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EFFICIENT SHOOT INDUCTION AND PLANT REGENERATION OF PIGEON PEA [*CAJANUS CAJAN* (L.) MILLISP.] ICPL 87 VARIETY USING LEAF PETIOLE EXPLANTS

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ABSTRACT: Pigeon pea or red gram is a high protein legume crop of semi-arid tropics and sub-tropics, which ranks sixth in production among the other legumes. It is used as a major source of protein (21%) in many countries like India. In the present study, an efficient *in vitro* regeneration protocol was developed for ICPL 87 variety of pigeon pea (*Cajanus cajan* L.) using leaf petiole explants. Leaf petiole explants of six day old seedlings were cultured on MS media supplemented with different concentrations of 6-Benzylaminopurine and α-napthaleneacetic acid for multiple shoot bud induction. MS media supplemented with 0.5 mg/l α-napthalene acetic acid + 2 mg/l 6-Benzylaminopurine hormonal concentrations induced highest number of shoot buds compared to other combinations. Induced multiple shoots were elongated on MS media fortified with 6-Benzylaminopurine, α-napthaleneacetic acid and Gibberellic acid hormonal combinations. Maximum number of shoots were elongated with hormonal combination of 1 mg/l 6-Benzylaminopurine + 0.2 mg/l α-napthaleneacetic acid + 3 mg/l Gibberellic acid. These well-elongated shoots were rooted on MS media enriched with different concentrations of Indole-3-butyric acid and maximum percentage of rooting was observed on MS media supplemented with 0.8 mg/l Indole-3-butyric acid concentration. Finally, these rooted plants were transferred to soil and vermiculate mixture in 1:1 ratio for hardening and acclimatization. The protocol standardized can be used for genetic transformation of pigeon pea to generate transgenic pigeon pea plants.

Key words: Pigeon pea, Organogenesis, Auxins, Cytokinins, Gibberellins

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INTRODUCTION

Pigeon pea (*Cajanus cajan* L., Mill sp.) is an important food legume crop (Family: Fabaceae) cultivated across 50 countries in Asia, Africa and the Americas. It ranks fifth in area after soybean, common bean, peanut and chickpea. Mainly, it is a tropical crop, which is cultivated as secondary crop or mixed crop with cereal grains like maize, millet and sorghum etc. It is an important source of protein (Singh et al., 1990), which caters to the protein requirement of the population of the Indian subcontinent. It contains 22.3 percent of protein content and the seeds contain important constituents like two globulins, cajanin and concojanin, which accounts for 58 percent and eight percent of the total nitrogen. It is the second most important food legume crop of India, which accounts for more than 80 % of the total world production. Over the past several years, production of pigeon pea had increased, but later the production and yield of pigeon pea was declined due to lack of better varieties, biotic, abiotic stresses and poor crop management. Out of these, cultivation of this crop is constrained mainly by several biotic and abiotic stresses, which are posing a big threat to its yield. Efforts to address this problem through conventional breeding methods are not completely successful due to non-availability of germplasm within the cross-compatible species. In this situation, regeneration of plants through tissue culture offers an efficient strategy to produce better varieties of pigeon pea through genetic transformation.

Due to its recalcitrance nature regeneration is not easy and sufficient numbers of standard protocols for regeneration of pigeon pea are not available for genetic transformation to produce better varieties. In the present study, an efficient protocol for *in vitro* regeneration of pigeon pea through multiple shoot induction was developed in high yielding; short duration variety ICPL 87 using leaf petiole explants.

MATERIALS & METHODS

Plant material and explant preparation

Seeds of pigeon pea variety ICPL 87 were taken from plant breeding Department of International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India. Mature and uniform seeds were selected and surface sterilized with 70 % ethanol (v) for 1 min followed by treatment with solution of 0.1% (w/v) $HgCl_2$ containing 1-2 drops of Tween-20 for 8 min. These seeds were rinsed thoroughly with sterile double distilled water for 4-5 times. Then the sterilized seeds were soaked in sterile double distilled water for 24 h and are germinated aseptically in sterile test tubes containing Murashige and Skoog (MS) media with 3 % (W/V) sucrose and 0.8 % (W/V) agar. The leaf petioles were excised from six-day-old germinated seedlings and were inoculated on MS media.

Shoot induction

Leaf petiole explants were taken from 6 day old germinated seedlings and inoculated on MS media with 6-Benzylaminopurine (1, 2 and 4 mg/l concentrations of BAP) and α-napthaleneacetic acid (0.1, 0.2, 0.3, 0.5 mg/l concentrations of NAA), where the abaxial surface of the leaf lamina is in contact with the medium. A minimum number of 50 explants were taken for each experiment and was repeated thrice. The explants were sub cultured on fresh medium with similar combination of plant growth hormones for every two weeks. The number of explants responded and the number of shoot buds proliferated were counted at regular intervals.

Shoot elongation & proliferation

Elongated and well-developed shoots were separated from the shoot clump and were inoculated on MS media containing various combinations of 6-Benzylaminopurine (BAP), α-napthaleneacetic acid (NAA) and gibberellic acid (GA3) for further elongation and proliferation of shoots. After two weeks, the shoots were sub-cultured on the fresh media by using similar combination of plant growth hormones.

Rooting and acclimatization

The elongated shoots more than 3 cm were transferred to MS and half MS media containing 3 % sucrose and various concentrations of Indole-3-butyric acid (IBA). After 2-3 weeks the rooted plantlets were transferred to small pots containing autoclaved soil and soilrite in 1:1 ratio and the plants were acclimatized in the green house.

RESULTS

The surface sterilized seeds inoculated on MS media showed 65-75 % germination, (Figure 1a) where as the seeds inoculated on sterile wet filter paper showed only 35-45% germination. Complete leaf along with the petiole excised from 6-day old *in vitro* germinated seedlings (Figure 1b) were taken for shoot induction. As plant growth regulators play a vital role in *in vitro* regeneration, different combinations and concentrations of 6-Benzylaminopurine (BAP) along with α-napthaleneacetic acid (NAA) were used for shoot induction. In the first week of inoculation, the size of leaf lamina was increased and petiolar region was bulged. From the bulged end of the leaf, shoot bud induction was initiated and after two weeks the multiple shoots were differentiated (Figure 1c). Among the other combinations, MS basal media containing 0.5 mg/l α-napthaleneacetic acid (NAA) + 2 mg/l 6-Benzylaminopurine (BAP) resulted in 76 % of shoot bud induction (Table 1, Figure 1d). After four weeks, the well-proliferated shoots were transferred to the shoot elongation medium containing different concentrations of (1-2 mg/l) 6-Benzylaminopurine (BAP) + (0.1-0.5 mg/l) α-napthaleneacetic acid (NAA) + (1-5 mg/l) Gibberellic acid (GA3). Out of different concentrations tested, maximum number of shoots were elongated on MS media supplemented with 1 mg/l 6-Benzylaminopurine (BAP) + 0.2 mg/l α-napthaleneacetic acid (NAA) + 3 mg/l Gibberellic acid (GA3) (Table 2). Within three weeks, the shoots were elongated up to 3 cm (Figure 1(e)) and the percentage of elongation was 78 %. These shoots were transferred to root induction medium containing different concentrations of Indole-3-butyric acid (IBA) for rooting and adventitious roots (Figure 1(f)) were developed within 3 weeks of sub culture. Out of different concentrations tested, MS media with 0.8 mg/l Indole-3-butyric acid (IBA) showed 75 % of rooting (Table 3). Then the *in vitro* raised plantlets with profusely developed roots were transferred to small pots containing autoclaved soil and soilrite mixed in 1:1 ratio (Figure 1(g)) for hardening followed by acclimatization.

Table-1: Effect of different concentrations of NAA and BAP on shoot bud induction.

Concentration of Plant growth regulators (mg/l)	Total no. of explants cultured	No. of explants producing shoot buds	Shoot bud induction frequency (%)
0.2 NAA + 1 BAP	50	12	24
0.3 NAA + 1 BAP	50	18	36
0.5 NAA + 1 BAP	50	22	44
0.2 NAA + 2 BAP	50	25	50
0.3 NAA + 2 BAP	50	32	64
0.5 NAA + 2 BAP	50	38	76
0.2 NAA + 4 BAP	50	4	8
0.3 NAA + 4 BAP	50	7	14
0.5 NAA + 4 BAP	50	9	18

Table-2: Effect of different plant growth regulators on shoot elongation.

Concentration of Plant growth regulators (mg/l)	No. of regenerated shoots kept for elongation	No. of shoots elongated	Shoot elongation frequency (%)
1 BAP + 0.2 NAA	18	9	50
1 BAP + 0.3 NAA	12	5	41
1 BAP + 0.5 NAA	9	3	33
1 BAP + 0.2 NAA + 2 GA3	32	22	68
1 BAP + 0.2 NAA + 3 GA3	38	29	78
1 BAP + 0.2 NAA + 4 GA3	25	14	56

Table-3: Effect of different concentrations of IBA on rooting.

Concentration of Plant growth regulators (mg/l)	No. of elongated shoots kept for rooting	No. of shoots producing roots	Rooting frequency (%)
0.2 IBA	9	4	44
0.4 IBA	14	8	57
0.6 IBA	22	15	68
0.8 IBA	32	24	75

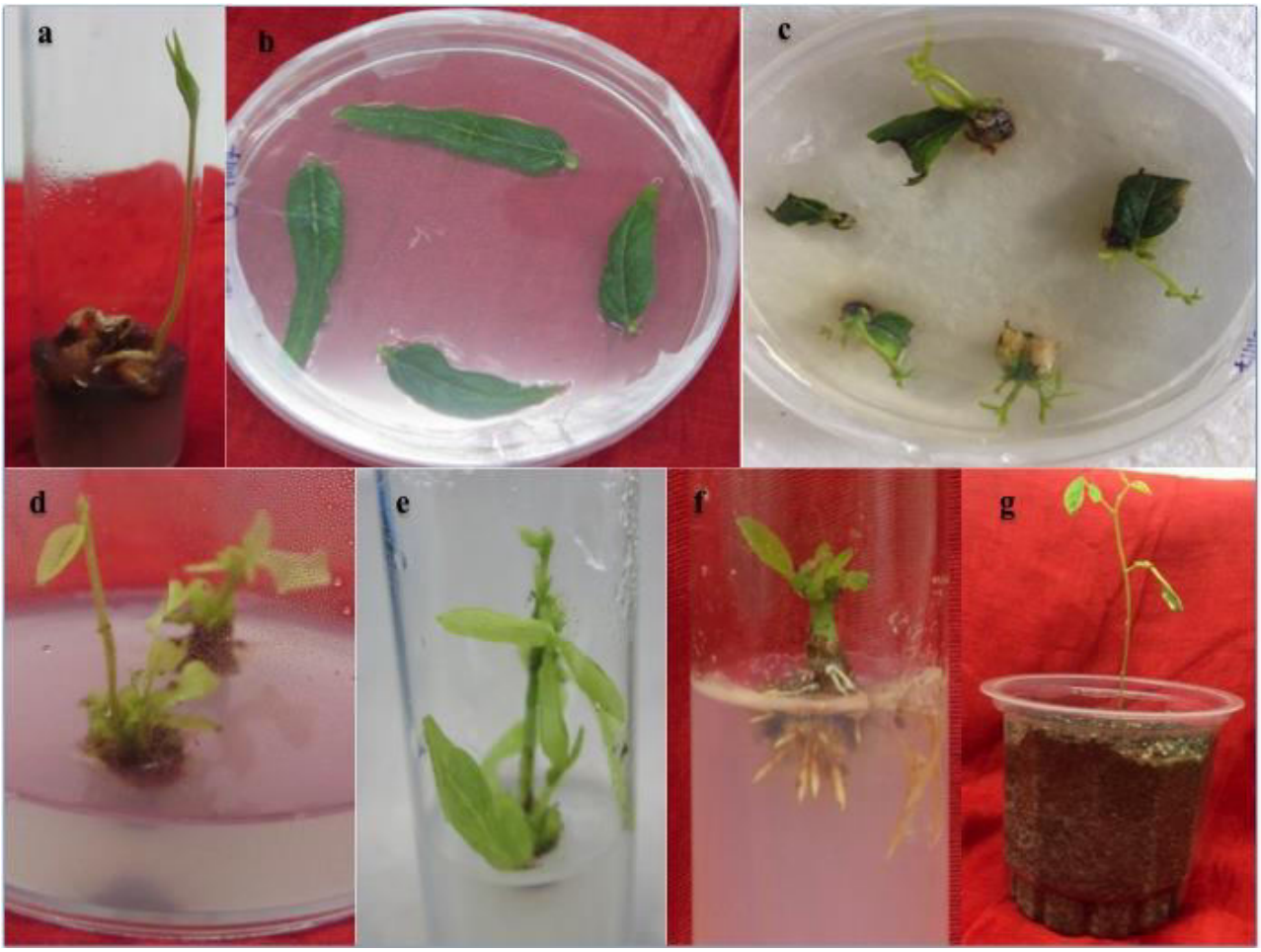

Fig.1 *In vitro* shoot bud induction and plant regeneration of ICPL 87 variety of pigeon pea. a) Three day old germinated seedlings of ICPL 87 pigeon pea variety on MS media. b) Six day old leaf petiole explants inoculated on Shoot Induction Media. c) Shoot bud induction from the petiolar end on Shoot Induction Media d) Multiple shoot regeneration & proliferation on Shoot Induction Media. e) Well elongated shoots on Shoot Elongation Media. f) Shoots showing profused rooting on Root Induction Media. g) Well rooted plants hardened in soil and soilrite mixture.

DISCUSSION

Selection of an explant is an important factor for direct organogenesis to achieve high percentage of shoot bud induction in pigeon pea regeneration. In ICPL 87 variety, leaf petiole was used as an explant for direct organogenesis to produce more number of shoots. Explants cultured on plain MS medium did not respond due to the lack of growth hormones. In our experiment, we have noticed that the age of the explant plays an important role in shoot induction, where the regeneration efficiency decreases with the increase of the age of the explant. For better shoot bud induction and regeneration, BAP was reported as an important growth hormone along with NAA compared to other growth regulators (Geetha et al.,) 1998. The regeneration frequency increased with the addition of 6-Benzylaminopurine (BAP) and α-napthalene acetic acid (NAA). In our experiment 76 % of shoot bud induction was noticed in MS media supplemented with 2 mg/l 6-Benzylaminopurine (BAP) + 0.5 mg/l α-napthaleneacetic acid (NAA). The percentage of shoot induction was low in the case of explants inoculated on the media composed of other concentrations of 6-Benzylaminopurine (BAP) and α-napthaleneacetic acid (NAA). Mehta and Mohan Ram (1980) reported direct shoot regeneration from the cotyledonary surface by using BAP. When high concentrations of BAP were used, callus formation was observed, which is similar to the results reported by Srinivasan et al. (2004). Earlier reports on pigeon pea regeneration specified that the low concentrations of BAP favored the development of shoot buds from the pre-existing meristems (Shiva Prakash et al., 1994; Ignacimuthu et al., 1997; Eapen and George, 1993; George and Eapen, 1994; Mohan and Krishnamurthy, 1998; Geetha et al., 1998; Shiva Prakash et al., 1994), which is compatible with our results.

The regeneration of pigeon pea shoot buds was reported from various explants such as leaf (Yadav and Padmaja, 2003), distal cotyledonary segments (Mohan and Krishnamurthy, 1998), leaves (Eapen and George, 1993; Eapen et al., 1998; Geetha et al., 1998), cotyledonary node (Shiva Prakash et al., 1994; Geetha et al., 1998), apical and axillary meristems (Franklin et al., 1998) and shoot tips (Geetha et al., 1999). In case of shoot bud regeneration from primary leaves, only 36 % of the callus responded (Eapen and George, 1993). Eapen et al. 1998 [8] reported that frequency of shoot bud regeneration was delayed with primary leaf explants. Shoot regeneration using leaf explants was observed with an intervening callus stage where the percentage of shoot induction was low (Asande et al. 2016). Around 81 % of explants showed regeneration in case of in *vitro* regeneration from cotyledonary explants, but the number of shoots induced per explant and the percentage of conversion of shoot buds to fully developed shoots was less in number (Raghavendra and Sudhakar, 2014). Vijay Kumar et al. 2016 reported that regeneration potential varies among different varieties of pigeon pea based on plant regeneration studies from axillary buds and the same was reported earlier by various researchers (Eapen et al., 1998; Geetha et al., 1998; Mohan and Krishnamurthy, 2002; Majumdar and Baneerjee, 2004; Aboshama, 2011; Krishna et al., 2011; Vandana et al., 2011; Vijay Kumar et al., 2016). Some researchers reported that regeneration is also possible from cotyledonary node explants cultured on charcoal containing media (Kaur et al., 2012).

Shoot elongation depends on the various factors like substitution of plant growth regulators (Mohamed et al., 1991; Nagi et al., 1997), alteration in media composition (Malik and Saxena, 1992; Shiva Prakash et al., 1994) and changes in light conditions. Gaurav Krishna et al. 2011 reported that the use of high concentration of BAP would reduce the percentage of shoot elongation. MS media comprising BAP and IAA or BAP alone (1.0 mg/l) failed to elongate shoots within the same variety (George and Eapen, 1994). Out of different concentrations tested, 78 % elongation was noticed using 1 mg/l 6-Benzylaminopurine (BAP) + 0.2 mg/l α-napthaleneacetic acid (NAA) + 3 mg/l Gibberellic acid (GA3). When the same concentration of BAP used for induction was used for elongation, shoot clumps were formed, which failed to elongate further. On the other hand, use of low concentrations of BAP did not result in yellowing and leaf fall.

When the elongated shoots were transferred to half MS media for rooting, leaf fall was noticed due to lack of sufficient nutrients for the growth. So, we used full MS media fortified with different concentrations of IBA for rooting. Out of different concentrations tested, MS media with 0.8 mg/l Indole-3-butyric acid (IBA) showed 75 % of rooting. The well-rooted plants were hardened initially in culture room by transferring to pots containing autoclaved soil and soilrite in 1:1 ratio and the plants were finally acclimatized in green house.

CONCLUSION

In the present study, an effective and reliable in vitro regeneration protocol for ICPL 87 pigeon pea variety through direct organogenesis was standardized, which can be used further to produce transgenic pigeon pea plants through genetic transformation.

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