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Research Article

TRI-DIRECTIONAL *IN VITRO* MULTIPLICATION ON A SINGLE MEDIUM OF *LEUCAENA LEUCOCEPHALA*, A MEDICINALLY IMPORTANT LEGUMINOUS TREE

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ABSTRACT
Cotyledonary nodes and shoot apices of *Leucaena leucocephala*, a multipurpose economically important leguminous tree were cultured on Murashige and Skoog's basal medium supplemented with various concentrations of N^6- benzyl aminopurine or kinetin (0.2 - 8 mg/l). Both explants showed a tri-directional multiplication on a single medium, viz primary shoot formation, secondary axillary branches and shoot buds on N^6- benzyl aminopurine or kinetin supplemented media. The formation of shoot buds was observed only at relatively higher level of cytokinin used. Shoot buds, produced at higher concentrations of either N^6- benzyl aminopurine or kinetin, when subcultured at lower level of either N^6- benzyl aminopurine or kinetin alone or in combination with naphthalene acetic acid or without plant growth regulators, developed leafy shoots. It was revealed that cotyledonary node was better explant than shoot apex in terms of direct shoot regeneration, axillary branching and shoot bud formation and N^6- benzyl aminopurine also proved its supremacy over kinetin.

Keywords: *Benzyl Aminopurine, Cotyledonary Nodes, Kinetin, Naphthalene Acetic Acid, Shoot Apex*

Abbreviations: ANOVA- analysis of variance, BAP- N^6- benzylaminopurine, DMRT- Duncan's multiple range test, Kn- Kinetin, MS- Murashige and Skoog's medium, NAA- α-naphthaleneacetic acid, PGR- Plant growth regulator, SE- standard error

INTRODUCTION
Plantation forestry is important to the world economy which provides renewable energy, fibre and timber as well as maintains and preserves the ecological balance with the control of soil erosion (Dijkman, 1950; Maity *et al.*, 2007; Banerjee, 2013). To ensure sustainable and equitable use of resources for meeting the basic needs of present and future, the best option is to practice massive tree plantation of forest species. Seed propagation has a number of constraints such as prolonged dormancy and poor viability (Venkateswaran and Gandhi, 1982). Thus large scale clonal propagation of superior clones along with accelerated tree improvement programs are necessary for successful reforestation and forest management programs. Propagation of tree species through in vitro techniques is regarded as a reliable means for rapid multiplication of such plants (Sreedevi and Pullaiah, 1999). Regeneration of forest trees in general and legumes in particular has been a difficult task as the seeds of such taxa are considered to be recalcitrant in nature (Ravishankar and Jagadishchandra, 1989).
Leucaena leucocephala is a fast growing leguminous tree offers enormous economic importance as a source of pulpwood. Apart from being a wood crop, it has good heating value and relieving the pressure on the consumption of fossil fuel (Negi *et al.*, 1995). The plant has been used by human beings since many centuries ago for herbal medicine. It has antidiabetic (Syamsudin *et al.*, 2010) and antinematicidal (Adekunle and Aderogba, 2008) activity. *Leucaena leucocephala* is an important forage legume of the tropics, used in animal nutrition as the leaves are rich in protein, minerals and vitamin content. However, it does contain the alkaloid mimosine which is toxic and may cause death if fed in excess, particularly to mono-gastric animals. Attempts have been made to develop transgenic *Leucaena leucocephala* with reduced amounts of mimosine (Jube and Borthakur, 2009, 2010; Negi *et al*., 2013). However,

development of an efficient and reproducible regeneration protocol is very important for the improvement programme of any plant by utilizing the power and potential of genetic engineering.

So far the literature review is concerned, *in vitro* studies of *Leucaena leucocephala* have been done by a number of workers (Ghosh and Bandyopadhyay, 1984; Dutta and Dutta, 1985; Hossain *et al.*, 1992; Maity *et al*., 2005; Pal *et al.*, 2012), a detailed morphogenetic study using different explants and different PGRs in relation to tri-directional *in vitro* multiplication on a single medium of *Leucaena leucocephala* have not been reported yet. The present investigation is therefore, undertaken to determine the optimal conditions for rapid *in vitro* regeneration of uniform plantlets from cotyledonary node and shoot apex of the elite germplasm which would be an effective measure for conservation.

MATERIALS AND METHODS

Seeds of *Leucaena leucocephala* were collected from elite trees growing in dry deciduous forest. The seeds were washed with 2% (w/v) detergent solution (Teepol) for 10 minutes, rinsed with 70% ethanol for 1 minute and surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride for 10 mins followed by thorough washing with sterilized distilled water.

The surface sterilized seeds were cultured on moist absorbent cotton bed in 250 ml Erlenmeyer flasks and incubated in the culture room for 10 days under 10 hours of photoperiod of 37.5 μ mol. $m^{-2}s^{-1}$ light intensity to develop axenic seedlings. Explants like cotyledonary nodes and shoot apices were excised from axenic seedlings of 10-12 days old and were aseptically cultured on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of N^6- benzylaminopurine (BAP) or kinetin (Kn) (0.2 - 8 mg L^{-1}). Shoot buds produced at higher concentrations of cytokinin were sub-cultured on MS solid media supplemented with lower levels of cytokinin (0.5 - 1 mg L^{-1}) alone or in combination with NAA (0.1 mg L^{-1}) or without PGR for subsequent leafy shoot emergence. The control set was devoid of any plant growth regulator. The pH of the media was adjusted to 5.7 using 1N HCl prior to addition of 0.8%w/v agar (BDH, India). Approximately 20 ml of medium was dispensed in 25×150 mm glass tubes capped with non-absorbent cotton. The cultures were sterilized at 121°C temperature in 1.06 Kg cm^{-2} pressure for 15 min.

The regenerated shoots were excised from the parent culture and transferred to half strength MS semi-solid medium with different concentrations of NAA (0.1 - 1 mg L^{-1}) for induction of roots. *In vitro* grown plants with well developed root systems were first washed under running tap water and transferred to plastic cups containing sterile sand-soil mixture (1:1) with adequate water. Surviving plants were finally transplanted to the soil on earthen pots. The process of transfer and gradual acclimatization to the natural conditions were stringently monitored.

The experimental units were assigned to "randomized complete block design" with single replicate per block. The mean values of different morphogenetic responses were shown along with their respective standard errors (SE) and were analyzed by analysis of variance (ANOVA). After obtaining a significant F value ($\alpha = 0.05$) the treatment means were separated by Duncan's Multiple Range Test (DMRT) in case of single Factor ANOVA only. Statistical analyses were performed according to Little and Hills (1978).

RESULTS AND DISCUSSION

The cotyledonary node explants and shoot apices of *Leucaena leucocephala* when cultured in presence of various concentrations of BAP or Kn, showed a tri-directional multiplication, viz primary shoot formation, secondary axillary branches and shoot buds (Table: 1 & 2). Direct regeneration of multiple shoots and the differentiation of shoot buds were monitored in cotyledonary node (Figure 1 A) and shoot apex (Figure 1 B) explants both on the PGR-free and PGR supplemented media.

In the PGR free control, both the explants did not show the production of secondary axillary branching and shoot bud formation. The response in terms of multiple shoot and shoot bud formation improved considerably with the application of either BAP or Kn. Prudent application of PGRs was necessary to achieve *de novo* differentiation of shoot buds and further development of plantlets. Therefore, it appears

that the inactiveness of explants in the control could be due to lack of adequate endogenous growth regulators (Roy and Banerjee, 2000).

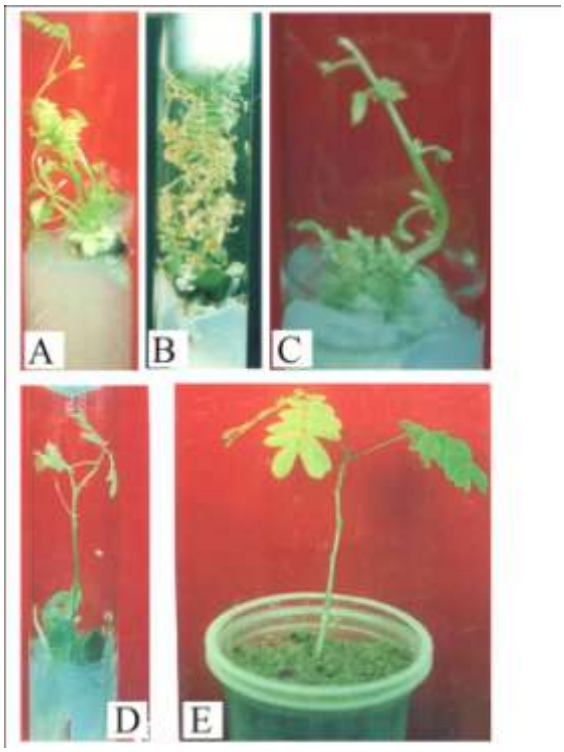

Figure 1: Figure 1 A- D: *In vitro* multiple shoot regeneration of *Leucaena leucocephala*
A. Multiple shoots from cotyledonary node, B. Multiple shoots from shoot apex, C. Leafy shoot emergence from shoot bud, D. Induction of roots on the regenerated plants, E. Plantlet established in pot

Direct regeneration of shoot buds has also been reported in a number of tree legumes viz., *Albizia lebbeck, Dalbergia paniculata, Leucaena leucocephala and Acacia auriculiformis using* different cytokinins at various levels (Raju *et al.*, 1984; Goh *et al.*, 1990; Maity *et al.*, 2005, 2007). Thus it was observed that exogenous PGRs were crucial for improved development of shoot buds as well as for plantlet formation which is in conformity with the observation of Banerjee *et al.*, (2007, 2011) in another leguminous species.

Although organ formation results from quantitative interactions between auxin and cytokinin (Skoog and Miller, 1957), the present investigation showed direct shoot regeneration in presence of cytokinin (BAP or Kn) which supports the observations of Gupta *et al.*, (2001) and Banerjee *et al.*, (2011).

In terms of primary shoot formation, the best response in the cotyledonary node explants has been achieved at 4 mg L^{-1} BAP (11.8 ± 0.55). Beyond 4 mg L^{-1} BAP the production of primary axillary shoots decreased. The number of secondary axillary shoots per explant was increased up to a certain level (4 mg L^{-1} BAP) and beyond this level of BAP exhibited an inhibitory effect. However, the pattern of formation of the primary and secondary axillary shoots observed in this study has striking similarity with the earlier work of Banerjee *et al.*, (2007) in a herbaceous legume, groundnut. It is further corroborated that BAP alone could induce multiple shoot formation (Banerjee *et al.*, 1999; Agarwal and Gupta, 1999; Banerjee *et al.*, 2007).

The formation of shoot buds was observed only at relatively higher concentration of BAP. The number of shoot buds (24.80 ± 0.80) per explant attained its peak at 8 mg L^{-1} BAP which is the maximum level used

in this experiment. The increase in the number of shoot bud generation was directly related to the level of BAP in the media. These multiplication of shoot bud took place both by adventitious as well as by axillary shoot bud proliferation. It is known that in an intact plant the apical bud exerts an inhibitory influence on axillary buds, preventing their development into leafy shoots (Street and Opik, 1986). Moreover, the adventitious production of multiple shoot buds could be directly controlled by the exogenous cytokinin concentration in other legumes like *Vigna radiata* (Gulati and Jaiwal, 1994) and in groundnut (Banerjee *et al.,* 2007).

A more or less similar trend has also been observed in the Kn supplemented media, the only difference which has been noticed was the magnitude of the response, which is slightly lower in Kn supplemented media compared to BAP supplemented. Apart from this, the response in terms of shoot bud production initiated at 1mg L^{-1} BAP which was 2 mg L^{-1} with Kn supplemented media (Table: 1 & 2). BAP proved better than KN in terms of induction of shoot bud and subsequent multiplication, which corroborated the results of Vishwanath and Jayanthi (1997), Gupta *et al.,* (2001), Banerjee *et al.,* (2011) and Banerjee (2013).

Table 1: Response of cotyledonary node of *Leucaena leucocephala* cultured on MS agar media supplemented with various concentrations of BAP after 30 days of incubation

BAP (mg L^{-1})	**Mean no. of primary shoots ± SE**	**Mean no. of secondary axillary branches ± SE**	**Mean no. of shoot buds ± SE**	**Mean Shoot length ± SE (mm)**
0	1.10 ± 0.36^{e}	0	0	8.20 ± 0.10^{e}
0.2	3.50 ± 0.42^{d}	1.3 ± 0.26^{d}	0	23.0 ± 0.42^{c}
0.5	4.60 ± 0.45^{c}	2.7 ± 0.33^{c}	0	27.3 ± 0.44^{b}
1	6.20 ± 0.38^{b}	3.3 ± 0.47^{b}	2.40 ± 0.62^{d}	37.4 ± 0.92^{a}
2	11.40 ± 0.60^{a}	4.0 ± 0.14^{a}	11.6 ± 0.50^{c}	33.5 ± 0.41^{a}
4	11.80 ± 0.55^{a}	4.3 ± 0.21^{a}	21.1 ± 1.15^{b}	32.9 ± 0.72^{b}
8	5.60 ± 0.45^{b}	1.8 ± 0.20^{c}	24.8 ± 0.80^{a}	13.0 ± 0.11^{d}
KN (mg L^{-1})				
0.2	2.50 ± 0.16^{c}	1.0 ± 0.00^{d}	0	20.20 ± 0.32^{d}
0.5	4.20 ± 0.32^{b}	1.4 ± 0.16^{c}	0	33.24 ± 0.45^{c}
1	5.60 ± 0.45^{b}	1.6 ± 0.16^{b}	0	41.56 ± 0.84^{a}
2	10.2 ± 0.64^{a}	2.0 ± 0.00^{a}	1.36 ± 0.42^{a}	39.25 ± 0.44^{b}
4	10.0 ± 0.36^{a}	2.2 ± 0.41^{a}	9.86 ± 0.58^{a}	34.22 ± 0.72^{c}
8	8.10 ± 0.73^{a}	1.1 ± 0.10^{c}	17.3 ± 1.45^{a}	23.22 ± 0.33^{d}

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT).

Table 2: Response of shoot apex of *Leucaena leucocephala* cultured on MS agar media supplemented with various concentrations of BAP after 30 days of incubation

BAP (mg L^{-1})	Mean no. of primary shoots ± SE	Mean no. of secondary axillary branches ± SE	Mean no. of shoot buds ± SE	Mean Shoot length ± SE (mm)
0	0.90 ± 0.23^{e}	0	0	15.6 ± 0.42^{d}
0.2	2.90 ± 0.37^{d}	0.9 ± 0.23^{d}	0	16.4 ± 0.44^{d}
0.5	4.20 ± 0.38^{c}	2.3 ± 0.21^{b}	0	31.8 ± 0.10^{b}
1	5.40 ± 0.45^{b}	2.5 ± 0.26^{b}	2.10 ± 0.22^{d}	38.0 ± 0.46^{a}
2	10.4 ± 0.68^{a}	2.8 ± 0.35^{a}	8.62 ± 0.45^{c}	32.6 ± 0.52^{b}
4	10.1 ± 0.38^{a}	3.3 ± 0.77^{a}	22.10 ± 2.15^{b}	26.4 ± 0.42^{c}
8	4.00 ± 0.36^{c}	1.6 ± 0.16^{c}	26.32 ± 0.44^{a}	11.5 ± 0.36^{e}
KN (mg L^{-1})				
0.2	2.70 ± 0.42^{d}	1.0 ± 0.00^{c}	0	21.00 ± 0.32^{d}
0.5	4.00 ± 0.73^{c}	2.2 ± 0.20^{b}	0	31.02 ± 0.34^{b}
1	5.20 ± 0.38^{b}	2.6 ± 0.30^{a}	0	40.22 ± 0.44^{a}
2	9.60 ± 0.44^{a}	3.1 ± 0.23^{a}	1.90 ± 0.36^{c}	38.28 ± 0.24^{a}
4	9.80 ± 0.49^{a}	2.1 ± 0.10^{b}	10.26 ± 0.44^{b}	26.04 ± 0.44^{c}
8	7.40 ± 0.54^{b}	2.0 ± 0.14^{b}	21.38 ± 1.66^{a}	22.90 ± 0.32^{c}

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT)

Table 3: Response of shoot buds of *Leucaena leucocephala* on MS agar media supplemented with different plant growth regulators after 30 days of incubation

Plant growth regulators (mg L^{-1})	Percentage of leafy shoot emergence ± SE	Shoot length (mm) ± SE	Number of leaves per shoot
0	57.10 ± 4.61^{c}	7.04 ± 0.41^{d}	1.64 ± 0.12^{c}
0.5 BAP	72.62 ± 2.59^{a}	9.25 ± 0.22^{b}	3.23 ± 0.21^{a}
1 BAP	51.14 ± 1.76^{d}	8.12 ± 0.32^{c}	2.34 ± 0.16^{b}
0.5 BAP + 0.1 NAA	61.11 ± 2.88^{b}	9.28 ± 0.24^{b}	2.82 ± 0.24^{a}
0.5 Kn	70.25 ± 4.31^{a}	10.15 ± 0.66^{ab}	2.04 ± 0.31^{b}
1 Kn	62.35 ± 3.54^{b}	10.92 ± 0.26^{a}	2.32 ± 0.44^{b}
0.5 Kn + 0.1 NAA	56.43 ± 3.29^{c}	11.45 ± 0.33^{a}	2.02 ± 0.14^{b}

Mean values followed by same letter are not significantly different at 0.05 levels (DMRT)

The mean primary shoot length observed in the PGR free control was 8.2 ± 0.1 mm and the length increased considerably with the addition of either BAP or Kn. The shoot elongation was recorded as 37.4 ± 0.92 and 41.56 ± 0.84 mm at 1mg L^{-1} BAP and 1 mg L^{-1} Kn respectively.

Further enhancement of cytokinin suppressed the shoot length considerably which is in conformity with the observation of Banerjee *et al.,* (2001 and 2007) in groundnut.

The results of shoot apex culture of *Leucaena leucocephala* in the presence of various levels of either BAP or Kn are shown in Table 2. The highest mean number of primary axillary shoot was 10.4 ± 0.68 and 9.8 ± 0.49 at BAP (2 mg L^{-1}) and Kn (4 mg L^{-1}) respectively. The number of secondary axillary branches was increased up to a certain level in both media supplemented with either BAP or Kn and beyond that level decreased. Maximum number of shoot buds was produced at the concentration of 8 mg L^{-1} either BAP or Kn.

The response of shoot apex culture of *Leucaena leucocephala* in presence of various concentrations of either BAP or Kn showed similar trend as in the cotyledonary node explant.

The differential response in the regeneration capacity of cotyledonary node and shoot apex might be due to the fact that cotyledonary node contained two preformed lateral shoot buds while the shoot apex contained a single apical bud. Such differential response could also be due to different levels of endogenous PGRs within the explants (Banerjee *et al.,* 2007). Levels of endogenous growth regulators in the explants are influenced by the duration of light, its quality and the intensity and also by the chemical environmental factors (Kefeli, 1978). Further, the effect of a particular PGR depended not only on the concentrations applied, but also on the presence of the other PGRs as well as the interaction with the endogenous growth regulators (Roy and Banerjee, 2000). Therefore, the regeneration frequency as well as the number of regenerated shoots per culture depended not only upon the concentration and type of cytokinin and the explant type used for the experiment but also on the various other physical and chemical factors. However, the number of axillary branch and shoot bud increased with the increase in the BAP level and very high concentration of BAP showed an inhibitory effect, which is in accordance with Badere *et al.,* (2002), Banerjee *et al.,* (2011) and Banerjee (2013).

Shoot buds, produced at higher concentrations of either BAP or Kn, when subcultured at lower level of cytokinin alone or in combination with NAA or without PGR, developed leafy shoots (Figure 1 C). It is evident that there was a distinct difference in response in terms of the leafy shoot emergence from tiny shoot buds in between the treatments of complete omission of cytokinin and presence of cytokinin (0.5-1.0 mg L^{-1}) either BAP or Kn alone or in combination with 0.1 mg L^{-1} NAA (Table: 3). Relatively higher level of cytokinin (1.0 mg L^{-1}) either BAP or Kn was not beneficial. The percentage of leafy shoot emergence in the basal medium without PGR was 57.10 ± 4.61 and at 0.5 mg L^{-1} BAP was 72.62 ± 2.59. The addition of NAA (0.1 mg L^{-1}) with BAP had no beneficial effect in leafy shoot emergence from the tiny shoot buds rather it proved inhibitory. Kn (0.5 mg L^{-1}) supplemented media also proved better than PGR free control in respect of leafy shoot emergence. The addition of NAA (0.1 mg L^{-1}) with Kn had also no beneficial effect likewise, rather inhibitory. Similar response was reported by Maity *et al.,* (2005). It is interesting to note that BAP clearly proved its supremacy over Kn which is in contrast to the observation of Banerjee *et al.,* (2011) in *Arachis hypogaea* L var AK 1224.

The presence of NAA was essential for the induction of roots (Figure 1 D) from the base of the excised *in vitro* grown shoots, which was initiated after 12-15 days of incubation. The frequency of root induction in 0.1 mg L^{-1}NAA was only 50% where as 100% root induction was observed in the presence of 0.5-1 mg L^{-1}NAA. It could be mentioned that the roots developed at the base of the isolated shoots did not turn into callus. Plantlets, successfully transferred to plastic pots (Figure 1 E) containing sand-soil mixture (1:1), showed 85.71% survival rate. Finally, the plantlets grew normally in the experimental garden.

In conclusion, the present investigation revealed that cotyledonary node is better explant than shoot apex in terms of direct shoot regeneration, axillary branching and shoot bud formation on a single medium. On the other hand BAP proved better than Kn in terms of the above response from cotyledonary nodes and shoot apex explants. The results of the present study of *in vitro* multiplication could be useful in

improving this medicinally important tree legume and effectively applied for conservation purposes and is important for future programs of genetic transformation of this species.

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