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M Gopalakrishna
Agri Biotech Foundation, PJTSAU
Campus, Hyderabad, Telangana,
India

Bindu Prathyusha
Agri Biotech Foundation, PJTSAU
Campus, Hyderabad, Telangana,
India

Vijaya Naresh Juturu
Heartfulness Tree Conservation
Centre, Kanha Shanti Vanam,
Hyderabad, Telangana, India

Corresponding Author:
M Gopalakrishna
Agri Biotech Foundation, PJTSAU
Campus, Hyderabad, Telangana,
India

Efficient *in vitro* regeneration of *Leucaena leucocephala* through direct organogenesis from cotyledonary node explants

M Gopalakrishna, Bindu Prathyusha and Vijaya Naresh Juturu

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Abstract

The present study reports a reliable regeneration protocol for the commercially important genotype K-8 using hypocotyl and cotyledonary node explants derived from four-day-old aseptically germinated seedlings. Hypocotyl explants cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzylaminopurine (BAP) or kinetin (Kn) produced friable callus but failed to regenerate shoots. In contrast, cotyledonary node explants showed direct organogenesis with 100% regeneration frequency on BAP-containing media. Maximum shoot multiplication (17.13 shoots per explant) was achieved on MS medium supplemented with 11.11 μ M BAP, while higher concentrations reduced shoot length and number. Kinetin induced fewer shoots compared to BAP. Efficient rooting (100%) was obtained on half-strength MS medium supplemented with 2.5 μ M indole-3-butyric acid (IBA). The regenerated plantlets were successfully acclimatized with a survival rate of approximately 90%. This protocol provides a simple and efficient regeneration system suitable for large-scale propagation and future genetic transformation studies in *L. leucocephala*.

Keywords: *Leucaena leucocephala*, *in vitro* regeneration, BAP, subabul

Introduction

Subabul (*Leucaena leucocephala*) is an important underutilized multipurpose tree legume widely used for fodder, fuelwood and pulp production. It is native to central America & Mexico and was introduced to most tropical areas worldwide. *Leucaena* foliage is especially useful as fodder for livestock animals because of its high protein (15-18%) values. *Leucaena* wood is used as a raw material for pulp and paper industry (Lopez *et al.*, 2008) [6]. Global paper and paperboard consumption touched almost 420 million tons in 2023, and it is expected to rise to over 476 million tons by 2032 (Statista, 2026) [13]. This growth is being led, in large part, by developing countries such as China and India. Demand for paper is ever increasing in India. *Leucaena* is a potential alternative to conventional source of pulp (Owofadeju and Onilude, 2005) [7]. However, it is relatively rich in lignin, which adversely affects the quality of paper produced (Casler *et al.*, 2002) [3]. Lignin removal during pulp processing is very expensive besides creating environment pollution. Developing transgenics with altered or reduced lignin will be a potential boost to paper and pulp industry (Rastogi and Dwivedi, 2006) [10]. However, there are only a few reports on the genetic transformation of these plants moreover they are not reproducible. Development of simple, reliable and reproducible regeneration methods for subsequent utilization in genetic transformation studies is the need of the hour. Hence, this present work was undertaken to refine and standardize an efficient regeneration protocol for a commercially popular variety 'K-8' of subabul.

Materials and Methods

Selection of explant

Hypocotyl and cotyledonary node explants prepared from 4-day-old aseptically grown seedlings (K-8 genotype) were used in this present study.

Seed surface sterilization

Few equal size seeds of K-8 genotype with good texture were selected and treated with 25% H₂SO₄ solution for 10 minutes to break the seed dormancy. Then seeds were washed 2-3 times with distilled water to remove the traces of H₂SO₄. Then the seeds were surface sterilized with 0.1% (w/v) Mercuric Chloride (HgCl₂) solution for 2 minutes and thoroughly washed 3-4 times with sterile distilled water to remove the traces of mercuric chloride. Then the seeds were kept for overnight (16 hr) soaking in sterile distilled water. The imbibed seeds were kept for germination on sterile wet cotton and incubated for 4-days under 12/12 hr light/dark photoperiod. These 4-day old aseptically germinated seedlings were used as source for hypocotyl and cotyledonary node explants.

Explants preparation and inoculation

Hypocotyl and cotyledonary node explants were prepared from 4-day-old seedlings aseptically germinated on sterile wet cotton. Hypocotyl explants (5 mm in size) were dissected by making two cuts at 2 mm and 5 mm distance below the node on embryonic axis. Cotyledonary node explants were prepared by making a cut at the proximal and distal end of the seedlings selecting only 2 mm above and 2 mm below of the cotyledonary node leading to the production of each explant comprising basal half of the two cotyledons on either side of the cotyledonary node. Both these explants were inoculated on to shoot bud induction media-Murashige & Skoog (MS) basal medium supplemented with 0.0, 2.22, 11.11 & 22.22 µM 6-BAP & kinetin and cultured for 30 days in growth racks with one subculture after 15 days on to the fresh media with same composition. After 30 days the cultures were transferred on to shoot proliferation media- MS medium supplemented with half reduced concentrations (of Initial concentrations) of BAP & kinetin (1.11, 5.55 & 11.11 µM) in combination with NAA (0.25 & 0.50 µM) and cultured for another 30 days. Percentage regeneration efficiency of explants in each treatment was recorded for every 3days interval for 30days whereas mean shoot length, shoot number was noted after 30d of inoculation.

In vitro rooting

For *in vitro* rooting the regenerated shoots of approximately 3-4 cm height were separated and kept for rooting on half strength MS medium supplemented with different concentrations of IBA (0, 2.5 and 5.0 µM) and cultured for four weeks in the growth racks. Cultures were examined for every 5d intervals to observe root initiation, percentage rooting response, and mean root number & root lengths were recorded after 30d of inoculation on rooting media.

Culture medium and conditions

MS medium with 3% sucrose was used for all the experiments. pH of the medium was adjusted to 5.8 ± 0.1 prior to the addition of 0.8% agar and autoclaved at 121 °C for 15 min at 15 psi pressure. The cultures were maintained at 25 ± 2 °C under a 12/12 h photo period with light provided by cool white fluorescent tubes at an intensity of 85 µmol photons m⁻²s⁻¹. All experiments were repeated three times with 15 replicates each time.

Hardening

Well rooted plantlets were washed carefully under running tap water to remove traces of agar. The plantlets were transferred in to the plastic cups containing soil: sand (1:1) mixture and covered with polypropylene covers to maintain humidity and

kept in growth racks for one week. After two weeks holes were made to the polythene bags to acclimatize the plants to the ambient environment. After 4 weeks polythene bags were removed and plantlets were transferred in to pots containing Soil: Sand: Farm yard manure (FYM) (10:4:4) and kept in the green house at 28±2 °C temperature with 50-60% relative humidity.

Results and Discussion

Seed surface sterilization and germination

The surface sterilised seeds were kept for germination on sterile wet cotton and the seedlings thus obtained were normal, healthy and greenish in colour. After 4 days of incubation, seedlings showed 100% germination (Fig-1).

Effect of BAP, kinetin (Kn) & NAA on hypocotyl explants

The type, combination and concentration of plant growth regulators used strongly influenced the callusing and regeneration potential of the explants of variety K-8 of subabul. Initial culturing of the hypocotyl explants (Fig 2a) on shoot bud induction medium resulted in enlargement of the explants twice to their original size by the seventh day. Callus was developed from two cut ends of the hypocotyls explants (Fig 2b). After 30 days of culture, friable creamish green callus was developed on MS medium supplemented with different concentrations (2.22, 11.11 & 22.22 µM) of BAP (Fig 2c) and Kn (Fig 2d). Control cultures showed very less amount of callus. BAP favoured formation of more amount of callus than kn. Further culturing on half reduced concentrations of BAP and Kn (1.11, 5.55 & 11.11 µM) and addition of lower concentrations of NAA (0.25 & 0.50 µM), calli become friable and necrotic (Fig 2). The size of the callus increased with the increase in NAA concentration. These calli developed on all combinations and concentrations of hormones failed to regenerate.

Effect of BAP and Kinetin on cotyledonary node explants

Cotyledonary node explants (Fig 3) when cultured on shoot bud induction medium containing different levels of BAP and Kn resulted in varied response (Table 1). Emergence of multiple shoot buds from axillary meristematic regions was observed by seventh day onwards from 100 percent cotyledonary node explants which were cultured on BAP (Fig 4a). Multiple shoot number was increased when the explants were transferred after 30 days on to MS medium containing half reduced concentrations of hormones. The explants cultured on MS medium supplemented with 11.11 µM BAP gave highest number (17.13) of multiple shoots when they were further cultured on MS medium containing half reduced concentration of BAP. Dhawan and Bhojwani (1985)^[4], Puthur *et al.*, (1998)^[8] and Sirisha *et al.*, (2008)^[15] also observed that BAP was best for shoot proliferation in Subabul. The efficacy of BAP to induce multiple shoots from explants is well documented in other tree legumes also (Sonia *et al.* 2000, Anis *et al.* 2005)^[12, 1]. Further increase in BAP concentration (22.22 µM) shoot number was decreased (13.6). Shoot length was decreased with the increase in BAP concentration (Table-1, Fig 4b). Similar type of results was observed in cultures on Kinetin. Among different concentrations of Kinetin examined, explants cultured on 11.11 µM Kinetin concentration produced highest number of shoots (3.63) per explants (Table-1). With the increase in kinetin concentration to 22.22 µM shoot number was decreased (3.33). In contrast to BAP, with the increase in kinetin concentration, shoot length was increased (Fig-5).



Fig 1: Surface sterilization and germination of subabul seed (K-8) on sterile wet cotton

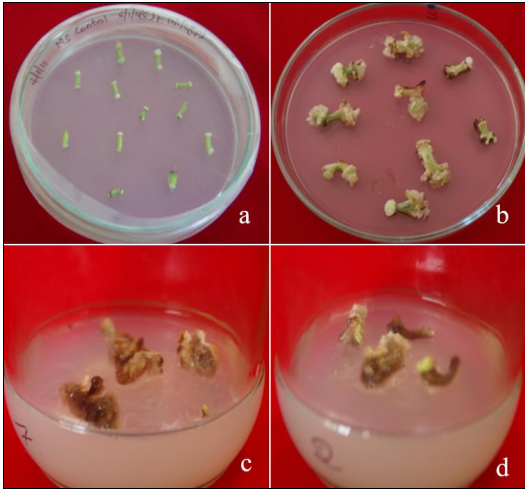


Fig 2: Hypocotyl explants inoculation (a), callus formation from both the cut ends (b) and non-regenerative friable callus formation on BAP (c) & Kinetin containing media

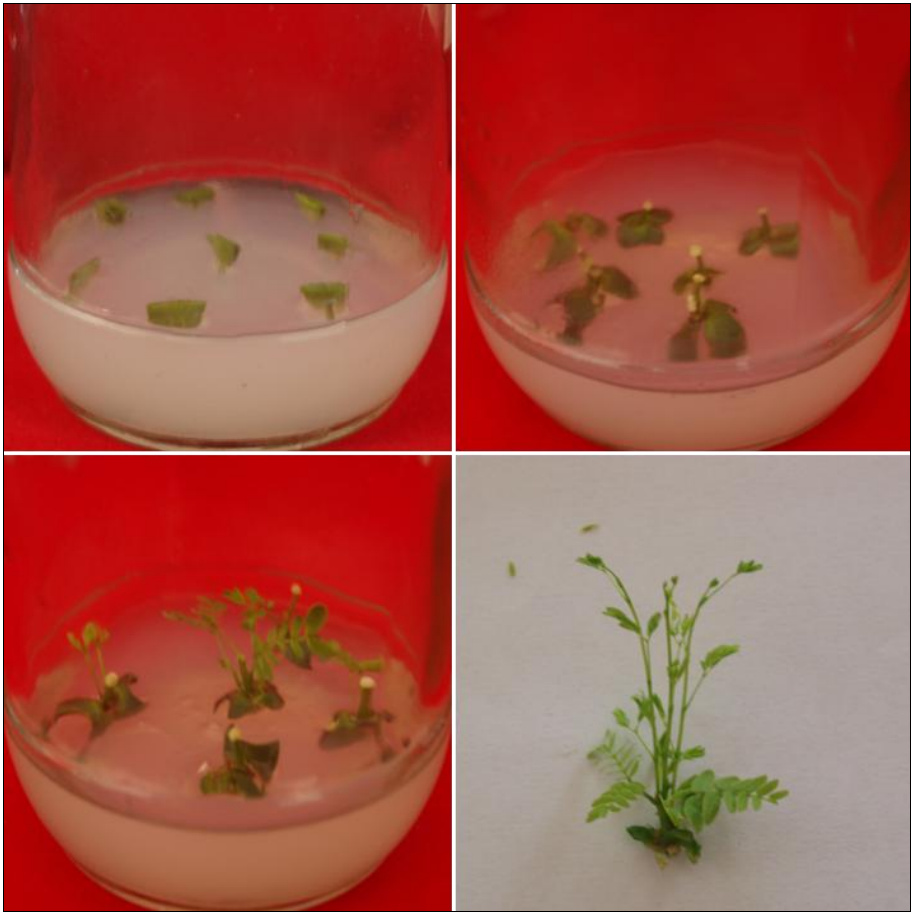


Fig 3: Different stages of direct organogenesis from cotyledonary node explants on MS medium supplemented with BAP and Kinetin

Table 1: Effect of different concentrations of BAP and Kinetin on% of regeneration, shoot number and shoot length from cotyledonary node explants of *Leucaena leucocephala*

Treatment(μ M)	% Regeneration frequency	Mean Shoot Number	Mean Shoot Length(cm)
MS	83.33%	1.88 $\pm$ 0.11	4.51 $\pm$ 0.57
MS+2.22 μ M BAP	100%	3.86 $\pm$ 0.17	4.17 $\pm$ 1.84
MS+11.11 μ M BAP	100%	17.13 $\pm$ 1.15	3.42 $\pm$ 0.51
MS+22.22 μ M BAP	100%	13.6 $\pm$ 0.72	3.39 $\pm$ 0.71
MS+2.22 μ M Kn	66.66%	2.16 $\pm$ 0.22	4.53 $\pm$ 0.26
MS+11.11 μ M Kn	50%	3.63 $\pm$ 0.13	6.65 $\pm$ 0.75
MS+22.22 μ M Kn	50%	3.33 $\pm$ 0.33	7.09 $\pm$ 2.57

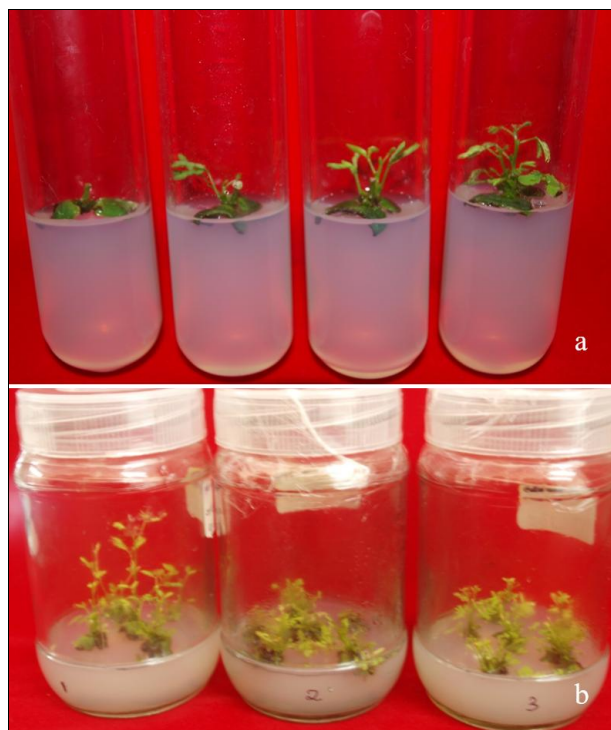


Fig 4: Cotyledonary node explants showing multiple shoot buds after 15 days of inoculation on MS + 0.0, 2.22, 11.11 & 22.22 µM BAP (a) and further proliferation half reduced concentrations of 1.11, 5.555 & 11.11µM BAP (b) after 30 days of culture respectively

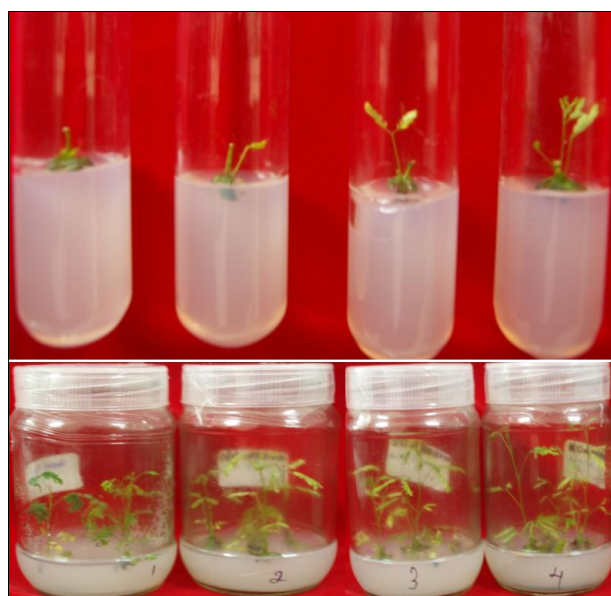


Fig 5: Cotyledonary node explants showing multiple shoot buds after 15 days of inoculation on MS + 0.0, 2.22, 11.11 & 22.22 µM Kn (a) and further proliferation half reduced concentrations of 0.0, 1.11, 5.555 & 11.11µM Kn (b) after 30 days of culture respectively

In vitro rooting

Low rooting response was observed on MS basal medium alone (33.3%). Addition of 2.5µM IBA hormone to the basal medium significantly improved the rooting response to 100% with 2.57 mean number of roots. Root initiation started after 10 days and within 21 days full-fledged roots were developed (Fig-6b). Increase in IBA concentration to 5.0 µM decreased the mean number of roots per shoot (0.77) with a rooting response below 85% (Table 3). Reduced strength of MS or B5 has been reported to favour rooting in case of other legumes also (Veltcheva and Svetleva 2005, Anis *et al.* 2005)^[14, 1]. The suitability of IBA for

improving the rooting frequency has also been suggested by Saradhi and Alia (1995)^[11], Puthur *et al.*, (1998)^[8] and Rastogi and Dwivedi (2003)^[9] in *Leucaena leucocephala*.

Table 3: Effect of different concentrations of IBA on% of rooting and Root number

Treatment(mg/l)	% of Rooting response	Mean Root Number
MS	33.33%	0.33±0.98
MS+2.5µM IBA	100%	2.57±0.15
MS+5.0µM IBA	84.42%	0.77±0.05

Acclimatization

Well rooted plantlets were gradually acclimatized for two weeks inside the culture room. High humidity was provided with polypropylene covers for the first 2 weeks and the humidity was gradually lowered by pricking hole to the covers in the 3rd & 4th weeks (Fig-6c). After 4 weeks the polypropylene covers were removed and plantlets were successfully established in soil in green house (Fig 6d). This procedure of hardening of plantlets ensured a survival rate of 90%. In contrast, Dhawan and Bhojwani (1985)^[4] reported a survival rate of only 40%. This low survival rate was a consequence of transplantation shock. In order to circumvent the problem of high mortality rate during transplantation, few procedures including *in vitro* nodulation by Rhizobium (Dhawan and Bhojwani 1987)^[5] and use of VAM fungi (Puthur *et al.* 1998)^[8] were reported. In contrast to these tedious and time consuming procedures, our procedure was simple and fast. Similar method was also followed by Bashir Khan *et al.* (2009)^[2].

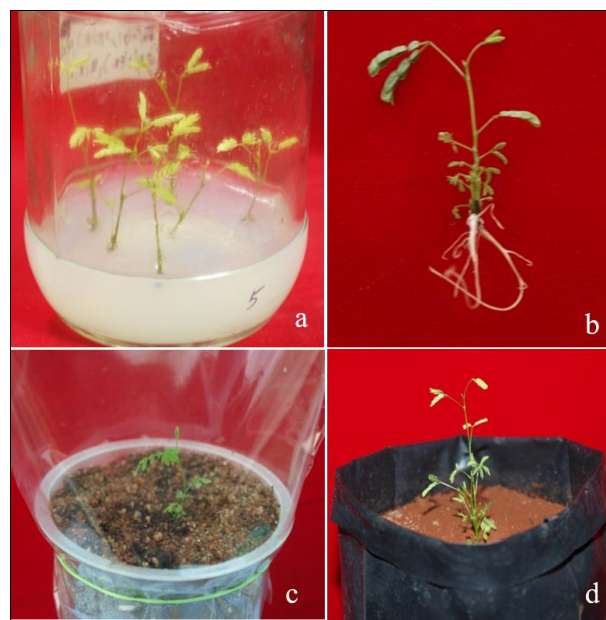


Fig 6: a) Isolated shoots kept for rooting on ½ MS + 0, 2.5 & 5.0 µM IBA, b) profuse rooting on ½ MS + 2.5 µM IBA, c) well rooted shoot kept for primary hardening, d) completely hardened plantlet in secondary hardening.

Conclusion

The present study successfully standardized an efficient *in vitro* regeneration protocol for subabul (*Leucaena leucocephala*) Genotype K-8 using cotyledonary node explants through axillary shoot proliferation. Among the two explants, cotyledonary node explants were found to be more efficient in producing multiple shoots. Hypocotyl explants resulted in only callusing and failed to regenerate even a single shoot. Highest shoot multiplication (17.3) was obtained from cotyledonary node explants on MS

medium supplemented with 11.11 μ M BAP (Table-2). BAP induced highly efficient multiple shoot development than Kinetin. Rooting of these elongated shoots was best in ½ MS medium supplemented with 2.5 μ M IBA. Present results proved that the addition of the cytokinin, BAP induced highly efficient multiple shoot development.

Thus, an efficient and reproducible *in vitro* regeneration system by direct organogenesis has been established for the tree legume *Leucaena leucocephala*. This system is very simple and could pave the way for genetic manipulation of the tree in order to acquire useful traits.

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