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Assessment of *in vitro* Multiple Shoot Bud Induction from Leaf Explants among Eleven Indian Cultivars of Pigeon Pea (*Cajanus cajan* L. Mill sp.)

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Abstract: *In vitro* multiple shoot bud induction from leaf explants of eleven Indian cultivars of pigeon pea (*Cajanus cajan* L. Mill sp.) under the influence of variable concentration of three different cytokinins namely BAP, KIN and TDZ on MS media has been studied. Callus of variable morphology and nature was observed for most of the cultivars except four cultivars IPA-2013, Pusa-9, T-7 and IPA-98-3 showing multiple shoot bud induction. Among cytokinins used BAP was found to be most effective. Further elongation of multiple shoot buds was achieved in the same media while rooting of regenerated multiple shoots were attempted in MS media supplemented with variable concentration of three different auxins namely NAA, IAA and IBA. Efficient rooting was achieved in the presence of NAA. Regenerated plants were successfully established in soil with acclimatization frequency varying from 25-50% with cultivar IPA-2013 showing the best response.

Key words: Pigeon pea, multiple shoot bud induction, MS medium, acclimatization, regeneration, cultivars

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Mill sp.) is an important high-protein grain legume of semi-arid tropics and sub-tropics cultivated in more than 50 countries of Asia, Africa and the Americas with India showing more than 80% of the world production (Nene and Sheila, 1990; Popelka *et al*., 2004; FAO, 2008). The overall production of pigeon pea is hampered by its susceptibility to number of biotic and abiotic stresses. The efforts to raise stress-resistant genotypes have met with limited success because of limited genetic variability amongst cultivated accessions and sexual incompatibility with wild relatives. The potential of transgenic technology for pigeon pea improvement has been visualized though there is paucity of efficient regeneration protocol due to its inherent recalcitrance to regeneration under *in vitro* tissue culture conditions.

In vitro regeneration by organogenesis and embryogenesis from callus or directly from different explants along with recent developments in transformation technology has been recently reviewed (Krishna *et al*., 2010). Reports of organogenesis from an unorganized callus are rare (Kumar *et al*., 1983). Multiple shoot production and regeneration via organogenesis from explants namely cotyledons, embryonic axes (Sarangi and Gleba, 1991), cotyledonary nodes from mature seeds (Prakash *et al*., 1994; Franklin *et al*., 1998) and seedling petioles (Srinivasan *et al*., 2004) has been reported. There have been attempts to initiate *in vitro* cultures from different tissue sources in pigeon pea (Geetha *et al*., 1998). The genotype dependent morphogenic potentiality of various explants of pigeon pea has also been demonstrated (Naidu *et al*., 1995), followed by genotype dependence of organogenesis from distal half of cotyledon explants without pre-existing meristem (Mohan and Krishnamurthy, 1998).

There have been reports of *in vitro* regeneration protocols suitable for genetic transformation of pigeon pea in recent years (Lawrence and Koundal, 2001; Satyavathi *et al*., 2003; Dayal *et al*., 2003; Thu *et al*., 2003; Mohan and Krishnamurthy, 2003; Prasad *et al*., 2004) revealing that the crop is not as recalcitrant as assumed earlier however, limited to some genotypes or cultivars. There is a need to assess the regeneration potential of different explants of existing cultivars to develop efficient regeneration protocol amenable for genetic transformation.

This study deals with assessment of *in vitro* regeneration from leaf explants for multiple shoot bud induction for selected eleven Indian cultivars of pigeon pea in the presence of different growth hormones.

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MATERIALS AND METHODS

Seeds of pigeon pea cultivars viz., IPA-2013, IPA-3088, Pusa-9, IPA-34, IPA-204, IPA-242, T-7, IPA-61, IPA-337, IPA-341 and IPA-98-3 were procured from Indian Institute of Pulses Research, Kanpur.

Seeds were surface sterilized with 1% cetrimide for 10 min followed by 0.2% $HgCl_2$ for 5 min and thoroughly rinsed 4-5 times with sterile distilled water and germinated aseptically on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 0.3% sucrose under cool white fluorescent light at 25±2°C. The morphogenic potential of leaf explants of 10, 15 and 20 days old seedlings were analyzed on different concentration of growth hormones viz., BAP, Kinetin and TDZ. For multiple shoot bud induction about 0.5 cm^2 section from petiolar end of the leaf was cultured on MS media supplemented with variable concentration of growth hormones. The pH of all media was adjusted to 5.8 before adding agar and sterilized by autoclaving at 1.08 kg cm^2 for 20 min. Cultures were incubated under light intensity of 1500l ux with 16 h light and 8 hs dark period at 25±20°C. For each experimental set up 10 explants were used with each concentration and experiment was repeated twice. After each successive subculture within 15 days, the well developed shoots were rooted on MS media augmented with three different concentration i.e., 0.1, 0.2 and 0.3 mg L^{-1} of three growth regulators namely NAA, IAA and IBA. Finally few plants of each cultivar were successfully acclimatized to soil.

RESULTS AND DISCUSSION

In vitro regeneration via organogenesis has been extensively attempted as compared to somatic embryogenesis in pigeon pea using explants like apical meristem, undifferentiated callus, leaves, hypocotyls, cotyledons, cotyledonary nodes, epicotyls and embryonal axes for more than 51 diverse genotypes/cultivars (Krishna *et al*., 2010). Efforts have been made for developing *in vitro* regeneration protocol via organogenesis using leaf and leaf petiole as explants for more than 17 cultivars of pigeon pea (Eapen and George 1993; Eapen *et al*., 1998; Geetha *et al*., 1998; Singh *et al*., 2002; Dayal *et al*., 2003; De Villiers *et al*., 2008).

In the present study eleven cultivars of pigeon pea not attempted for regeneration studies earlier (Krishna *et al*., 2010) were assessed for multiple shoot bud induction and regeneration using leaf explants under the influence of different cytokinins namely 6-Benzylaminopurine (BAP), Kinetin (KIN) and Thidiazuron (TDZ) cultured on Murashige and Skoog (MS) media. The leaf explants obtained from 10 days old *in vitro* germinated seedlings were found to be better for multiple shoot bud induction on MS media supplemented with different concentration of BAP, KIN and TDZ. *In vitro* regeneration using leaf lamina as explants with leaves from 5-12 -day-old seedlings has been reported (Eapen and George, 1993; Geetha *et al*., 1998).

The response of eleven cultivars for multiple shoot bud induction on MS media supplemented with variable concentration of BAP ranging from 0.5 to 4.0 mg L^{-1} revealed callus of diverse nature and morphology for most of the cultivars (Table 1). Only four cultivars namely IPA-2013, Pusa-9, T-7 and IPA- 98-3 showed appreciable multiple shoot bud induction (Fig. 1a-d). In case of cultivars IPA-2013 and T-7 higher concentration of BAP i.e., 3.0 mg L^{-1} was found to be effective for multiple shoot bud induction resulting with 6 and 5 buds per explants, respectively. In case of Pusa-9 and IPA- 98-3 a total of 6 buds per explants were obtained in 2.0 mg L^{-1} of BAP. Other cultivars did not responded for multiple shoot bud induction in variable concentration of BAP. Among different hormones used BAP has been reported to be most effective for *in vitro* regeneration of pigeon pea (Geetha *et al*., 1998; George and Eapen 1994). BAP alone has been used for regeneration via organogenesis using leaf explants of cultivars ICPL 93115 (Yadav and Padmaja, 2003) and one for unknown cultivar (Kumar *et al*., 1983). The response of these cultivars for multiple shoot bud induction in the presence of variable concentration of KIN ranging from 0.5 to 4.0 mg L^{-1} was very poor. *In vitro* regeneration via organogenesis of leaf explants is not reported in the presence of KIN alone though combination of BAP and KIN has been preferred by several workers (Tyagi *et al*., 2001; De Villiers *et al*., 2008).

The response of these eleven cultivars for multiple shoot bud induction in the presence of variable concentration of TDZ ranging from 0.05-0.4 mg L^{-1} is shown in Table 1. Only one cultivar IPA-2013 showed multiple shoot bud induction resulting in 6 buds per explants in the presence of TDZ at 0.05 mg L^{-1} while other cultivars revealed callus formation with variable morphology and nature (Table 1). TDZ-induced shoot regeneration in pigeon pea cultivars ICPL 161, ICPL 88039, UPS 120 has been reported (Eapen *et al*., 1998).

Multiple shoot buds obtained from leaf explants were subjected to rooting in full strength MS basal medium supplemented with three different hormones viz., NAA, IAA and IBA at three different concentrations namely 0.1, 0.2 and 0.3 mg L^{-1}. NAA was found to be best for inducing rooting in multiple shootlets derived from leaf explants. The rooting response at different concentration of NAA for the cultivars IPA-2013, Pusa-9, T-7 and

Table 1: Multiple shoot bud induction, callus morphology from leaf explants of 10 days *invitro* germinated seedlings cultured on MS media supplemented with different concentration of BAP and TDZ after 8 weeks of culture

	Number of shoots (Mean±SD) at different concentration of BAP (mg L^{-1})							
Cultivars	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
IPA-2013	NR	NR	YWCC	YWCC	4.0±0.0	5.0±0.0	3.5±0.5	NR
IPA-3088	WGCC	GCC	GCC	GCC	BCC	BCC	BCC	BCC
Pusa-9	NR	NR	NR	2.7±1.3	WCC	WCC	WCC	WCC
IPA-34	NR	YWFC	YWFC	WCC	WCC	WGFC	WCC	WCC
IPA-204	NR	WCC	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC
IPA-242	NR	NR	WGCC	BCC	GCC	GCC	WGCC	WGCC
T-7	YBCC	YBCC	WGCC	WGCC	3.5±0.5	4.5±0.5	1.4±0.4	WGCC
IPA-61	NR	NR	GCC	GCC	YGCC	YBCC	YBCC	YBCC
IPA-337	NR	NR	NR	YGCC	YGCC	BCC	BCC	BCC
IPA-341	NR	NR	YWCC	YWCC	YGFC	YGFC	YWCC	YWCC
IPA-98-3	NR	3.3±1.7	NR	5.0±1.0	NR	YGCC	YGCC	NR
	Number of shoots (Mean±SD) at different concentration of NAA (mg L^{-1})							
Cultivars	0.05	0.1	0.15	0.20	0.25	0.30	0.35	0.40
IPA-2013	2.7±0.5	NR	NR	NR	NR	NR	NR	NR
IPA-3088	NR	NR	NR	NR	NR	NR	NR	NR
Pusa-9	NR	NR	NR	NR	YWCC	NR	NR	YWCC
IPA-34	NR	WGCC	WGCC	WGCC	NR	NR	NR	WGCC
IPA-204	NR	NR	YGCC	WGCC	WGCC	YGCC	YGCC	YGCC
IPA-242	NR	WGFC	WGFC	WGFC	WGFC	WGFC	WGFC	WGFC
T-7	NR	NR	NR	WGCC	WGCC	NR	NR	NR
IPA-61	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC
IPA-337	NR	NR	NR	WGCC	YBCC	YBCC	WGCC	WGCC
IPA-341	NR	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC	WGCC
IPA-98-3	YGCC	NR	YGCC	YGCC	NR	YGCC	YGCC	YGCC

NR: No response, YWCC: Yellowish white compact callus, WGCC: Whitish green compact callus, GCC: Green compact callus, BCC: Brown compact callus, WCC: White compact callus, YWFC: Yellowish white friable callus, WGFC: Whitish green friable callus, YBCC: Yellowish brown compact callus, YGCC : Yellowish green compact callus, YGFC: Yellowish green friable callus

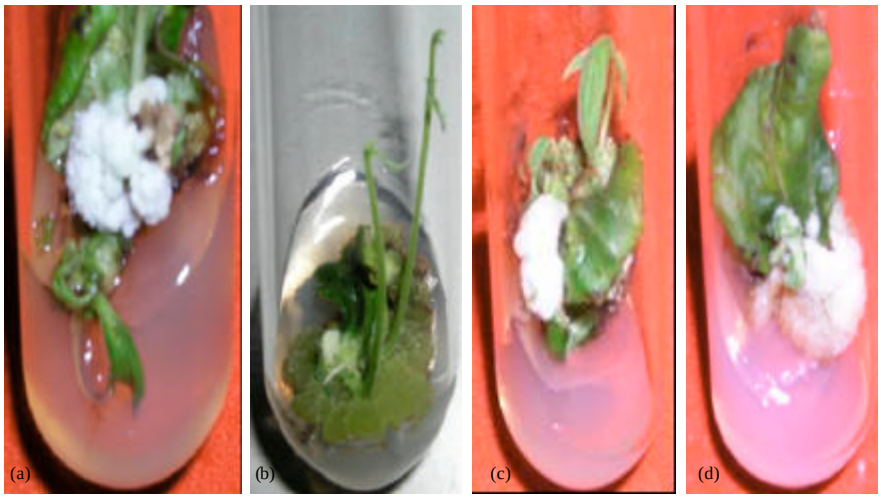

Fig.1(a-d): Multiple shoot bud induction from leaf explants of different cultivars of *Cajanus cajan* on MS media supplemented with different concentration of BAP (a) IPA2013 (3.0 mg L^{-1}), (b) Pusa-9 (2.0 mg L^{-1}) and (c) T-7 in (3.0 mg L^{-1}) and (d) IPA-98-3 (2.0 mg L^{-1})

IPA- 98-3 showing efficient multiple shoot bud induction on MS media supplemented with BAP and TDZ is shown in Fig. 2a-d, respectively. Cultivar IPA-2013 showing efficient multiple shoot bud induction in the presence of BAP and TDZ revealed efficient rooting with 0.1 mg L^{-1} NAA (Table 2). Efficient rooting of multiple shoot bud induction from leaf explants in the presence of IBA, NAA and IAA has been reported (Srinivasan *et al*., 2004).

The rooted plantlets were hardened in small pots and transferred to the soil for acclimatization. The percentage

Table 2: Rooting responses of *in- vitro* regenerated multiple shoot buds from leaf explants of four cultivars of pigeonpea at different concentration of NAA recorded after 4 weeks of culture

		NAA (0.1 mg L^{-1})		(NAA 0.2 mg L^{-1})		(NAA 0.3 mg L^{-1})
		Number of primary roots		Number of primary roots		Number of primary roots
Cultivars	% of rooting	Mean±SD	% of rooting	Mean±SD	% of rooting	Mean±SD
IPA-2013	100	8.7±0.6	70	1.4±0.9	NR	NR
Pusa-9	100	1.0±0.0	100	1.0±0.0	NR	NR
T-7	100	2.0±0.0	90	4.5±1.5	80	3.1±1.6
IPA-98-3	100	2.8±0.4	100	1.9±0.5	NR	NR

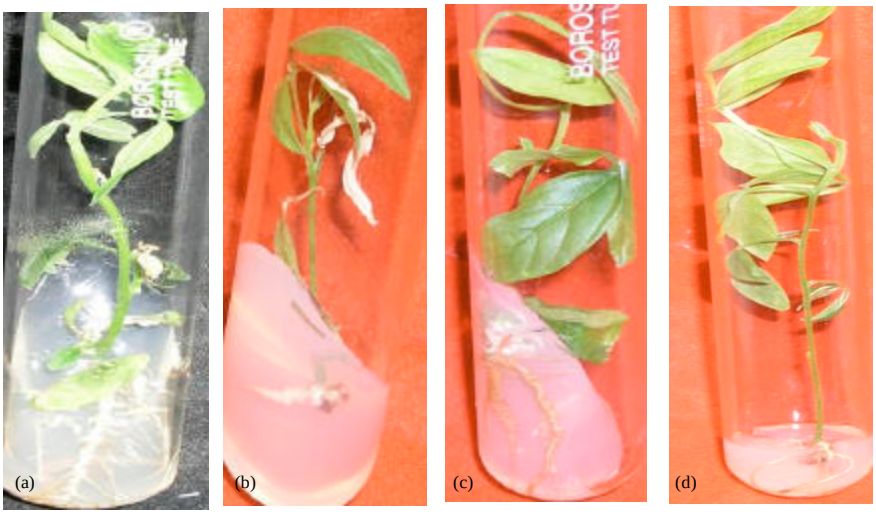

Fig. 2(a-d): Rooting response for multiple shoot buds from leaf explants of different cultivars of *Cajanus cajan* on MS media supplemented with different concentration of NAA (a) IPA-2013 (0.1 mg L^{-1}), (b) Pusa-9 (0.1 mg L^{-1}), (c) T-7 (0.2 mg L^{-1}) and (d) IPA-98-3 (0.1 mg L^{-1})

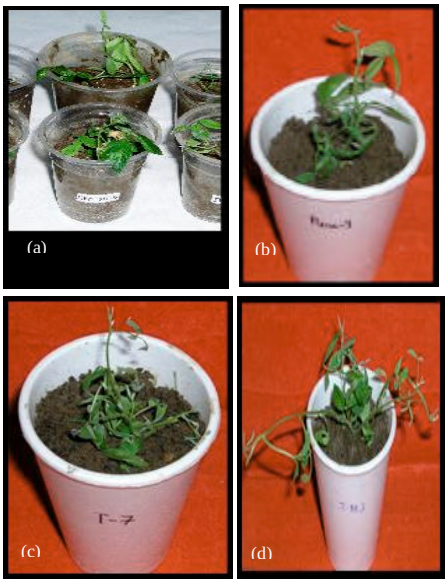

Fig. 3(a-d): Acclimatized plants of the cultivars (a) IPA 2013, (b) Pusa-a, (c) T-7 and (d) IPA-98-3

acclimatization ranged from 25 to 50% with cultivar IPA-2013 showing best response. The acclimatized plants of cultivars IPA2013, Pusa-9, T-7 and IPA-98-3 are shown in Fig. 3a-d, respectively.

CONCLUSION

The response of eleven cultivars not attempted earlier for regeneration via organogenesis using leaf explants has been assessed for multiple shoot bud induction. The age of the explants donor seedling greatly influenced the differentiation of shoot buds with leaves from 10 days old seedling gave better response as compared to the leaves of 15, 20 and 25 days seedling explants. Further higher concentration of BAP (3.0 mg L^{-1}) and lower concentration of TDZ (0.05 mg L^{-1}) responded best for multiple shoot induction. NAA was found to be best for rooting of multiple shootlets derived from leaf explants as compared to IAA and IBA. Among the eleven cultivars studied the response of IPA-2013 for multiple shoot bud induction was best which could be further standardized

for developing efficient *in vitro* regeneration protocol via organogenesis.

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