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BRIEF COMMUNICATION

In vitro regeneration of *Leucaena leucocephala* by organogenesis and somatic embryogenesis

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Abstract

In the present study, *in vitro* regeneration system for a recalcitrant woody tree legume, *Leucaena leucocephala* (cvs. K-8, K-29, K-68 and K-850) from mature tree derived nodal explants as well as seedling derived cotyledonary node explants was developed. Best shoot initiation and elongation was found on full-strength Murashige and Skoog (MS) medium supplemented with 3 % (m/v) sucrose, 100 mg dm^{-3} myoinositol, 100 mg dm^{-3} glutamine, 20.9 μM N^6-benzylamino-purine (BAP) and 5.37 μM 1-naphthalene acetic acid (NAA). Rooting was induced in half-strength MS medium containing 2 % (m/v) sucrose, 100 mg dm^{-3} myoinositol, 14.76 μM indole-3-butyric acid (IBA) and 0.23 μM kinetin. The cultivar K-29 gave the best response under *in vitro* conditions. Rooted plantlets were subjected to hardening and successfully transferred to greenhouse. Further, somatic embryogenesis from nodal explants of cv. K-29 *via* an intermittent callus phase was also established. Pronounced callusing was observed on full-strength MS medium containing 3 % (m/v) sucrose, 100 mg dm^{-3} myoinositol, 40.28 μM NAA and 12.24 μM BAP. These calli were transferred to induction medium and maximum number of globular shaped somatic embryos was achieved in full-strength MS medium fortified with 3 % (m/v) sucrose, 100 mg dm^{-3} myoinositol, 15.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5.0 μM BAP and 1.0 mM proline. Moreover, an increase in endogenous proline content up to 28th day of culture in induction medium was observed. These globular shaped somatic embryos matured in full-strength MS medium with 3 % (m/v) sucrose, 100 mg dm^{-3} myoinositol, 10.0 μM BAP, 2.5 to 5.0 μM IBA and 0.5 mM spermidine.

Additional key words: callus, root induction, shoot formation and multiplication, subabul, tissue culture, tree legume.

Leucaena leucocephala (*Fabaceae*), commonly known as subabul, is a commercially valuable multipurpose tree with tremendous applications as forage, raw material for pulp and paper industry, timber, firewood, fuel, gum, organic fertilizer and depilatory agent (Rastogi and Dwivedi 2003, 2008). The conventional methods of vegetative propagation for the tree are unsatisfactory owing to hard seed coat, low seed viability and long period between successive generations. Moreover, regeneration of forest trees in general and legumes in particular has been a formidable problem. Although attempts directed towards tissue culture of *L. leucocephala* have been made, of these only a few deal with micropropagation but with low survival rate, while others report only shoot regeneration or plantlet regeneration with a weak root system (for review see Mascarenhas and Muralidharan 1989, Rastogi and Dwivedi 2003). The present paper describes a simple, efficient and reproducible system for organogenesis and somatic embryogenesis in *L. leucocephala*.

Leucaena leucocephala (Lam.) de Wit cvs. K-8, K-29, K-68 and K-850 growing in departmental garden were used for explant procurement. The juvenile shoots

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Abbreviations: BAP - N^6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxy acetic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; NAA - 1-naphthalene acetic acid.
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of the mature trees were cut into single node segments containing axillary buds, without leaves and used as nodal explants. These explants were thoroughly washed and then treated with absolute alcohol for 5 s, 1 % (v/v) sodium hypochlorite for 15 min and 0.5 % (m/v) mercuric chloride for 5 min [0.1 % (m/v) for 5 min in case of K-68]. Extensive washing with sterile water followed each treatment. Surface disinfected explants were decapitated to remove proximal meristematic ends and used for plant regeneration or callus induction. For seedling derived cotyledonary node explants, seeds of *L. leucocephala* cv. K-8, K-29, K-68 and K-850 were soaked in boiling water for 5 min and surface sterilized as described for nodal explants of mature tree. Seeds were allowed to germinate aseptically for two days in sterile Petri plate with moistened filter paper at 29 ± 0.2 °C in dark. The cotyledonary nodes without cotyledons and radicles were used as explants for organogenesis. The culture conditions of 16-h photoperiod, 60 μmol m^{-2} s^{-1} irradiance, 55 % relative humidity, and 29 ± 0.2 °C temperature were maintained for all the stages of organogenesis and somatic embryogenesis.

For shoot regeneration, the nutrient medium consisted of major and minor salts as well as vitamins of full-strength Murashige and Skoog (1962; MS) medium, 100 mg dm^{-3} myoinositol, 3 % (m/v) sucrose and 0.8 % (m/v) agar (pH 5.7). The effects of cytokinins such as N^6-benzylaminopurine (BAP) and kinetin, auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) on shoot regeneration were investigated. The surface sterilized mature tree derived nodal explants and seedling derived cotyledonary node explants were placed on shoot regeneration medium. After 2 d of culture, the explants were subcultured on fresh medium. A proliferating shoot culture was established by repeated subculturing on shoot regeneration medium after each harvest of the newly formed shoots at 3-week intervals. Single shoots with 3 - 4 nodes were excised from the multiple shoot cultures and transferred in root induction medium comprising of half-strength MS medium (pH 5.7), 100 mg dm^{-3} myoinositol, 2 % (m/v) sucrose and 0.8 % (m/v) agar. The medium was supplemented with different combinations and concentrations of 2,4-dichlorophenoxy-acetic acid (2,4-D), IAA, IBA, NAA with BAP and kinetin. Roots were thoroughly washed to remove agar, immersed in sterile water for 2 d and the plantlets were subjected to acclimatization in culture room in pots containing soil and covered with polythene sheet having pinholes for air passage. After 2 weeks of hardening, the plants were transferred to soil in greenhouse, watered twice a day to field capacity and exposed to natural lighting.

For profuse callusing in different cultivars of *L. leucocephala*, surface sterilized mature tree derived nodal explants were implanted horizontally on callus induction medium, comprising of full-strength MS medium (pH 5.7) with 3 % sucrose, 100 mg dm^{-3} myoinositol, 0.8 % agar, varying combinations and concentrations of auxins (NAA and IAA) and cytokinins (BAP and kinetin). The explants were transferred to fresh medium after 2 d. The optimal concentrations of the plant growth regulators for callusing were standardized for different cultivars. Calli, once formed, were subcultured at 3-week intervals. The K-29 calli were induced for somatic embryogenesis in callus induction medium supplemented with proline and different concentrations of 2,4-D and BAP. The globular shaped somatic embryos were transferred after 4 weeks to maturation medium, which comprised of callus induction medium fortified with spermidine and different concentrations of BAP and IBA. Proline content was estimated during somatic embryogenesis according to the method of Bates *et al.* (1973) with slight modifications. Thus, 500 mg of callus tissue was homogenized in 10.0 cm^3 of 3 % (v/v) sulpho-salicylic acid under cold conditions. The homogenate was filtered through *Whatman No. 1* filter paper. To 2.0 cm^3 filtrate, 2.0 cm^3 12.5 % (m/v) solution of ninhydrin (in glacial acetic acid) was added and boiled for 1 h in boiling water bath. The solution was allowed to cool in ice followed by addition of 4.0 cm^3 toluene. The aqueous layer (red) was separated and proline was measured at 520 nm.

In the present study, we report an *in vitro* regeneration system *via* organogenesis from mature tree derived nodal explants and seedling derived cotyledonary node explants of *L. leucocephala* cvs. K-8, K-29, K-68 and K-850 under defined nutritional, hormonal and environmental conditions. Moreover, an efficient tissue culture system for callus induction in cv. K-29 and formation of somatic embryos is also reported. The mature tree derived nodal explants of *L. leucocephala* cv. K-29, callus formation and various stages of organogenesis are depicted in Fig. 1*A-F*.

For *in vitro* regeneration *via* organogenesis, the mature tree derived nodal explants as well as seedling derived cotyledonary node explants of all the four cultivars of *L. leucocephala* were implanted in shoot regeneration medium and the effects of various plant growth regulators on shoot proliferation were investigated. The percentage of explants that differentiated into shoots was the highest in sho regeneration medium containing BAP and NAA, while shoot formation was almost negligible in the presence of other combinations of plant growth regulators. The incorpo-ration of 20.9 μM BAP and 5.37 μM NAA in the full-strength MS medium promoted rapid and prolific shoot induction and multiplication in all the four cultivars of *L. leucocephala*. Thus, for cv. K-29, the number of responsive explants was found to be about 90 %. In this medium, shoot multiplication culminated in 3 - 4 weeks, when the shoots developed to a height of 4 - 6 cm. A combination of BAP and NAA at other concentrations led to differentiation of only 30 - 60 % explants. No shoot initiation was achieved on MS medium devoid of plant growth regulators. Similarly, Goyal *et al.* (1985) had reported shoot differentiation from lateral bud explants of mature trees of *L. leucocephala* cv. K-67 in the presence of 3.0 mg dm^{-3} BAP and 0.05 mg dm^{-3} NAA in the half-

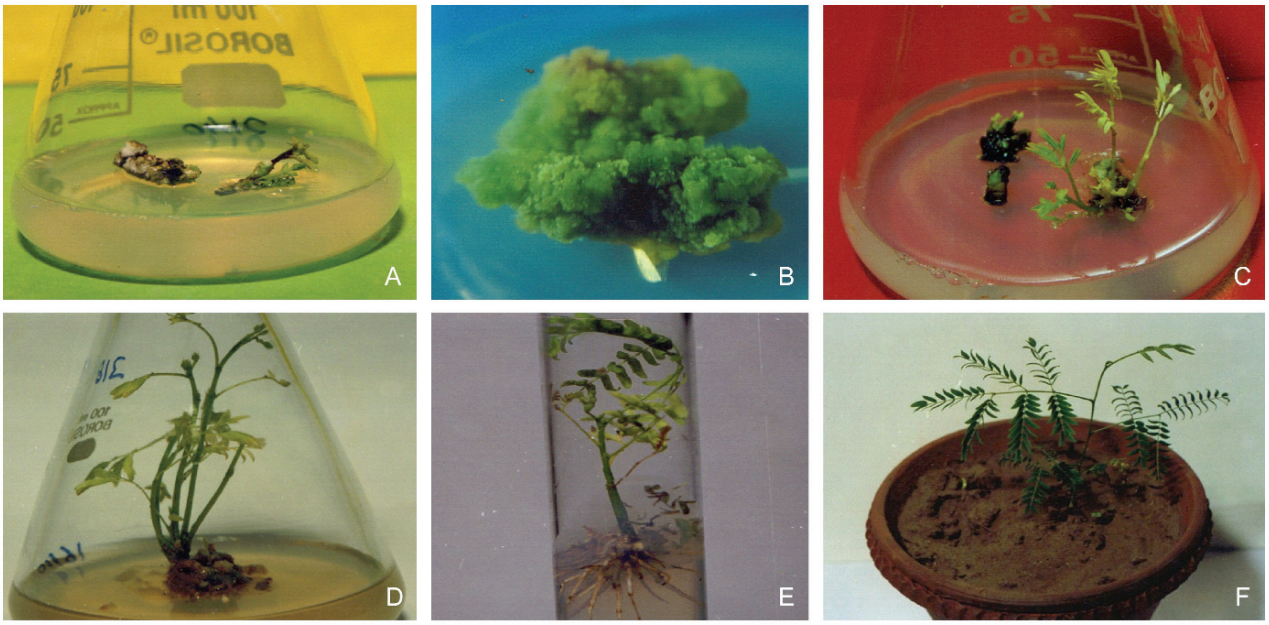

Fig. 1. Various stages of plant regeneration from mature tree derived nodal explant of *Leucaena leucocephala* cv. K-29. *A* - nodal explant; *B* - callus formation; *C* - shoot initiation; *D* - multiple shoots regeneration; *E* - rooting stage; *F* - F_0 plant growing in greenhouse.

strength MS medium. Dhawan and Bhojwani (1985) also reported shoot multiplication on MS medium containing 3.0 μM BAP with a 6 - 7 fold increased rate of multiplication every three weeks. 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). A marked variation in the shoot initiation response of different plant cultivars and types of explants was also observed indicating the dependence of *in vitro* regeneration on the genotype of the plant. Within 3 - 4 weeks, about 5 - 6 shoots per explant developed in case of K-29, while number ranged from 3 - 4 shoots per explant in case of K-8 and K-850 and 2 - 3 in case of K-68. Thus, K-29 was the most responsive among all the four cultivars analyzed. A comparison of regeneration potential of nodal explants and cotyledonary node explants cultivated on the full-strength MS medium containing 20.9 μM BAP and 5.37 μM NAA revealed nodal explants of all the four cultivars of *L. leucocephala* to be more responsive (forming 2 - 6 shoots per explant) than the cotyledonary node explants (forming only 2 - 3 shoots per explant). Moreover, the use of mature tree derived nodal explants for plant regeneration bypasses the juvenile phase and shortens the period of plant development. At the initial stages of *in vitro* culture, two major problems deleterious to the growth, *viz*. precocious leaf fall and excessive leaching of phenolics were confronted. Later, the problem of premature leaf fall was effectively circumvented by inclusion of glutamine at a concentration of 100 mg dm^{-3} in the shoot regeneration medium. Glutamine also enhanced regeneration ability of explants and complemented in greening of leaves and increase in number and vigor of shoots. Similarly, the inclusion of adenine and phloroglucinol in the medium could also help in circumventing this problem (Dhawan and Bhojwani 1985). Amongst the concentrations of glutamine tested the concentrations below and above 100 mg dm^{-3} were not very effective. The other problem of release of phenolics from cut ends of the explants was overcome by subculturing the explants on fresh medium after 2 d of implantation. The shoots thus developed were either proliferated by repeated excision from basal explant tissue and subculturing on fresh medium or subjected to rooting in root induction medium. Root initiation medium comprised of half-strength MS medium, 100 mg dm^{-3} myoinositol, 2 % (m/v) sucrose and different plant growth regulators at varying concentrations. Such reduced strength of MS or B5 medium has been reported to favor rooting in case of other legumes also (Veltcheva and Svetleva 2005, Anis *et al*. 2005).

A half-strength MS medium fortified with 14.76 μM IBA and 0.23 μM kinetin was found to be best for rooting in all the cultivars of *L. leucocephala*. In this medium, root initiation started after 15 - 21 d and within 4 - 6 d after root initiation, full-fledged roots were developed. More than 80 % shoots subjected to rooting could successfully develop roots in this medium and the number of roots per shoot was 7 - 9 in this medium. The frequency of rooting was the highest in cv. K-29 followed by K-8, K-850 and the least in K-68. Moreover, the roots developed in K-68 were very tender and posed problems in successful transfer to soil conditions. Though the rooting was achieved in the presence of other concentrations of IBA and kinetin, any alteration in the concentration of IBA and kinetin (lower or higher than

14.76 μM and 0.23 μM, respectively) decreased the number of roots per shoot with a rooting response below 70 %. Rooting response in presence of IAA was only 40 % and the number of roots per shoot ranged from only 1 to 3. Moreover, the rooting period extended to 4 - 4.5 weeks and the few plantlets formed under *in vitro* conditions were unable to adapt successfully to soil conditions. The suitability of IBA for improving the rooting frequency has also been suggested by Saradhi and Alia (1995) in *L. leucocephala*, while IAA was found to be least effective. The results suggest that the concentration of plant growth regulators and MS salts have to be properly manipulated before successful rooting could be expected.

Table 1. Effect of plant growth regulators on colour of callus, number of somatic embryos of globular stage and proline content [μmol g^{-1}(f.m.)] on day 28 of culture in induction medium of *L. leucocephala* cv. K-29. Proline concentration in induction medium was 1.0 mM.

2,4-D [μM]	BAP [μM]	Callus	Somatic embryos	Proline content
15.0	5.0	whitish green	15 - 17	7.192 ± 0.03
10.0	5.0	light green	2 - 3	1.146 ± 0.05
10.0	10.0	yellowish green	7 - 8	2.143 ± 0.12

Inside the culture room, the rooted plantlets were gradually acclimatized for two weeks in a pot containing soil and covered with pin-bored polythene sheets to avert water stress induced transplantation shock. This procedure of hardening of micropropagated plants of *L. leucocephala* ensured a survival rate of more than 75 % following their transfer to soil. In contrast, Dhawan and Bhojwani (1985) reported a survival rate of only 40 % during the final stage of micropropagation. This low survival rate was a consequence of transplantation shock inducing abnormal morphology and physiology in plants such as reduced epicuticular wax content, deficient starch grains in leaf cells, defective photosynthetic machinery and a poor control of water loss (Dhawan and Bhojwani 1987a). In order to circumvent the problem of high mortality rate during transplantation, few procedures including *in vitro* nodulation by *Rhizobium* (Dhawan and Bhojwani 1987b) and use of vesicular arbuscular mycorrhizal fungi (Puthur *et al*. 1998) were reported. In contrast to these tedious and time-consuming procedures, our procedure was simple and fast. After a short period of acclimatization the rooted plantlets were successfully established in soil in greenhouse. On an average, it took just 1.5 - 2 months from explant inoculation to establishment in soil. These *in vitro* raised plants set seeds and were morphologically indistinguishable from field grown plants.

Besides *in vitro* regeneration by organogenesis, the study was also aimed at induction of somatic embryos from callus cultures raised from mature tree derived nodal explants. To our knowledge, somatic embryogenesis of few leguminous tree species has been reported (Sinha and Mallick 1991, Arrillaga *et al*. 1994, Roberts *et al*. 1995, Garg *et al*. 1996, Chand and Singh 2001, Kumar *et al*. 2002, Singh and Chand 2003, Arockiasamy *et al*. 2006). Callus formation was achieved from mature tree derived nodal explants of all the four cultivars of

Table 2. Effect of plant growth regulators and spermidine on maturation of globular stage somatic embryos after four weeks of subculture.

BAP [μM]	IBA [μM]	Spermidine [mM]	Maturation [%]
10	2.5	0	4
10	2.5	0.5	23
10	4.0	0.5	22
10	5.0	0.5	24
15	2.5	0.5	10
15	5.0	0.5	9

L. leucocephala on callus induction medium. The rate of callus formation was modest to profuse depending on the concentrations of plant growth regulators as well as plant genotype. Thus, callus induction was most prolific in the full-strength MS medium supplemented with NAA and BAP. No callusing was achieved in the absence of any plant growth regulator, while some signs of callusing in case of all the cultivars, except K-68, were observed in the presence of other plant growth regulators investigated. Moreover, a cultivar dependent response to varying concentrations of plant growth regulators was also observed. For K-29 and K-850, callusing was much pronounced on full-strength MS medium containing 40.28 μM NAA and 12.24 μM BAP, while for K-8 and K-68 luxuriant growth was observed on full-strength MS medium fortified with 26.85 μM NAA and 12.24 μM BAP. Thus, the required concentration of NAA for callus formation was higher for cv. K-29 and K-850 as compared to cv. K-8 and K-68. In contrast to a high cytokinin/auxin ratio, which favored shoot induction, a high auxin concentration relative to cytokinin favored callus induction in all the four cultivars. The earliest visible signs of callus growth from explants were noticeable within 4 - 5 d of culture in all the cultivars. Further growth of callus was fast and after 20 - 30 d of culture, the relative size of callus to the original explant was of the order of 15 to 20. The frequency of explants forming calli was quite high (75 - 80 %). Upon subculturing on fresh medium, a one-inch callus doubled in just 15 d in the medium. For induction of somatic embryos, cv. K-29 calli were transferred to somatic embryogenesis induction medium. Best response was achieved in full-strength MS medium containing 15.0 μM 2,4-D, 5.0 μM BAP and 1.0 mM proline, where the number of globular somatic embryos per callus clump ranged from 15 - 17. The use of 2,4-D in MS medium for generation of globular shaped somatic embryos has been

reported for high frequency direct somatic embryogenesis in other tree and forage legumes (Jadhav and Hedge 2001, Singh and Chand 2003, Huang and Wei 2004, Arockiasamy *et al.* 2006). Only those calli that turned whitish or yellowish showed somatic embryogenesis, while green or brownish calli did not bear somatic embryos. Moreover, friable calli gave better response than compact calli. As proline, a stress-associated amino acid and a source of organic nitrogen supply, is known to provide protection against possible stress associated with *in vitro* culture and embryogenesis, the inclusion of 1.0 mM L-proline showed a promotive effect on somatic embryogenesis. Moreover, an increase of up to 7.0 μmol g^{-1}(f.m.) in endogenous proline content on the 28^{th} day of culture on induction medium containing 1.0 mM L-proline was also observed (Table 1). Such increase in endogenous proline content during embryogenesis has been reported for other plant species also (Chowdhury *et al*. 1993). Addition of 50 mg dm^{-3} L-glutamine in the induction medium also enhanced the frequency of formation of somatic embryos by about 10 %. Such stimulation of somatic embryogenesis by exogenous supply of L-proline, L-serine, and L-glutamine into the medium has been demonstrated in other plant species also (Claparols *et al*. 1993, Sen *et al*. 2002). The globular shaped somatic embryos were then subcultured on maturation medium and about 23 - 24 % maturation was acquired in full-strength MS medium fortified with 10.0 μM BAP, 2.5 to 5.0 μM IBA and 0.5 mM spermidine (Table 2).

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

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