±1.20	9.00±0.58	4.30±0.67
EIM 3	8.60±1.20	5.50±0.88	0	10.30±1.86	6.30±0.33	1.60±0.33	12.30±0.58	8.60±0.58	3.30±0.33
EIM 4	4.30±0.67	0	0	4.60±0.88	0	0	5.60±1.67	0	0
EIM 5	4.00±0.44	3.10±0.29	2.60±0.29	4.50±1.00	0	0	5.40±0.40	0	0
EIM 6	4.00±0.87	0	0	3.10±0.44	0	0	5.00±0.76	0	0
EIM 7	4.60±1.20	2.40±0.88	2.00±0.33	5.30±0.88	0	0	6.60±0.33	0	0
EIM 8	3.30±0.88	0	0	4.00±0.58	0	0	5.40±1.86	0	0
EIM 9	3.50±0.29	0	0	3.00±0.58	1.80±0.17	0	4.20±0.44	3.30±0.44	2.80±0.17
EIM 10	3.20±0.60	0	0	4.30±0.44	0	0	5.10±0.60	0	0
EIM 11	5.00±0.44	0	0	5.60±0.67	3.60±0.67	2.60±0.33	8.00±0.58	4.60±0.66	3.40±0.88
EIM 12	4.30±0.88	0	0	6.30±1.45	0	0	8.60±0.67	0	0
EIM 13	6.30±1.45	3.40±0.67	1.40±0.33	7.40±1.66	0	0	9.60±0.33	0	0
EIM 14	5.30±1.20	0	0	7.60±0.33	4.30±1.86	2.60±0.17	10.40±0.88	7.00±1.15	3.00±0.57
EIM 15	5.60±1.20	0	0	8.00±0.57	5.30±1.20	2.30±0.88	12.70±0.33	8.60±0.33	4.00±0.57
EIM 16	12.30±0.33	8.50±0.50	0	23.30±1.20	18.40±0.88	8.60±0.66	26.00±0.67	20.00±0.58	14.70±0.67
EIM 17	13.40±1.20	10.70±0.67	9.40±0.88	26.50±0.88	20.50±0.57	18.30±0.88	39.00±0.58	26.30±0.88	21.00±0.33
EIM 18	11.00±0.57	9.70±0.88	0	21.70±0.33	18.70±0.33	9.70±0.33	26.30±0.33	19.40±0.33	12.40±1.20
EIM 19	10.40±0.33	6.70±0.33	0	21.60±1.20	12.30±0.33	7.60±0.66	24.60±0.33	18.30±1.20	1.30±0.88
EIM 20	2.30±0.17	5.80±0.58	3.10±0.17	4.60±0.33	9.00±0.58	6.60±0.33	6.00±0.58	13.30±0.67	9.00±0.57
EIM 21	10.30±0.40	6.90±0.62	2.60±0.12	15.30±0.66	10.60±0.67	5.00±0.88	19.60±1.45	7.00±1.15	8.00±0.57
EIM 22	9.30±1.86	5.30±1.33	2.30±0.88	12.30±0.88	8.00±1.00	3.30±0.88	15.60±1.45	11.30±1.20	6.70±0.88
EIM 23	9.60±0.33	5.60±0.88	1.60±0.33	13.00±1.15	9.00±1.53	3.30±0.88	16.30±1.20	10.30±1.33	3.30±0.33
EIM 24	8.60±1.20	4.30±0.67	0	11.60±0.66	6.70±1.20	2.60±0.44	12.20±0.58	7.30±0.67	1.60±0.66
EIM 25	8.60±1.45	3.30±1.33	0	10.40±1.20	6.40±1.45	0	13.30±0.88	8.30±0.33	2.00±0.58
EIM 26	3.30±1.33	0	0	5.00±1.45	3.00±0.57	0	6.00±1.20	3.50±1.45	2.00±0.66

et al. 2003) and cowpea (Ramakrishnan *et al.* 2005). In our case, BA or Kin when applied along with 2,4-D induced only low frequency of embryogenic calli. In contrast, Ganesh Kumari *et al*. (2008) reported combined cytokinin (BA) and auxin (NAA) effect for the best organogenic callus induction in castor-bean. Our results in mung bean showed that the addition of YE, ME, CH and Put either alone or in 2,4-D (2 mg dm^{-3}) containing MMS medium did not enhance embryogenic callus induction. Acquisition of embryogenic competence of callus was greater in MMS medium (88.40 %) containing 2.0 mg dm^{-3} 2,4-D and 150 mg dm^{-3} Gln. Similar effect of Gln mediated embryogenic callusing was reported in cowpea (Ramakrishnan *et al.* 2005). Embryogenic calli induced on three different media (CIM 6, 22 and 23) at varying degree of competence, were chosen for induction of somatic embryos in 2,4-D containing liquid medium.

Maximum viable embryogenic clusters were observed in EIM 17 (MMS with 0.5 mg dm^{-3} 2,4-D + 50 mg dm^{-3} Pro). Continuous culturing in this medium led to globular stage somatic embryos on day 14 after initiation. On the other hand, more nitrogen in the form of CH and Gln was required for the embryo induction in cowpea and pigeon pea (Ramakrishnan *et al.* 2005). The increased induction frequency of somatic embryos and their transition depends on the low concentration of 2,4-D in suspension culture containing Pro. Girija *et al.* (2000) reported different stages of somatic embryos of mung bean at 1.5 mg dm^{-3} 2,4-D and 50 mg dm^{-3} Pro in MS liquid medium. In our experiments, the presence of 50 mg dm^{-3} Pro in MMS medium produced all stages of somatic embryos up to 21 d. There was approximately 3-fold increase in the embryo formation in the presence of proline in mung bean in comparison with blackgram (Sen *et al.* 2002). The embryo induction medium containing CH and Gln did not favour transition of somatic embryos.

Maturation is an important phase for the maximum plantlet conversion. The influence of ABA on somatic embryos maturation has already reported in different plant species (Ackerson 1984, Girija *et al.* 2000, Torres *et al.* 2001, Ramakrishnan *et al.* 2005). However, we report for the first time in mung bean the combination of KNO_3^-, PEG 6000, Pro, and ABA that showed enhanced production of mature embryos (46.5 %) when cultured in half-strength B5 medium. These results differ from the observations made by Prem Anand *et al.* (2000) in

cowpea that the combined use of ABA and mannitol only induced embryo maturation in MS medium. In the present study, 3 % mannitol is an efficient sugar alcohol for somatic embryos maturation. Mannitol-mediated somatic embryo maturation was also observed in cowpea (Prem Anad *et al*. 2000, Ramakrishnan *et al.* 2005).

The rate and degree of desiccation of mature embryos are crucial for their ability to germinate (Obendorf *et al.* 1998). The mature embryos incubated in B5 medium synchronously germinated to uniform plantlets in MMS medium containing maltose and BA. Enhanced extension of hypocotyl and plantlet development was observed by increasing KNO_3 concentration. Similar response was reported in cowpea somatic embryo germination and plantlet development (Ramakrishnan *et al.* 2005). The half-strength MMS medium with 3 % maltose, 0.5 mg dm^{-3} BA and 500 mg dm^{-3} KNO_3 greatly favoured germination of somatic embryo into a plantlet. However, BA was essential for both germination and conversion of embryos of *Dalbergia latifolia* (Rao and Lakshmi Sita 1996) and mung bean **(**Devi *et al.* 2004). KNO_3 (500 mg dm^{-3}) was effective for enhancement of somatic embryo germination for both cowpea (Ramakrishnan *et al.* 2005) and mung bean in this study. Further experiments showed that mature mung bean embryos turned brown and recallused when BA was replaced by TDZ in the germination medium which is in contrast to our earlier observation on cowpea (Ramakrishnan *et al.* 2005).

Table 5. Effect of different media and plant growth additives used for maturation of somatic embryos in mung bean. Means ± SE, n = 3. All media supplemented with 5 µm ABA and 20 mg dm^{-3} Pro. Additionally EMM 1 to 5 and 10 to 12 - 3 % sucrose, 6 to 9 and 14 to 19 - 3 % mannitol, 20 - 3 % sorbitol; 19 and 20 also supplemented with 50 mg dm^{-3} PEG 6000 and 1000 mg dm^{-3} KNO_3.

Code	Medium	2,4-D [mg dm^{-3}]	Maturation [%]
EMM 1	MMS basal	-	8.0 ± 0.76
EMM 2	MMS	0.05	12.8 ± 0.60
EMM 3	MMS	0.10	10.8 ± 1.01
EMM 4	Half MMS	0.05	16.6 ± 1.09
EMM 5	Half MMS	0.10	14.0 ± 1.00
EMM 6	MMS	0.05	18.0 ± 1.15
EMM 7	MMS	0.10	17.0 ± 0.58
EMM 8	Half MMS	0.05	19.0 ± 1.53
EMM 9	Half MMS	0.10	18.1 ± 1.01
EMM 10	B5	0.05	21.0 ± 1.53
EMM 11	B5	0.10	19.5 ± 0.87
EMM 12	Half B5	0.05	24.1 ± 0.93
EMM 13	Half B5	0.10	23.0 ± 1.15
EMM 14	B5	0.05	26.5 ± 0.87
EMM 15	B5	0.10	24.8 ± 0.60
EMM 16	Half B5	0.05	31.4 ± 0.93
EMM 17	Half B5	0.10	27.3 ± 1.17
EMM 18	Half B5	0.05	43.0 ± 0.58
EMM 19	Half B5	0.05	46.5 ± 0.76
EMM 20	Half B5	0.05	38.6 ± 0.44

Table 6. The effect of plant growth regulators and additives [mg dm^{-3}] on somatic embryos germination from primary leaf explants of mung bean. Only recallusing and browning was observed on basal media and on media with TDZ. Means ± SE, n = 3.

Medium	BA	KNO_3	Regeneration [%]
MMS	0.5		30 ± 0.71
MMS	1.0		20 ± 0.62
MMS	0.5	500	40 ± 0.50
MMS	1.0	500	30 ± 0.87
½ MMS	0.5		40 ± 0.67
½ MMS	1.0		30 ± 0.58
½ MMS	0.5	500	60 ± 0.76
½ MMS	1.0	500	40 ± 0.93

Table 7. Effect of NAA and IBA [mg dm^{-3}] on root induction of mung bean on ½ MMS media (RM 1 - 9). Means ± SE, n = 3.

Code	NAA	IBA	Rooting [%]	Root number	Length [cm]
RM 1			68.40 ± 0.67	4.00	3.50
RM 2	0.5		70.40 ± 0.58	5.00	3.90
RM 3	1.0		70.80 ± 0.76	5.00	3.60
RM 4		0.5	88.20 ± 0.44	6.00	5.40
RM 5		1.0	90.40 ± 0.29	7.00	5.50
RM 6	0.5	0.5	72.30 ± 0.67	5.00	4.00
RM 7	1.0	0.5	73.75 ± 0.88	5.00	4.30
RM 8	0.5	1.0	80.10 ± 0.60	5.50	4.80
RM 9	1.0	1.0	77.30 ± 0.93	5.30	4.50

Mung bean plantlets with initial two leaves and profuse lateral roots were established in half-strength MMS containing 1.0 mg dm^{-3} IBA after 7 d of culture. Similar IBA-mediated root induction has been reported in *Ricinus communis* (Ganesh Kumari *et al.* 2008). NAA is lesser effective for rooting than IBA in mung bean whereas the combination of NAA (0.5 mg dm^{-3}) and 2.5 mg dm^{-3} IBA enhanced the production of lateral roots in cowpea (Ramakrishnan *et al.* 2005). Plants were successfully acclimatized in soil and subsequently transferred to the field. The harvested grains are morphologically normal and fertile.

The process of somatic embryogenesis and plant regeneration of mung bean reported here is a highly reproducible protocol, which could be well exploited for genetic manipulation and crop improvement of a recalcitrant legume, mung bean.

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