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MICROPROPAGATION OF *PONGAMIA PINNATA*- A SUSTAINABLE BIOFUEL PLANT SPECIES FOR SEMI ARID CLIMATIC CONDITIONS

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ABSTRACT
In vitro technique has been standardized successfully for clonal propagation of identified elite Pongamia tree using leaf and axillary buds. Role of 2, 4- Dichlorophenoxy acetic acid (2, 4-D), Benzylaminopurine (BAP), Kinetin (Kin) at different concentrations is studied in combination with Murashige and Skoog's (MS) media for callus initiation. The higher concentrations of BAP, Kin cause an increase in shoot induction, 42 % and 59 % of shoots were developed from leaf and auxiliary buds grown in 13.28 µM BAP with 9.24 µM Kin. On an average, 15.7 shoots were grown using axillary buds in media supplemented with 13.28 µM BAP and 16.26 µM Adenine sulphate (AdS). Prolonged supplementation of 13.28 µM BAP in combination with 9.1 µM Thidiazuron (TDZ) for 8-10 weeks results in shoot elongation of about 8.3 cm, the shoots were sub-cultured on MS media supplemented with 9.8 µM Indoyl butyric acid (IBA) showed 64 % and 59% of rooting from leaf and axillary buds respectively. The standardized protocols will be helpful for mass propagation of identified elite Pongamia species in afforestation program especially in semiarid conditions, genetic engineering research on plant transformation and over expression studies to improve oil synthesis in seeds.

KEYWORDS: Axillary buds, Biodiesel plant, Leaf, Micropropagation, Pongamia, Plant growth regulators, Plant tissue culture.

INTRODUCTION

Pongamia pinnata L. Pierre, commonly known as karanja or honge, is a medium-sized glabrous, leguminous tree species indigenous to Indian subcontinent and also found in South-East Asia and has successfully introduced in other parts of the world. Pongamia is an out breeding species, thus seed populations exhibit high levels of heterogeneity. Pongamia seed contain non-edible oil (30-40 %) used for tanning leather, making soap, treatment of various ailments (Meera *et al*., 2003) and also as an illuminating oil in some parts of rural India. There has been an expansion of Pongamia plantations in India within the last few years, mainly for its non-edible oil for biodiesel production and it has impressive growth performance in tropical, arid and semi arid climatic conditions. Pongamia can help in restoration of soil fertility especially in degraded soils owing to its nitrogen fixing ability. To increase the biodiesel production, it is important to have unlimited feedstock with high oil yielding seeds. Thus, there is a crucial need to develop efficient methods for mass production of improved quality planting stocks identified by tree breeding method. The large scale plantation of clonal stocks of elite genotype needs to be done to encourage a forestation program. Tree breeding can be done both by sexual and asexual means, sexual method of propagation through seeds and it has limitation that the seedlings raised do not fully resemble the mother plant and often fails to transmit the essential superior qualities or plus traits of a mother plant to the young ones. Further, there are chances that seeds exhibit dormancy or they have poor viability and long regeneration time before attaining maturity (Somashekar and Sharma, 2002). On contrary, in asexual or vegetative mode of propagation through vegetative parts such as leaf, stem cuttings, the progeny always resembles the mother plant in all respects and helps in maintaining the quality of elite plants which exhibit special characters or plus qualities in the progeny (Henrique *et al*., 2006). However, commercial scale planting of Pongamia is hampered by several factors: (1) shortage of elite planting material, (2) low viability of the seeds, (3) insufficient seed germination due to fungal contamination during their storage, (4) seedling susceptibility to *Rhizoctonia hiemalis* leading to premature defoliation, blight and retarded growth and (5) presence of hard seed coat that reduces germination capability (Edwards and Naithani, 1999). The major application of plant tissue culture lies in the production of true-to-type high quality planting material that can be multiplied under aseptic conditions irrespective of seasonal variations and weather conditions. Protocols for micropropagation of Pongamia from seedling derived axillary meristem (Sugla *et al*., 2007; Shrivastava and Kant, 2010), de novo organogenesis (Sujatha *et al*., 2008) and mature tree derived nodal meristem (Sujatha and Hazra, 2006) are available. However, there are no reports on *in vitro* plant regeneration from leaf or axillary buds explants. Adenine sulphate is a form of adenine used as additive in plant tissue culture media to stimulate cell growth and enhance shoot formation (Pierik, 1987). The response of regeneration and proliferation increases with the addition of Adenine sulphate (AdS) and Thidiazuron (TDZ) to pre-culturing media induces multiple shoots and regeneration of whole plant from a leaf or axillary buds as explants. It is also reported that adenine alters the level of endogenous phytohormones and results in regeneration (Thorpe, 1994). Adenine sulphate in combination with

other cytokinin favor shoot formation (Skoog and Miller, 1957), especially in combination with BAP is effective in multiple shoot regeneration in many species such as *A. nilotica* and *A. Senegal* (Venkadesan *et al*., 2002). TDZ is identified as the most active cytokines substance for woody plant tissue culture; it is more efficient for shoot elongation in woody plants (Sujatha *et al*., 2008). Before the discovery of TDZ as a plant growth regulator, the requirement of extremely low concentrations of TDZ against high concentrations of other cytokinins to stimulate shoot proliferation was not known (Huetteman and Preece, 1993). The activity of TDZ varies depending on the concentration, exposure period, cultured exposing and plant species (Murthy *et al*., 1988). TDZ has been found to be effective at very low concentrations for micropropagation of several species (Lu, 1993). However, it has been used at higher concentrations for micro propagation of some forest tree species. Moreover, continuous supply of planting material throughout the season demands needs development of mass multiplication techniques. Regeneration protocols of Pongamia will find application in clonal propagation for mass propagation, germplasm preservation and development of transgenic. Genetic engineering and plant transformation technology can be applied to enhance the fatty acid profile, silencing of the genes responsible for the production of toxins pongamol and karanjin in seeds, efficient regeneration system preferably from explants amicable for Agrobacterium mediated transformation.

MATERIALS & METHODS

Selection and sterilization of explants

Identified elite Pongamia trees were selected from North Karnataka region based on evaluation through bioproductivity (Patil *et al*., 2015), morphological (Patil and Naik, 2016), biochemical (Patil and Naik, 2015, 2015a) and molecular (Patil *et al*., 2016) marker studies. Young healthy leaves and axillary buds from identified elite Pongamia trees were selected for clonal propagation.

The explants (leaves and axillary buds) were collected and surface sterilized by washing thoroughly under running tap water for 30 min with 0.1 % (v/v) aqueous solution of teepol and rinsed three to five times with distilled water to reduce the level of microbes. The explants were then surface sterilized with 0.2 % w/v aqueous $HgCl_2$ solution for 5 min and rinse with sterile distilled water, followed by 70 % ethanol wash for 2 min and finally rinsed in distilled water. The explants were cut into appropriate sizes of 1 cm^2 (leaf) and 1 cm (axillary buds) using a sterile forceps in a laminar air flow and inoculated in culture bottles containing 60 ml of solid Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with 3 % sucrose, pH 5.8±0.2 and 0.8 % w/v agar was added before autoclaving at 121°C for 20 min. The cultures were incubated at 25 ±2°C under a 16 hr photoperiod with 16 µE $m^{-2}s^{-1}$ photon flux density provided by cool white fluorescent tubes.

Induction of callus and shoots from explants

The MS media was supplemented with various concentrations of 2,4-D (0.5 and 1.0 mg/l), BAP (0.5, 1, 2 and 3 mg/l) and Kin (0.5, 1, 2 and 3 mg/l); 2.4-D were treated alone and also with different concentrations of BAP and Kin for callus initiation. The induced callus was co-cultured on MS media supplemented with plant growth regulators (PGR's) at different concentrations including BAP (1, 2, 3 and 5 mg/l), Kin (1, 2, 3 and 5 mg/l) for proliferation of shoots. The proliferated shoots were initially observed at 3mg/l of BAP, thus a same concentration of BAP was used in combination with different concentrations of Adenine Sulphate (1, 2 and 3 mg/l) for shoot elongation and 3 mg/l BAP with different concentrations of TDZ (0.5, 1 and 2 mg/l) for multiple shoot induction. The shoots were given two passages of 15 days. The sprouting frequency, shoot length and number of shoots per explants were studied for every 2 weeks of culture. Ten replicates were used per treatment and all the experiments were repeated in thrice.

Rooting and strengthening of shoots

The elongated shoots (4-5 cm) were excised from clusters and cultured individually on half strength MS medium supplemented with different concentrations of IAA (0.5, 1 and 2 mg/l), IBA (0.5, 1 and 2 mg/l) and NAA (0.5, 1 and 2 mg/l). The number of roots and root length were noted after every three days. Plantlets with open leaves and roots (3-4 cm in length) were transferred to a mixture of autoclaved sand and soil (1:1). Plantlets were hardened at 25±2 ^{o}C for 4 week in 70-80 % humidity and 24 hr photoperiod. The propagules raised were transferred to pots in greenhouse.

Statistical data analysis

All the experiments were repeated in thrice with ten replicates; mean, standard error of mean and variance were calculated by analysis of variance (ANOVA). The differences among the treatment means were tested using Duncan multiple range test (DMRT) at 5 % probability level ($P<0.05$).

RESULTS

Initiation of callus formation

The explants leaf and axillary bud were inoculated on MS media supplemented with different concentrations of 2,4-D separately and in combination with different cytokines like Kin and BAP for good callus initiation. There was no significant variation in response of explants with low concentrations of 2,4-D, but low concentrations of BAP and Kin in combination with 2,4-D initiated the callus formation. Low concentrations of BAP (2.22 µM) in combination with 2,4-D (4.52 µM) increased the response of callus initiation in leaf explants, but low concentration of Kin (2.32µM) have a moderate response for leaf explants and good response from axillary buds. The response of BAP and Kin for both the explants were same at low concentrations, however the leaf explants didn't respond and only few axillary buds respond in control medium (MS media) as shown in Fig. 1 (A, B).

The response time for callus initiation in MS media with 2,4-D was observed higher of about 20-24 days, but the explants when grown in media with 2,4-D and in combination with BAP and Kin responded within 8 days of inoculation, induced callus from leaf and axillary bud is depicted in Fig. 1 (C, D and E). Increase in callus formation was observed with increased concentration of BAP and Kin in combination with 4.52 µM 2,4-D. The highest response to callus initiation was at 4.52 µM 2,4-D in combination with 13.28 µM BAP were 95 % of the leaf

explants and 64 % of axillary buds induced callus as depicted in Table 1. However, it was also observed that the increase in BAP or Kin beyond this concentration has decreased response of callus initiation.

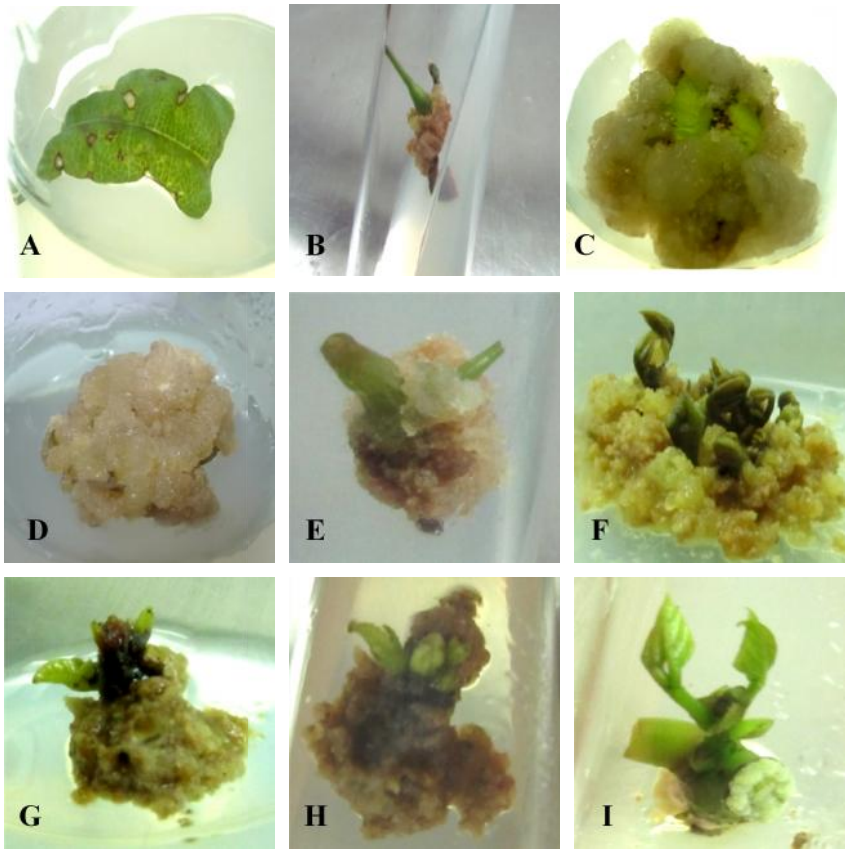

FIGURE 1. Micropropagation of Pongamia (A) leaf explant growth on MS media (control), (B) Axillary bud explants grown on MS media (Control), (C and D) Callus induced from leaf explants, (E) Callus induced from axillary bud explant, (F and G) Sprouting of shoots from callus induced from leaf explants at 13.28 µM BAP + 9.24 µM Kin, (H and I) Sprouting of shoots from callus induced from axillary bud at 13.28 µM BAP + 9.2 µM Kin.

TABLE 1: Effect of different growth hormones on induction of callus from leaf and axillary buds explants of *Pongamia pinnata*

Media formulation	% of explants response	
	Leaf	Axillary bud
Control (MS)	0.0^{a}	11^{a}
MS + 2.22 µM 2,4-D	07^{b}	12^{a}
MS + 4.52 µM 2,4-D	17^{c}	24^{b}
MS + 4.52 µM 2,4-D + 2.22 µM BAP	38^{d}	29^{c}
MS + 4.52 µM 2,4-D + 4.52 µM BAP	64^{e}	38^{d}
MS + 4.52 µM 2,4-D + 8.04 µM BAP	78^{f}	41^{d}
MS + 4.52 µM 2,4-D + 13.28 µM BAP	95^{g}	64^{e}
MS + 4.52 µM 2,4-D + 2.32 µM Kin	18^{c}	21^{b}
MS + 4.52 µM 2,4-D + 4.65 µM Kin	22^{c}	15^{a}
MS + 4.52 µM 2,4-D + 8.24 µM Kin	37^{d}	20^{b}
MS + 4.52 µM 2,4-D + 12.84 µM Kin	57^{e}	62^{e}

Effect of plant growth regulators on proliferation of multiple shoots

A 6-8 weeks old callus was transferred to shoot inducing medium containing various concentrations and combinations of BAP and Kin and observed sprouting of shoots after 3-4 weeks. BAP is reported to have favored axillary buds for proliferation of shoots as compared to leaf explants; incorporation of a cytokinin in the media greatly affected the differentiation of shoot proliferation from axillary bud induced callus. Increase in shoot development was observed with increasing concentration of cytokinins (BAP or Kin). The shoots proliferate on media supplemented with 13.28 µM BAP, so the shoots were sub-cultured on medium containing the same concentrations of BAP (13.28 µM) and different concentrations of AdS and TDZ for shoot elongation and for multiple shooting, this was repeated at 4 week interval. The frequency of regeneration and number of shoots from explants was maximum of 42 % and 59 % in MS medium supplemented with 13.28 µM BAP and 9.24 µM Kin which induce the highest shoot regeneration frequency,

number of shoots per explants (3.7±0.53) and shoot length (2.83±0.51) as depicted in Table 2.

Induction of multiple shoots was observed in media with 13.28 µM BAP and different concentrations of AdS, long term supplementation of AdS and TDZ also increased the shoot length (7.6± 0.15 cm) and number of shoots per explants (15.7± 0.1) at 16.26 µM AdS. However, the average number of shoots (12±0.46) and average shoot length (8.3±0.19 cm) in MS media with 13.28 µM BAP+9.1 µM TDZ is depicted in Table 3.

TABLE 2: Effect of different cytokinins on proliferation of shoot from callus induced from leaf and axillary buds of *Pongamia pinnata*

Concentration of hormones	% of shoot development		Average number of buds/ explants		Average shoot length (mm)	
	Leaf	Axillary bud	Leaf	Axillary bud	Leaf	Axillary bud
Control (MS)	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
MS + 4.45 µM BAP	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
MS + 8.84 µM BAP	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
MS + 13.28 µM BAP	08^{b}	11^{b}	1 ± 0^{b}	2.0 ± 0.4^{b}	0.68 ± 0.1^{b}	1.24 ± 0.64^{b}
MS + 22.12 µM BAP	15^{c}	14^{b}	1 ± 0^{b}	2.7 ± 0.23^{b}	0.73 ± 0.1^{b}	0.88 ± 0.14^{b}
MS + 4.6 µM Kin	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
MS + 9.24 µM Kin	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
MS + 13.88 µM Kin	8^{b}	12^{b}	1 ± 0^{b}	2.6 ± 0.14^{b}	0.74 ± 0.1^{b}	1.85 ± 0.32^{b}
MS + 23.12 µM Kin	9^{b}	16^{b}	1 ± 0^{b}	1.8 ± 0.29^{b}	0.81 ± 0.02^{c}	1.24 ± 0.43^{b}
MS + 13.28 µM BAP + 0.46 µM Kin	18^{c}	21^{c}	1 ± 0^{b}	2.1 ± 0.34^{c}	0.94 ± 0.1^{d}	1.43 ± 0.17^{b}
MS + 13.28 µM BAP + 2.32 µM Kin	23^{c}	43^{d}	1 ± 0^{b}	3.7 ± 0.23^{d}	1.1 ± 0.05^{e}	1.68 ± 0.15^{bc}
MS + 13.28 µM BAP + 4.6 µM Kin	37^{de}	47^{d}	1 ± 0^{b}	1.2 ± 0.27^{b}	1.4 ± 0.26^{fg}	2.83 ± 0.51^{c}
MS + 13.28 µM BAP + 9.24 µM Kin	42^{e}	59^{e}	1 ± 0^{b}	1.4 ± 0.63^{b}	1.3 ± 0.4^{f}	2.67 ± 0.19^{c}

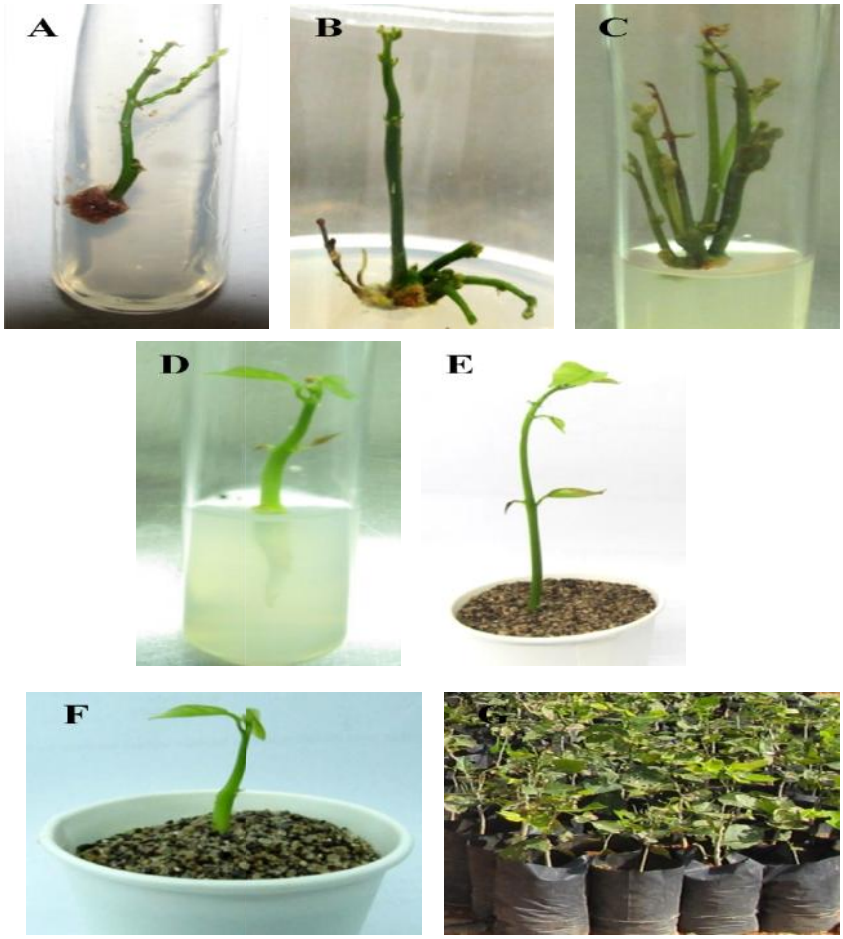

FIGURE 2: (A) Elongation of shoots from the cluster of buds in media containing13.28 µM BAP+ 16.26 AdS, (B and C) Induction of multiple shoots in media containing 13.28 µM BAP+ 9.1 µM TDZ, (D) Rooting of shoots grown in media with 9.82 µM IBA, (E and F) Hardening of plantlets in sterilized sand and soil (1:1) mixture, (G) Transfer of plantlet to greenhouse after acclimatization.

TABLE 3: Effect of Adenine sulphate and Thidiazuron on multiple shoot induction and shoots elongation regenerated from leaf and axillary bud of *Pongamia pinnata*

Concentration of hormones	% of shoot development		Average number of shoots/ explants		Average shoot length (cm)	
	Leaf	Axillary buds	Leaf	Axillary buds	Leaf	Axillary buds
Control (MS)	0^{a}	0^{a}	0^{a}	0a	0a	0^{a}
MS+13.28 µM BAP+ 5.42 µM AdS	08^{b}	11^{b}	5 ± 0.72^{b}	9.3 ± 0.2^{b}	1.0 ± 0.13^{b}	2.1 ± 0.38^{b}
MS+13.28 µM BAP+ 10.84 µM AdS	13^{c}	16^{c}	9 ± 0.53^{e}	13.3 ± 0.13^{c}	1.3 ± 0.56^{b}	4.7 ± 1.53^{c}
MS+13.28 µM BAP+ 16.26 µM AdS	17^{d}	23^{d}	12 ± 0.46^{e}	15.7 ± 0.1^{d}	2.0 ± 0.4^{b}	7.6 ± 0.15^{de}
MS +13.28 µM BAP+ 2.27 µM TDZ	11^{c}	16^{c}	3 ± 0.42^{c}	9.5 ± 0.05^{b}	1.2 ± 0.21^{b}	1.9 ± 0.52^{b}
MS + 13.28 µM BAP +4.55 µM TDZ	25^{e}	39^{e}	7 ± 0.83^{d}	10.3 ± 0.36^{bc}	1.5 ± 0.43^{b}	5.3 ± 1.37^{cd}
MS +13.28 µM BAP+ 9.1 µM TDZ	41^{f}	53^{f}	10 ± 0.46^{f}	12.7 ± 0.21^{c}	2.6 ± 0.14^{c}	8.3 ± 0.19^{e}

Rooting and hardening of elongated shoots:

The elongated shoots were transferred for rooting in half strength MS media containing different concentrations of auxins (IAA, IBA and NAA), initiation of roots was observed after 15-18 days and the root inducing hormones showed good results on rooting of elongated shoots, increased concentration of IAA, IBA and NAA increased the rooting efficiency (Table 4). However, the maximum rooting efficiency was observed in media supplemented with 9.82 µM IBA, 64 % and 59 % from leaf and axillary buds explants derived plants respectively (Fig. 2 (D)). Well rooted plantlets were washed and transplanted into plastic pots containing sterilized soil and sand (1:1) for acclimatization established in a greenhouse. The plant were gradually exposed to low humidity conditions and finally transferred for field trails.

TABLE 4: Effect of different concentration of IAA, IBA and NAA in half strength MS media on rooting of proliferated shoots of *Pongamia pinnata*

Concentration of hormones	% of rooting		Number of roots/shoot	
	Leaf	Axillary bud	Leaf	Axillary bud
Control (MS)	0.00	0.00	0.00^{a}	0.00^{a}
MS+2.85 µM IAA	13	18	1.1^{b}	1.3^{b}
MS+5.71 µM IAA	18	25	1.4^{b}	2.2^{b}
MS+11.41 µM IAA	26	48	1.5^{b}	1.8^{b}
MS+2.46 µM IBA	31	38	1.1^{b}	1.7^{b}
MS+4.93 µM IBA	58	53	2.8^{d}	2.8^{bc}
MS+9.82 µM IBA	64	59	2.3^{c}	2.1^{b}
MS+ 2.7 µM NAA	25	34	1.3^{b}	1.7^{b}
MS+ 5.4 µM NAA	37	49	1.4^{b}	1.9^{b}
MS+ 10.83 µM NAA	39	41	2.1^{c}	1.5^{b}

DISCUSSION

The presence of plant growth hormones initiated the changes in *Pongamia* leaf and axillary buds explants. The presence of low concentration of 2,4-D initiated minor changes in leaf and axillary buds, however, in control (MS) media the explants was unchanged for *Pongamia* leaf explants even after 6-8 weeks of incubation. The MS media supplemented with 2,4-D and different concentrations of BAP and Kin, the explants leaf (94 %) and axillary bud (64 %) initiated to respond for callus formation on 5^{th} day after incubation and callus growth was observed after 8 days of incubation. Similar kind of results was observed with 94 % of germination in decoated *Pongamia* seeds cultured on MS media supplemented with 1 µM gibberlic acid to single shoots in comparison with 28 % of regeneration on unsupplemented MS media; this demonstrates the beneficial effects of growth regulators for stimulation of growth in *Pongamia* explants (Sugla *et al*., 2007). The callus didn't proliferate into shoot in control (MS) media or with low concentration of BAP or Kin, but the combination of BAP and Kin with varying concentrations however initiated proliferation of shoots and BAP was found to be more effective than Kinetin. Sugla *et al*., 2007 also observed the changes in *Pongamia* cotyledonary nodes, when incubated on basal media without growth regulators showed no shoot proliferation and incorporation of cytokinin in the medium greatly affected the induction of axillary shoot proliferation from cotyledonary nodes. Shoot development increased with increasing concentration of cytokinin (BAP or Kin) upto 7.5 µM; cotyledonary node cultures of *Pongamia* required a period of exposure to BAP to accumulate sufficient cytokinins to induce shoot bud proliferation. Similar kind of results was also observed in zygotic embryos of *Larix* by Kim *et al*., 1999 and in the central meristem of *Pongamia* by Sujatha and Hazra (2007), which differentiated into a single shoot using various growth hormones like BAP, Kin, Z and TDZ optimize sprouting and to induce multiple shoot development (Sujatha and Hazra, 2007). Failure of meristems to form shoots in media containing TDZ suggested that plant growth hormones even suppresses differentiation of the existing meristem in the node including Tamarind, where the presence of TDZ in seed germination medium suppressed differentiated apical meristem, but undulations circling the cotyledon nodes differentiated into caulogenic buds and subsequently into shoots upon withdrawn of TDZ (Mehta *et al*., 2004). The shoots differentiated in a lower concentration of TDZ without intervening callus formation and in higher concentrations, the frequency of response was reduced and the explants turned brown and necrotic (Kim *et al*., 1997). Proliferation of meristematic cells in

response to TDZ exposure was reported in peanut (Joshi et al. 2003) and cotyledon node meristem of tamerind (Mehta *et al*., 2005). It was also observed that higher concentrations of TDZ suggest increased morphogenic cell proliferation leading to emergence of more number of shoots in explants. In our case, multiple shoot induction was observed in medium containing 13.28 µM BAP and different concentrations of AdS, long term supplementation of AdS and TDZ also increased the shoot length (7.6± 0.15 cm) and number of shoots per explants (15.7± 0.1) at 16.26 µM AdS. The elongated shoots were transferred for rooting in half strength MS media containing different concentrations of auxins (IAA, IBA and NAA), initiation of roots occurred within 25-30 days and all the auxins show good results for rooting of elongated shoots, increase in concentration of IAA, IBA and NAA increased the percentage of rooting (Table 4). However, the maximum rooting percentage was observed in media containing 9.82 µM IBA, 64 % and 59 % of leaf and axillary buds explants derived plants respectively (Fig. 2 (D)). Well rooted plantlets were washed and transplanted into plastic pots containing sterilized soil and sand (1:1) were gradually exposed to low humidity conditions and finally transferred for field trails.

We were successful in developing an *in vitro* plant regeneration protocol, for the first time in Pongamia using leaf and axillary buds. The regeneration system can be adopted for mass propagation of elite quality plants and in genetic engineering, especially in Agrobacterium mediated transformation in this species.

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