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RESEARCH ARTICLE

In vitro regeneration through organogenesis and somatic embryogenesis in pigeon pea [*Cajanus cajan* (L.) Millsp.] cv. JKR105

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Abstract In vitro regeneration of pigeon pea through organogenesis and somatic embryogenesis was demonstrated with pigeon pea cv. JKR105. Embryonic axes explants of pigeon pea showed greater regeneration of shoot buds on 2.5 mg L^{-1} 6-benzylaminopurine (BAP) in the medium, followed by further elongation at lower concentrations. Rooting of shoots was observed on half-strength Murashige and Skoog (MS) medium with 2 % sucrose and 0.5 mg L^{-1} 3-indolebutyric acid (IBA). On the other hand, the regeneration of globular embryos from cotyledon explant was faster and greater with thidiazuron (TDZ) than BAP with sucrose as carbohydrate source. These globular embryos were maturated on MS medium with abscisic acid (ABA) and finally germinated on half-strength MS medium at lower concentrations of BAP. Comparison of regeneration pathways in pigeon pea cv. JKR105 showed that the turnover of successful establishment of plants achieved through organogenesis was more compared to somatic embryogenesis, despite the production of more embryos than shoot buds.

Keywords Pigeon pea · Organogenesis · Somatic embryogenesis · Cytokinins · Auxins

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Introduction

Pigeon pea [*Cajanus cajan* (L.) Millsp.] (Family: Fabaceae) popularly known as red gram is a grain legume grown in the semi-arid tropics. Its production and productivity, however, are constrained by several abiotic and biotic stresses. Although wild species of pigeon pea may provide genetic diversity for tolerance traits to these stresses, not present in cultivated species, but often are associated with undesirable agronomic traits that are difficult to overcome in conventional plant breeding programs (Muehlbauer 1993). This has necessitated to explore in vitro regeneration protocols for exploitation of plant cell totipotent capacity to micropropagate elite plant clones from differentiated tissues through organogenesis or/and somatic embryogenesis (Davey et al. 1994). The early history of pigeon pea tissue culture has been comprehensively reviewed by Krishna et al. (2010).

In pigeon pea, the regeneration of plants by organogenesis has been reported via pre-existing meristems like apical meristem (Cheema and Bawa 1991), undifferentiated callus (Kumar et al. 1983; George and Eapen 1994), differentiated non-meristematic leaf tissue (Eapen and George 1993; Eapen et al. 1998; Geetha et al. 1998; Singh et al. 2002; Dayal et al. 2003; Villiers et al. 2008); and seedling tissues such as hypocotyl (Cheema and Bawa 1991; Geetha et al. 1998), cotyledon (George and Eapen 1994; Geetha et al. 1998; Mohan and Krishnamurthy 1998; Chandra et al. 2003), cotyledonary node (Mehta and Mohan Ram 1980; Kumar et al. 1983, 1984; Naidu et al. 1995; Shiva Prakash et al. 1994; Geetha et al. 1998; Singh et al. 2003), epicotyl (Kumar et al. 1984; Naidu et al. 1995; Geetha et al. 1998), and embryonal axes (Sarangi and Gleba 1991; George and Eapen 1994; Naidu et al. 1995; Franklin et al. 2000).

Similarly, the somatic embryogenesis has been reported through in vitro cultures from different tissue sources of various pigeon pea cultivars of Asian origin (Nalini et al. 1996; Anbazhagan and Ganapathi 1999; Mohan and Krishnamurthy 2002; Chandra et al. 2003; Singh et al. 2003). Plant regeneration has been achieved via callus (Nalini et al. 1996; Sreenivasu et al. 1998), suspension cultures from leaf-derived callus (Anbazhagan and Ganapathi 1999), and cotyledonary node (Singh et al. 2003). Despite several reports available on organogenesis and somatic embryogenesis using cultivars of Asian and African origin, highly reproducible protocols in the same genotype are quite few. Thus, the present investigation is an attempt to focus the regeneration response by organogenesis and somatic embryogenesis in pigeon pea cv. JKR105.

Materials and methods

Healthy seeds of pigeon pea cv. JKR105 (JK Agri Genetics Ltd, Hyderabad) were surface sterilized by agitating in Erlenmeyer flask containing mercuric chloride ($HgCl_2$), and Triton X-100 (10 μl) for 20 min, followed by 8–10 washes in sterile double distilled water. Subsequently, the seeds were soaked in sterile distilled water for 18 h at 26±2 °C in the dark, drained aseptically, and washed twice in sterile double distilled water. Pre-soaked seeds were inoculated aseptically on cotton wet-bed with MS liquid medium containing 2.5 mg L^{-1} BAP (6-benzylaminopurine) for germination. Three-day old germinated de-coated seeds were used to excise embryonic axes and cotyledons for studies in organogenesis and somatic embryogenesis, respectively. All the combinations tested for the organogenesis and somatic embryogenesis have been repeated thrice.

Organogenesis

In order to regenerate shoot buds through organogenesis, the embryonic axes explants were cultured on MS basal medium supplemented either alone with BAP, KIN (kinetin), and TDZ (thidiazuron) or in combination of BAP with NAA and IBA (3-indolebutyric acid)]. Scoring was recorded on the explant response, after 4 weeks, for shoot bud induction (Fig. 1). The regenerated shoot buds were cultured for elongation on MS medium by addition of different concentrations of BAP alone (0.1, 0.2, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, and 5.0 mg L^{-1}), BAP (1.0, 2.0, and 5.0 mg L^{-1}) combined with IAA (indole-3-acetic acid) alone (0.2 and 0.5 mg L^{-1}) or with GA3 (gibberellic acid-3) (2.0 mg L^{-1}) (Table 1). The elongated shoots were

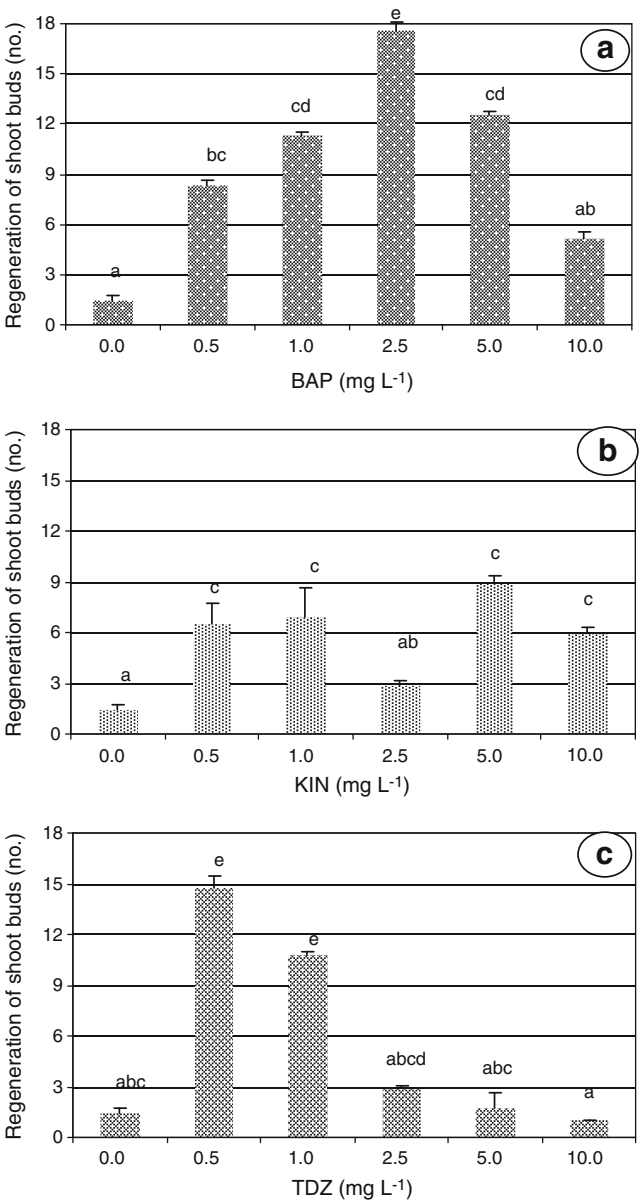

Fig. 1 Effect of BAP, KIN, and TDZ supplemented to MS medium, at various concentrations, on shoot bud regeneration in pigeon pea

transferred later on to the full- and half-strength MS medium, combined with IBA alone (0.25, 0.5, and 2.0 mg L^{-1}) and sucrose. The rooted shoots were finally transferred to plastic bags for hardening and grown to maturity in the greenhouse.

Somatic embryogenesis

Cotyledon explants were cultured on MS medium supplemented with different concentrations of NAA (0.1, 0.9, 1.8, 3.7, 7.4, 11.1, and 14.8 mg L^{-1}), 2,4-D (0.2, 1.1, 2.2, 4.4,

Table 1 Effect of elongation medium on the regeneration response of shoot buds in pigeon pea

Elongation medium [Plant growth regulators (mg L^{-1})]	Shoot length (cm) (Mean + SE)	Elongation (%)	Effect
BAP (0.1)	2.8 ± 0.20^{de}	64.12	No leaf falling and no yellowing of shoot and leaves
BAP (0.2)	3.2 ± 0.20^{cd}	68.10	No leaf falling and no yellowing of shoot and leaves
BAP (0.5)	5.5 ± 0.23^{a}	88.60	No leaf falling and no yellowing of shoot and leaves
BAP (0.7)	4.5 ± 0.35^{b}	71.20	Leaf falling
BAP (1.0)	2.1 ± 0.03^{fgh}	55.81	Leaf falling
BAP (1.5)	2.1 ± 0.08^{fgh}	37.17	Leaf falling
BAP (2.0)	2.2 ± 0.29^{fg}	23.00	Leaf falling
BAP (2.5)	2.2 ± 0.20^{fg}	21.80	Leaf falling
BAP (5.0)	1.5 ± 0.14^{ij}	18.50	Leaf falling
BAP (1.0)+IAA (0.2)	2.2 ± 0.05^{fg}	48.30	Yellowing of shoot and leaves
BAP (1.0)+IAA (0.2)+GA3 (2.0)	2.6 ± 0.11^{ef}	42.10	Leaf falling and yellowing of shoot and leaves
BAP (1.0)+IAA (0.5)	2.8 ± 0.05^{de}	35.20	Yellowing of shoot and leaves
BAP (1.0)+IAA (0.5)+GA3 (2.0)	3.7 ± 0.08^{c}	31.20	Leaf falling and yellowing of shoot and leaves
BAP (2.0)+IAA (0.2)	2.1 ± 0.03^{fgh}	40.07	Leaf falling and yellowing of shoot and leaves
BAP (2.0)+IAA (0.2)+GA3 (2.0)	2.2 ± 0.10^{fg}	21.03	Leaf falling and yellowing of shoot and leaves
BAP (2.0)+IAA (0.5)	1.6 ± 0.25^{fghi}	33.33	No leaf falling and no yellowing of shoot and leaves
BAP (2.0)+IAA (0.5)+GA3 (2.0)	2.2 ± 0.32^{fg}	21.02	Leaf falling and yellowing of shoot and leaves
BAP (5.0)+IAA (0.2)	1.3 ± 0.12^{ijk}	29.17	Yellowing of shoot and leaves
BAP (5.0)+IAA (0.2)+GA3 (2.0)	1.5 ± 0.08^{ij}	23.86	Leaf falling and yellowing of shoot and leaves
BAP (5.0)+IAA (0.5)	0.9 ± 0.08^{k}	31.11	No leaf falling and no yellowing of shoot and leaves
BAP (0.5)+IAA (0.5)+GA3 (2.0)	0.8 ± 0.20^{k}	29.09	Leaf falling and yellowing of shoot and leaves

Means followed by the same letter in the column are not significantly different ($P<0.05$; LSD)

8.8, 13.2, and 17.6 mg L^{-1}), TDZ (0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1}), and BAP (0.2, 1.1, 2.2, 3.3, 4.4, 6.6, and 11.2 mg L^{-1}); and in combination of either BAP (0.1 or 1.0 mg L^{-1}) with 2,4-D (0.2, 1.1, 2.2, 4.4, 8.8, 13.2, and 17.6 mg L^{-1}) or NAA (0.1, 0.9, 1.8, 3.7, 7.4, 11.1, and 14.8 mg L^{-1}). All the media concentrations were additionally mixed with 3.0 % sucrose/glucose. After 45 days of incubation period, the smooth nodular outgrowths evident on the adaxial surface of cotyledon explants enlarged into distinct globular pro-embryoids. The globular embryos derived from TDZ and BAP were transferred and incubated for 30 days on the same medium, hormone-free MS basal, and MS basal media with 1.8 mg L^{-1} ABA for maturation. Subsequently, the matured embryos were transferred to the hormone-free half-strength MS basal, and 0.5 or 0.1 mg L^{-1} BAP supplemented to half-strength MS basal media for germination over a period of 15 days. In vitro-germinated somatic embryo plantlets transferred from the culture bottles were gently washed under tap-water to remove the adhered agar and medium. Later, the emblings were transferred to the plastic cups containing autoclaved soils for hardening. They were grown till maturity for acclimatization, under greenhouse conditions.

Statistical analysis

The data was subjected to analysis of variance, and the treatment means were separated using least significant difference (LSD) test at $P>0.05$ (Gomez and Gomez 1984).

Results and discussion

Organogenesis

Successful germination was achieved on sterilized cotton-bed containing MS liquid medium with 2.5 mg L^{-1} BAP rather than MS liquid medium alone (Fig. 4a). This may be due to the triggering action of cytokinins not only for the growth of leaves and lateral buds but also for the development of secondary xylem. Similar results were obtained in pea (Kuraishi and Okumura 1956; Sugiura 2004) and tobacco (Medford et al. 1989). Additionally, the incubation under darkness may also be a key factor in realizing good germination frequency. Such a phenomenon has been attributed to involvement of PhyB noticed in

Arabidopsis (Shinomura et al. 1994). In contrast, the initial induction of cells with morphogenetic potential requires dark incubation, while plantlet formation resulted under light conditions in pigeon pea (Franklin et al. 2000). Present results are not in agreement with Romero et al. (2005), who reported high germination frequency in *Ehninaceae* sp. under 16–24 h light conditions.

Shoot bud regeneration

Explant incubation of embryonic axes showed significantly higher regeneration of shoot buds (17.5) with 2.5 mg L^{-1} BAP (Fig. 1 and Fig. 4b, c). But the regeneration responses of explants at other concentrations of BAP are significantly not different from 1.0 mg L^{-1}. Mehta and Mohan Ram (1980) reported that higher concentration of BAP induced direct shoot regeneration from the cotyledonary surface. While low concentrations of BAP favored the development of shoot buds from the pre-existing meristems (Shiva Prakash et al. 1994; Ignacimuthu et al. 1997). However, higher number of viable plants were regenerated on KIN medium in several cereal crops (Bhaskaran and Smith 1990). In contrast, the present studies demonstrated that 5.0 mg L^{-1} KIN had low shoot bud regeneration compared to 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} TDZ (Fig. 1a, b, c). Similar results were obtained with TDZ compared to 2,4-D, KIN, and NAA in Sugarcane (*Saccharum* spp.) (Gallo-Meagher et al. 2000), beans (*Phaseolus vulgaris*) (Malik and Saxena 1992a), chickpea (*Cicer arietinum*) (George and Eapen 1997), and peanut (*Arachis hypogaea*) (Kanyand et al. 1994). In pigeon pea, higher regeneration of shoot buds (14.7) from the embryonic axes explants was observed on 0.5 mg L^{-1} TDZ. Eapen et al. (1998) achieved similar results of high regeneration frequency of shoots from the leaf explants with TDZ. Comparison of regeneration response between 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} TDZ showed that the former enhanced greater number of shoots (17.5) than the latter with hyper-hydration. These results are in consonance with those reported in *Alfalfa* sp. and *Coleus* sp. with TDZ (Malathi et al. 2006). Further, this phenomenon was attributed to explant inability to degrade and leading to accumulation of TDZ (Mok and Mok 2001).

Better performance of BAP over auxins for shoot regeneration has been reported in pigeon pea (Franklin et al. 2000). Present study also showed that NAA and 2,4-D reduced the regeneration capacity of shoots. Despite higher number of shoots (17.5) retrieved by 2.5 mg L^{-1} BAP alone, but in combination with 0.5 mg L^{-1} NAA and 5.0 mg L^{-1} IBA resulted in poor regeneration by 5.60 and 1.67 shoot buds, respectively (Figs. 2 a–d and 3 a–d). These findings strongly support the multiple shoot regeneration under depleted auxin supplementation in legumes (Ignacimuthu et al. 1997; Franklin et al. 1998). In addition, it may be due to integration of BAP with endogenous auxin concentrations promoted calli regeneration but resulted in subsidized shoot bud formation.

Shoot bud elongation

Explants with a mass of shoot bud initials derived from 2.5 mg L^{-1} BAP sub-cultured on MS basal medium supplemented with BAP alone or combined with IAA and/or GA3, enhanced their elongation. Over an incubation period of 3 weeks, it was observed that 0.5 and 0.7 mg L^{-1} BAP responded better with a shoot length of 5.5 and 4.5 cm, respectively (Table 1). On the other hand, the explants cultured on <0.5 mg L^{-1} and >0.5 mg L^{-1} BAP showed significantly low shoot bud elongation. Furthermore, alteration in media composition (Malik and Saxena 1992b; Shiva Prakash et al. 1994), substitution of plant growth regulators (Mohamed et al. 1991; Nagi et al. 1997), and changes in light conditions had a significant effect on shoot elongation. Franklin et al. (2000) observed that the incubation of embryonal axes of pigeon pea, under light–dark conditions, though favored the shoot bud formation, was less efficient for elongation. IAA alone and combined with GA3 supplemented to 5.0 mg L^{-1} BAP showed significant reduction in shoot elongation, and conversely had greater elongation on the same combination(s) supplemented with 1.0 mg L^{-1} BAP. Katsumi and Kazama (1978) reported enhanced auxin biosynthesis by supplementation of GA3, which may become occasionally toxic for growth and shoot elongation (Soetikno 1981). Eapen and George (1993) obtained regeneration of calli from the leaf discs of pigeon pea and peanut, and indicated that BAP + IAA or IAA + aspartic acid are essential for shoot bud elongation. In addition, Kumar et al. (1983) reported regeneration from the leaf callus of pigeon pea on Blaydes (1966) medium with 0.5 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, and 0.01 mg L^{-1} GA3.

Elongated shoot rooting

Elongated shoots derived on BAP showed that all the combinations were ineffective for rooting, due to falling and yellowing of leaves, except half-strength MS supplemented by 0.5 mg L^{-1} IBA and 2 % sucrose (Table 2). Some pigeon pea genotypes (VBN1, VBN2, SA1, and CO5), however, have prolific rooting on the same medium with 3 % sucrose (Franklin et al. 2000). This genotypic variation may be due to carbon source concentration. Interestingly, in the present investigation, the nodal part embedded in the medium also displayed complete rooting of elongated shoots (Fig. 4d). Whereas

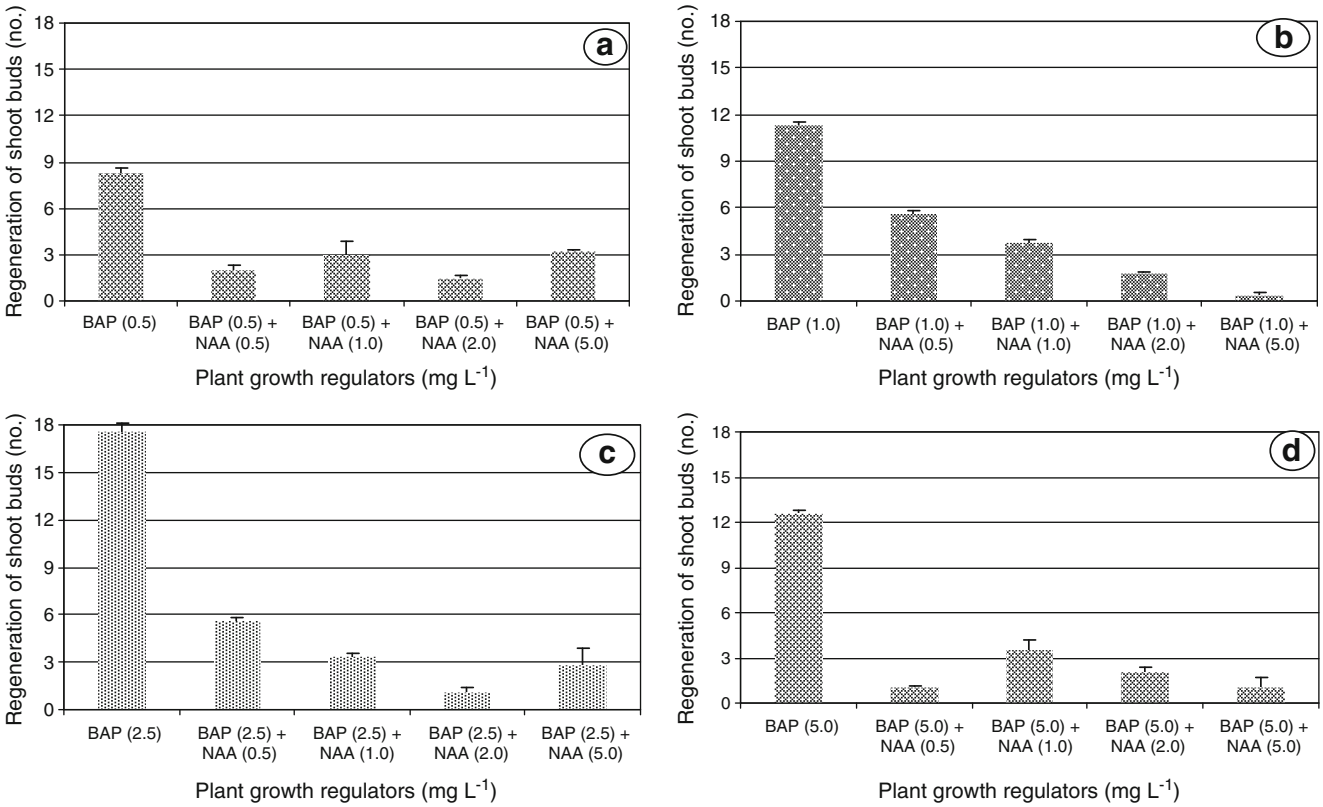

Fig. 2 Effect of BAP in combination with NAA supplemented to MS medium at various concentrations on shoot bud regeneration in pigeon pea

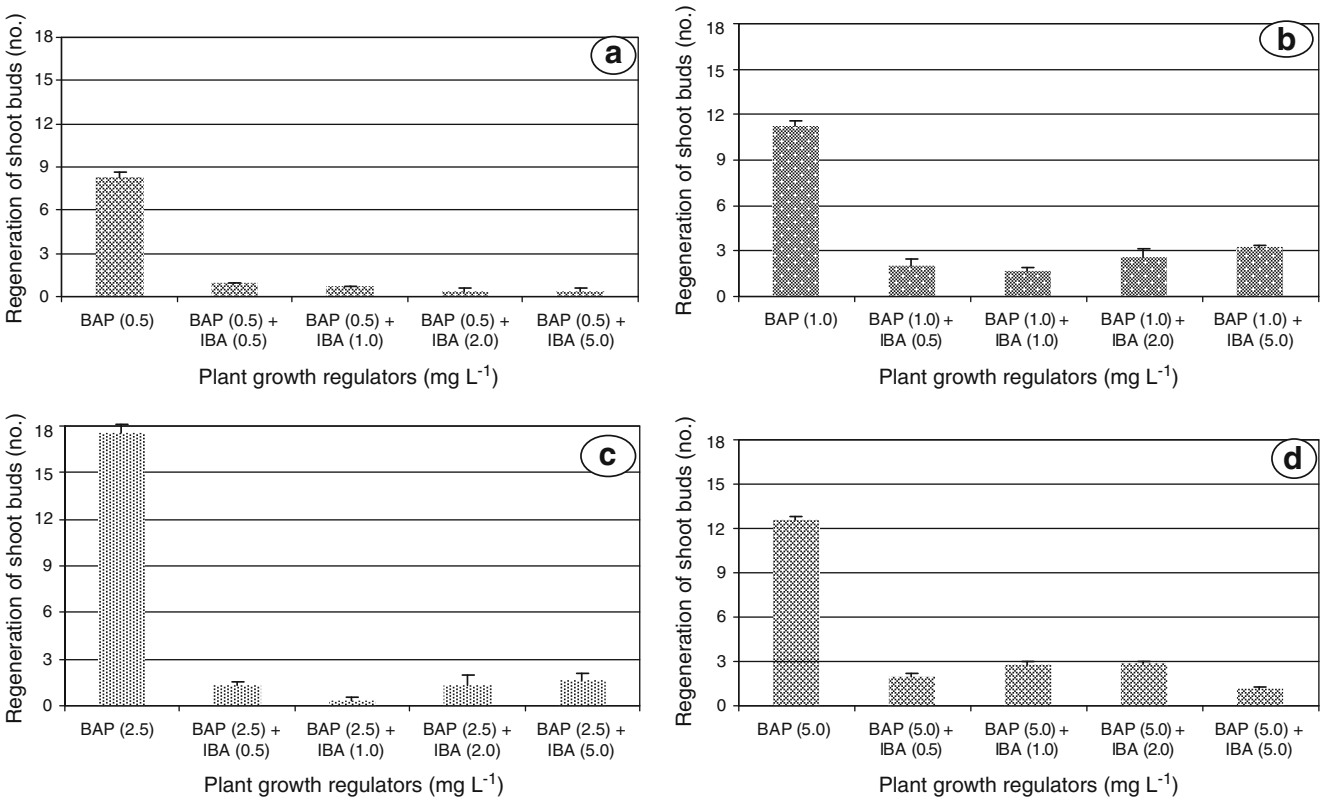

Fig. 3 Effect of BAP in combination with IBA supplemented to MS medium at various concentrations on shoot bud regeneration in pigeon pea

Table 2 Effect of rooting medium on the regeneration response of shoot explants in pigeon pea

Rooting medium			Root length (cm) (Mean ± SE)	Symptoms
MS medium	IBA (mg L^{-1})	Sucrose (%)		
MS	0.25	3.0	No response	Callus at shoot base
MS	2.00	3.0	No response	Callus at shoot base
half-strength MS	0.50	3.0	No response	Callus at shoot base
MS	0.25	2.0	No response	Leaf falling from shoot
MS	2.00	2.0	No response	Leaf falling from shoot
half-strength MS	0.50	2.0	5.9±0.3	Rooting
MS	0.25	1.5	No response	Leaf yellowing
MS	2.00	1.5	No response	Leaf yellowing
half-strength MS	0.50	1.5	No response	Leaf yellowing

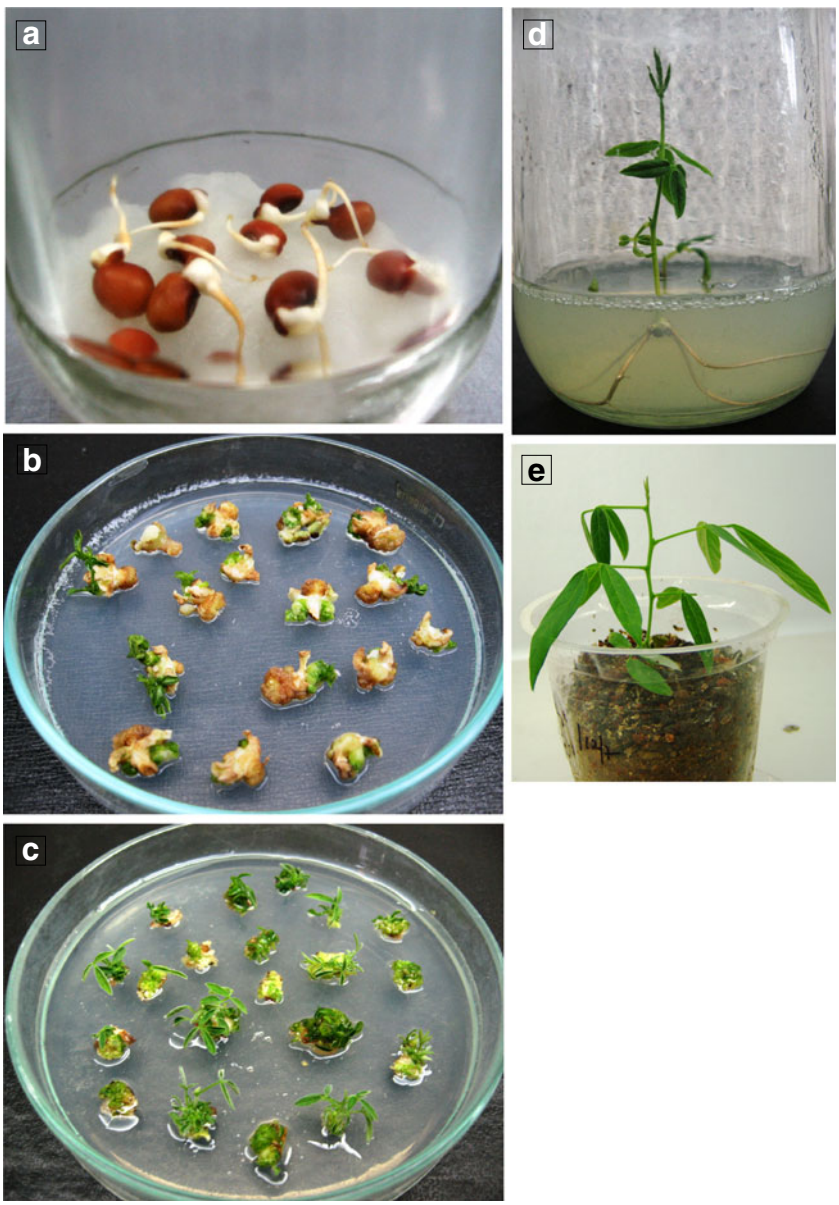

Fig. 4 Regeneration in pigeon pea through organogenesis: **a**-Seed germination on cotton bed with cytokinin; **b** and **c**-Multiple shoot regeneration from embryonic axes explants; **d**-Regenerated shoot on rooting medium; **e**-Plantlet during hardening

the rooting medium supplemented with 3 % sucrose showed vigorous callus growth at the shoot base. However, lower concentrations of sucrose (<1.5 %) in the medium caused leaf yellowing due to carbon deficiency. MS medium combined with 1.0 mg L^{-1} NAA resulted 90 % rooting (Eapen et al. 1998), and 1 mg L^{-1} IBA had 80–85 % rooting (Mohan and Krishnamurthy 1998). Finally the rooted shoots were transferred to the autoclaved soil for hardening and grown till maturity (Fig. 4e).

Somatic embryogenesis

Since embryonic calli exhibit prolific multiplication and somatic embryos originate from single cells, the regeneration approach through embryogenesis was considered as the most preferred pathway for genetic transformation (Hansen and Wright 1999), for which a genotype-independent protocol was standardized by using pigeon pea cv. JKR105. Induction of somatic embryogenesis in pigeon pea has been reported on EC6 basal (Patel et al. 1994; Mohan and Krishnamurthy 2002) and MS basal media (Nalini et al. 1996; Sreenivasu et al. 1998; Singh et al. 2003) for various Asian and African cultivars of pigeon pea.

In the present study, the cotyledon explants produced smooth nodular outgrowths on the adaxial surface were enlarged into distinct globular pro-embryoids. After 15-day incubation period, they began showing swelling in all combinations of the media (Fig. 5a). The induction of globular embryos was observed after 45-day incubation on MS basal medium supplemented with 0.2, 0.4, and 0.6 mg L^{-1} TDZ; and 0.2, 1.1, 2.2, 3.3, and 4.4 mg L^{-1} BAP, including addition of sucrose/glucose as a carbon source (Table 3). Present studies demonstrated that the globular embryos regenerated on the surface of cotyledon explants although higher (30.8) on the medium supplemented with 0.6 mg L^{-1} TDZ were significantly not different from 0.4 and 0.2 mg L^{-1} TDZ (Fig. 5b). Similarly, 1.1 mg L^{-1} BAP yielded 13.9 globular embryos, but did not vary irrespective of lower and higher concentrations (0.2,

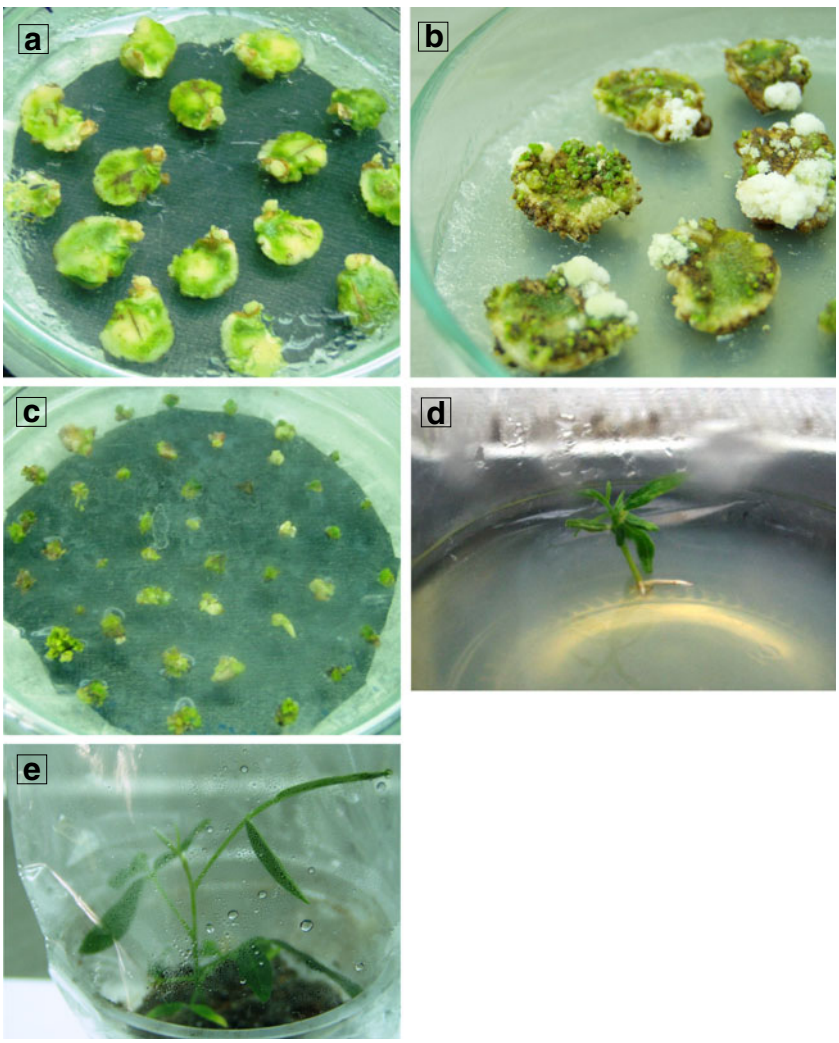

Fig. 5 Regeneration in pigeon pea through somatic embryogenesis: **a**-Cotyledon explants on TDZ medium; **b**-Somatic embryo regeneration on the surface of cotyledons; **c**-Somatic embryo on ABA maturation medium; **d**-Somatic embryo germination; **e**-Embling during hardening

Table 3 Combined effect of plant growth regulators and carbohydrates in MS medium on the response of somatic embryogenesis and globular embryos in pigeon pea

Plant growth regulator		Sucrose		Glucose	
Plant growth regulator	Concentration (mg L^{-1})	Type of response	Globular embryos explant^{-1} (no.) (Mean ± SE)	Type of response	Globular embryos explant^{-1} (no.) (Mean ± SE)
2,4-D	0.2	Callus	…..	Callus	…..
2,4-D	1.1	Callus	…..	Callus	…..
2,4-D	2.2	Callus	…..	Callus	…..
2,4-D	4.4	Callus	…..	Callus	…..
2,4-D	8.8	Callus	…..	Callus	…..
2,4-D	13.2	Callus	…..	Callus	…..
2,4-D	17.6	Callus	…..	Callus	…..
BAP	0.2	Somatic embryo	10.6±1.0^{d}	Somatic embryo	9.8±0.4^{de}
BAP	1.1	Somatic embryo	13.9±0.7^{d}	Somatic embryo	12.8±0.6^{d}
BAP	2.2	Somatic embryo	12.8±0.8^{d}	Somatic embryo	11.7±0.5^{de}
BAP	3.3	Somatic embryo	7.9±0.6^{d}	Somatic embryo	6.6±0.7^{e}
BAP	4.4	Somatic embryo	8.6±0.5^{d}	Somatic embryo	7.9±0.3^{de}
BAP	6.6	–		–	…..
BAP	11.2	–		–	…..
NAA	0.1	Callus	…..	Callus	…..
NAA	0.9	Callus	…..	Callus	…..
NAA	1.8	Callus	…..	Callus	…..
NAA	3.7	Callus	…..	Callus	…..
NAA	7.4	Callus	…..	Callus	…..
NAA	11.1	Callus	…..	Callus	…..
NAA	14.8	Callus	…..	Callus	…..
TDZ	0.2	Somatic embryo	26.4±0.7^{ab}	Somatic embryo	19.8±0.2^{abc}
TDZ	0.4	Somatic embryo	25.0±1.4^{abc}	Somatic embryo	21.1±1.1^{ab}
TDZ	0.6	Somatic embryo	30.8±0.7^{a}	Somatic embryo	23.1±0.7^{a}
TDZ	0.8	Callus	…..	Callus	…..
TDZ	1.0	Callus	…..	Callus	…..

Means followed by the same letter in the column are not significantly different ($P<0.05$; LSD)

2.2, 3.3, and 4.4 mg L^{-1}). TDZ has been used to elicit multiple shoot formation in a broad range of plant species (Malik and Saxena 1992a,b), and somatic embryogenesis in a few crop species (Gill and Saxena 1993; Gill et al. 1993; Lu 1993). While other studies indicated successful utilization of cytokinins for somatic embryogenesis not only in pigeon pea (Patel et al. 1994; Sreenivasu et al. 1998; Mohan and Krishnamurthy 2002; Chandra et al. 2003; Singh et al. 2003) but also other leguminous crops (Maheswaran and Williams 1984; Gill and Saxena 1992; Malik and Saxena 1992b; Murthy et al. 1996).

The MS medium comprising TDZ and BAP was found less effective for globular embryo formation with glucose than sucrose. This was evident when glucose supplemented to the MS medium by regenerating 19.8, 21.1, and 23.1 globular embryos with 0.2, 0.4, and 0.6 mg L^{-1} TDZ; and 12.8, 11.7, 6.6, and 7.9 embryos on 1.1, 2.2, 3.3, and 4.4 mg L^{-1} BAP (Table 3). Although it has been reported that among the carbohydrates, sucrose was highly effective for the induction of somatic embryogenesis (Anbazhagan and Ganapathi 1999), higher concentrations of cytokinins also promoted somatic embryogenesis in pigeon pea (Patel et al. 1994). In contrast, the present results demonstrated that higher concentrations of cytokinins did not favor embryo induction, but resulted in more callusing. On the other hand, somatic embryogenesis was effective with glucose in *Phaseolus coccineus* (Genga and Allavena 1991); maltose in *Alfalfa* sp. (Strickland et al. 1987; Denchev et al. 1991) and *Glycine max* (Finer and McMullen 1991); and the culture maintenance in darkness

for many crop species (Thorpe 1988). In contrast, somatic embryogenesis was predominantly seen when incubated at 16 L: 8D.

The combination of auxins (2,4-D and NAA) and cytokinin (BAP), at different concentrations, supplemented to the MS medium displayed the formation of white/green colored calli. In contrast, somatic embryogenesis has been reported on medium containing NAA and BAP (Nalini et al. 1996), and also in combination of BAP, KIN, and Ads (adenine sulphate) (Patel et al. 1994). This type of genotypic variability in response to induction of somatic embryos was reported in pigeon pea (Patel et al. 1994; Sreenivasu et al. 1998; Mohan and Krishnamurthy 2002; Singh et al. 2003), soybean (Barwale et al. 1986; Komatsuda and Ohyama 1988; Parrott et al. 1989; Bailey et al. 1993), and peanut (Sellars et al. 1990; Ozias-Akins et al. 1992; George and Eapen 1993; Baker et al. 1995; McKently 1995; Chengalrayan et al. 1998).

In pigeon pea, the somatic embryo formation has been observed on calli derived from cotyledon and leaf explants (Sreenivasu et al. 1998), and from suspension culture of leaf explants (Anbazhagan and Ganapathi 1999). Interestingly, the direct appearance of globular embryos on cotyledon explants has been noticed without callus formation. Normal embryos without plantlets were achieved from immature cotyledon and embryonal axes explants on auxin supplemented medium (George and Eapen 1994).

Cotyledon structure development

The globular embryos did not develop further when the cotyledon explants with globular embryos transferred to the same, hormone-free, and MS basal media. These observations suggest that TDZ may not be ideal for further development of globular embryos and considered as lethal for embryo development. Similar results were reported in *Geranium* sp. (Visser-Tenyenhuis et al. 1994). In contrast, Sreenivasu et al. (1998) observed the induction of somatic embryos via callus from the leaf explants on TDZ. Our results showed that the development of globular embryos to cotyledon structures was evident on the MS medium supplemented with 1.8 mg L^{-1} ABA (Fig. 5c). The maturation of normal cotyledonary embryos was 20 %, while the remaining embryos either formed callus or turned necrotic. The maturation of somatic embryos on ABA medium was reported for pigeon pea (Mohan and Krishnamurthy 2002), *Phaseolus* sp (Malik and Saxena 1992a), and chickpea (Suhasini et al. 1994).

Somatic embryo germination

Culturing of mature cotyledonary somatic embryos on MS basal medium supplemented with 1.8 mg L^{-1} ABA yielded good response for germination on half-strength MS medium augmented with 0.1 mg L^{-1} BAP (Fig. 5d). The development of shoots and roots was extremely poor on hormone free half-strength MS medium. Whereas Singh et al. (2003) observed good germination of somatic embryos on the same medium. While the addition of 0.5 mg L^{-1} BAP to half-strength MS medium has promoted the shoot formation and cotyledon swelling without root development; and 0.1 mg L^{-1} BAP produced both shoot and root systems with 20 % frequency (Fig. 5d). These results are in consonance with Mohan and Krishnamurthy (2002), who reported 22 % germination of somatic embryos on the same medium in pigeon pea cv. T-15-15, GAUT-82-90 and GAUT-82-99. Finally the embling with sufficient root and shoot system were transferred to the autoclaved soil for hardening (Fig. 5e).

Comparison of regeneration frequencies on embryo formation and shoot buds, respectively from cotyledon and embryonic axes explants of pigeon pea cv. JKR105 indicated that the former pathway was highly efficient (Fig. 1, Table 3). But subsequent in vitro development of embryos caused low yield in final emblings due to initial realization of 20 % cotyledonary stage embryos on ABA medium, followed by 20 % germination frequency. These studies suggest using any of these regeneration pathways with pigeon pea cv. JKR105, for further improvement through genetic transformation by using Bt gene(s) for resistance to the pod borer, *Helicoverpa armigera*.

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