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Micropropagation of *Dalbergia sissoo* from nodal explants of mature trees

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Abstract

A method for micropropagation of *Dalbergia sissoo* has been developed. Single node segments obtained from coppice shoots of a mature tree (20 - 25 year old) produced 3 - 4 shoots per explant on Murashige and Skoog (MS) medium containing 4.4×10^{-6} M benzylaminopurine (BAP) and 4.4×10^{-7} M of β-naphthoxy acetic acid (NOA) (shoot multiplication medium) within 4 weeks. The *in vitro* regenerated shoots were 3 - 4 cm in length and provided 2 to 3 culturable nodal segments which on shoot multiplication medium again produced 3 - 4 shoots. Following this procedure 18 - 24 shoots were produced from single nodal segment within 60 d. 80 % of the shoots directly produced five roots when they were firstly treated with MS medium supplemented with 10^{-5} M indole-3-butyric acid (IBA) and subsequently transferred to half strength liquid MS medium containing 1 % activated charcoal followed by half strength liquid MS free hormones, vitamins and activated charcoal. The *in vitro* raised plants were hardened for survival after transplantation to soil by exposing them to various humidity conditions, gradually from higher to low, with nearly 100 % transplant success.

Additional key words: acclimatization, *in vitro* culture, transplantation.

Introduction

Tissue culture techniques have been used for rapid clonal multiplication of selected genotypes of a number of forest trees including some woody legumes (see reviews by Dhawan 1989 and Trigiano *et al.* 1993). Most of the studies on leguminous trees have utilized seeds and juvenile tissues that are more amenable to *in vitro* multiplication than tissues from mature tree (Dhawan 1989). *Dalbergia sissoo* Roxb.,

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS - Murashige and Skoog (1962) medium; NAA - 1-naphthaleneacetic acid; NOA - β-naphthoxyacetic acid;

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a member of family *Fabaceae*, is one of the most valuable timber yielding trees of Indian subcontinent. Once widespread, the trees are not abundant now and all the natural stands fastly disappear. Conventional propagation (seeds, stem cuttings, root suckers) have low success. Induction of organogenesis from roots (Mukhopadhyaya and Mohan Ram 1981), axillary buds (Datta *et al.* 1983), hypocotyls (Sharma and Chandra 1988) and cell-suspension calli (Kumar *et al.* 1991) in *D. sissoo* has been reported but limited success has been achieved in transferring the *in vitro* regenerated plants to soil (Kumar *et al.* 1991). On the induction medium containing either an auxin or a cytokinin or both, the process of organogenesis was slow and frequency of shoot regeneration was low (Mukhopadhyaya and Mohan Ram 1981, Datta *et al.* 1983, Kumar *et al.* 1991). The main difficulties during multiplication of *D. sissoo* from nodal explants of a mature tree are excessive release of phenolic compounds in the medium from explants, inhibiting the growth of cultures and the loss of juvenility of explants (Datta *et al.* 1983). These difficulties can be removed by using explants from coppice shoots of a mature tree (Trigiano *et al.* 1993). The aim of the present report is to multiply a mature elite tree of *D. sissoo* through nodal explant culture.

Materials and methods

Explant preparation: Two superior trees of *D. sissoo* (20 - 25 years old) growing in the Forest Department Nursery, Rohtak, were selected. Coppice shoots were collected in February 1993. Nodal explants (1 cm long) excised from coppice shoots were washed 2 - 3 times with running tap water and then treated with 1 % (v/v) *Tepol* solution for 20 min with constant stirring. After washing 7 - 8 times with water, the explants were surface sterilized with 0.1 % (m/v) aqueous solution of mercuric chloride for 20 min in laminar air flow cabinet and then rinsed 5 - 6 times with autoclaved distilled water. The cut ends of the explants were trimmed with a sharp sterilized scalpel and implanted vertically on different media in culture tube with the cut end embedded upto 0.5 cm in the medium (Fig. 1).

Culture medium and conditions: In all experiments, MS basal medium was used. The MS medium was supplemented either with various auxins (NAA, IAA, IBA or NOA) or cytokinins (BAP or KIN) alone or in various combinations. The pH of the medium was adjusted to 5.8 and 0.7 % agar (*Hi-media*, Bombay, India) was added before sterilization at 121 °C for 20 min. All cultures were incubated under 16-h photoperiod (cool white fluorescent light, irradiance 80 $\mu mol\ m^{-2}\ s^{-1}$) at a temperature of 25 ± 2 °C. Each treatment consisted of 24 replicates and all experiments were repeated at least twice. The effect of different treatments was quantified on the basis of the percentage of explants showing response and the number of regenerants per culture after 28 d.

Rooting and transplantation: Regenerated shoots (2.5 - 3.0 cm in length) were excised from the explants and transferred aseptically to MS medium containing either NAA (2.7×10^{-6}, 5.4×10^{-6}, 1.08×10^{-5} M) or IAA (2.8×10^{-6}, 5.7×10^{-6}, 5.7×10^{-6}, 1.14×10^{-5} M) or IBA (2.5×10^{-6}, 5×10^{-6}, 10^{-5} M) separately for rooting.

Rooting response was also tested using three step culture procedure (*1*) MS solid medium containing IBA (10^{-5} M) only for 7 d, (*2*) half strength MS liquid medium with activated charcoal (1 %) for 14 d and (*3*) half strength MS liquid medium without vitamins, growth regulators and charcoal for 7 d.

Rooted shoots were removed from culture vessel and their roots were washed throughly in running tap water. One hundred rooted shoots were transferred to earthen pots containing a mixture of autoclaved vermiculite:sand:soil (1:1:2). Initially plants were covered with glass beakers and subsequently exposed to low air humidity for increasing period and finally the beakers were removed.

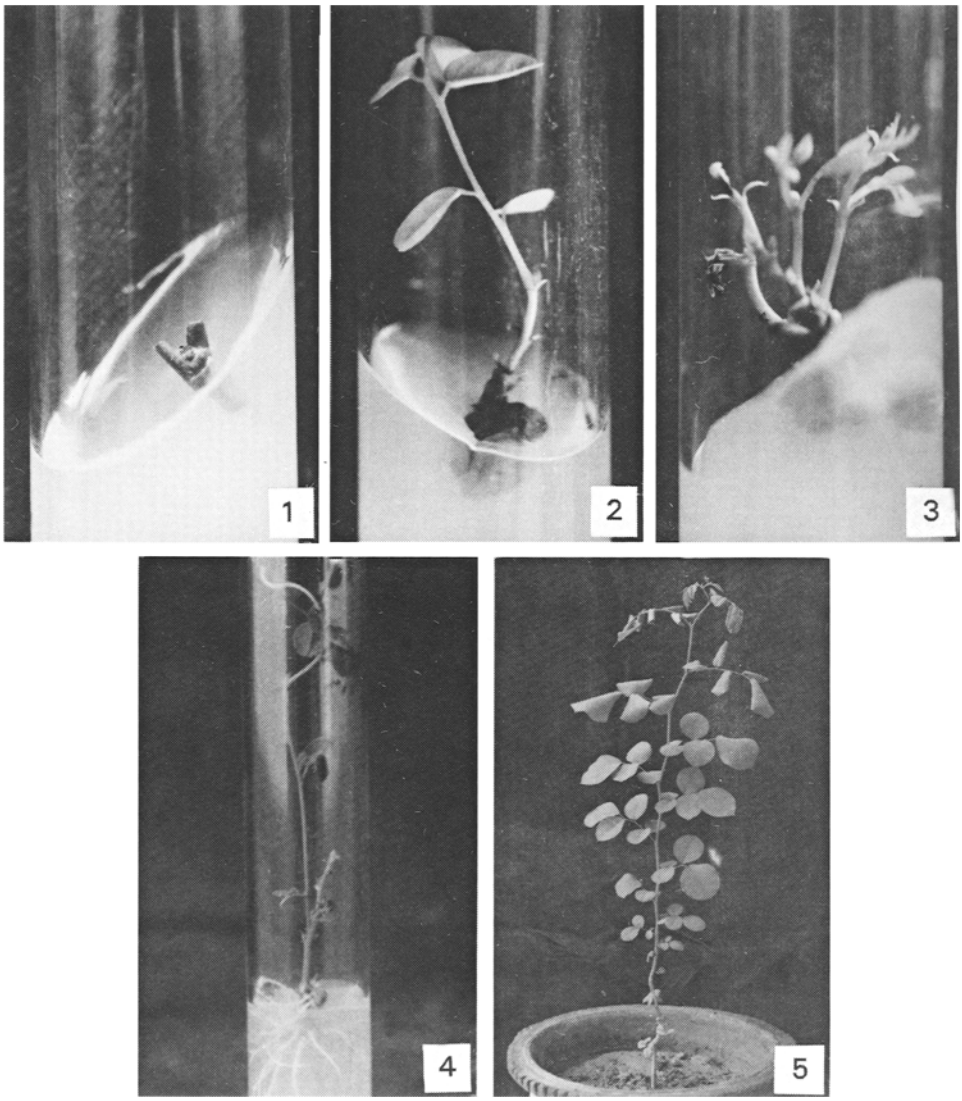
Results and discussion

The axillary buds on nodal explants became swollen and macroscopic within 7 d of culture on MS basal medium and directly developed into single shoots (1.3 cm in length) in 98 % of the cultures after 3 weeks (Fig. 2). Datta *et al.* (1983) have not reported shoot regeneration on basal medium from nodal explants excised from mature tree. The regenerated shoots showed premature leaf drop. A similar observation was made by Dhawan and Bhojwani (1985) in *Leucaena leucocephala* and Swamy *et al.* (1992) in *Dalbergia latifolia.*

Effect of BAP concentrations: Addition of BAP to MS basal medium induced a variable amount of callus at the embedded end of the explant within 10 - 15 d of culture followed by multiple shoot formation from nodal region. The presence of BAP in the medium drastically reduced the premature leaf drop. The proportion of explants forming shoots and the mean number and the average length of shoots per explant decreased and the amount of basal callus increased with increase in the concentration of BAP. BAP at concentration 4.4×10^{-7} M produced maximum number of shoots (1.8) in 73 % of the cultures (Table 1).

Effect of auxins: Auxins induced root formation at the embedded end of the explants with small amount of callus after 15 d of incubation. NAA (5.4×10^{-7}, 2.7×10^{-6} M), IBA (5×10^{-7}, 2.5×10^{-6} M), IAA (5.7×10^{-7}, 2.8×10^{-6} M) or NOA (4.4×10^{-7}, 2.2×10^{-6} M) showed 60 to 100 % bud sprouting. IAA (5.7×10^{-7} M) and IBA (2.5×10^{-6} M) induced in average one shoot in 100 % of the explants. However, only NAA at 2.7×10^{-6} M induced in average 2 shoots on 90 % of the explants (Table 1).

Interaction of BAP and KIN: The efficacy of BAP for shoot multiplication was improved when it was supplemented with KIN (Table 1). BAP at low concentration (4.4×10^{-7} M) in combination with KIN (4.7×10^{-6} M) produced maximum number of shoots (3) per explant in 100 % of the cultures. Use of the two cytokinins (BAP and KIN) has been found to be beneficial as in *Dalbergia latifolia* (Swamy *et al.* 1992).



Figs. 1 - 5: Plantlet formation from nodal explants of *Dalbergia sissoo.*

Fig. 1. Nodal explant of *D. sissoo* at the time of culture.

Fig. 2. Development of single shoot on MS basal medium after 4 weeks of culture.

Fig. 3. Differentiation of multiple shoots on MS + BAP (4.4×10^{-6} M) + NOA (4.4×10^{-7} M) after 4 weeks of culture.

Fig. 4. Root formation at the base of regenerated shoot by three step culture procedure (for details see Materials and methods) after 4 weeks of culture.

Fig. 5. A regenerated plant growing in pot containing normal garden soil, photographed two months after transplantation.

Table 1. Effect of different concentrations of BAP, KIN and auxins either alone or in various combinations added to MS basal medium on shoot regeneration from nodal explants of *Dalbergia sissoo* after 4 weeks. Means of 24 replications ± SE.

Hormones	Concentration [M]	Regenerating cultures [%]	Number of shoots [explant^{-1}]	Length of shoots [cm explant^{-1}]
	0	98	1.0 ± 0.0 a	1.4 ± 0.3
BAP	4.4×10^{-7}	73	1.8 ± 0.1 b	1.3 ± 0.3
	4.4×10^{-6}	58	1.1 ± 0.1 a	1.2 ± 0.2
	8.8×10^{-6}	33	1.0 ± 0.0 a	0.7 ± 0.1
NAA	5.4×10^{-7}	70	1.0 ± 0.0 a	0.8 ± 0.1
	2.7×10^{-6}	90	2.0 ± 0.1 b	0.5 ± 0.1
IAA	5.7×10^{-7}	100	1.0 ± 0.1 a	1.2 ± 0.3
	2.8×10^{-6}	64	1.0 ± 0.1 a	0.7 ± 0.1
IBA	5.0×10^{-7}	92	1.0 ± 0.0 a	0.9 ± 0.3
	2.5×10^{-6}	100	1.0 ± 0.0 a	1.2 ± 0.4
NOA	4.4×10^{-7}	83	1.0 ± 0.0 a	0.8 ± 0.2
	2.2×10^{-6}	85	1.0 ± 0.0 a	0.7 ± 0.1
BAP + KIN	$4.4 \times 10^{-7} + 2.3 \times 10^{-6}$	92	2.2 ± 0.4 b	3.0 ± 0.2
	$4.4 \times 10^{-7} + 4.7 \times 10^{-6}$	100	2.8 ± 0.4 b	2.9 ± 0.2
	$4.4 \times 10^{-6} + 2.3 \times 10^{-6}$	83	1.2 ± 0.2 a	2.6 ± 0.2
	$4.4 \times 10^{-6} + 4.7 \times 10^{-6}$	92	1.2 ± 0.1 a	2.4 ± 0.1
BAP + NAA	$4.4 \times 10^{-7} + 5.4 \times 10^{-7}$	100	2.8 ± 0.3 b	3.3 ± 0.4
	$4.4 \times 10^{-6} + 5.4 \times 10^{-7}$	75	3.6 ± 0.4 c	3.4 ± 0.4
BAP + IBA	$4.4 \times 10^{-7} + 5.7 \times 10^{-7}$	100	2.6 ± 0.2 b	4.2 ± 0.6
	$4.4 \times 10^{-6} + 5.7 \times 10^{-7}$	100	2.2 ± 0.2 b	2.8 ± 0.3
BAP + NOA	$4.4 \times 10^{-7} + 4.4 \times 10^{-7}$	100	3.0 ± 0.3 b	2.7 ± 0.2
	$4.4 \times 10^{-6} + 4.4 \times 10^{-7}$	100	3.3 ± 0.2 bc	3.2 ± 0.1
BAP + IAA	$4.4 \times 10^{-7} + 5.7 \times 10^{-7}$	75	2.0 ± 0.3 b	3.8 ± 0.4
	$4.4 \times 10^{-6} + 5.7 \times 10^{-7}$	100	2.5 ± 0.3 b	1.9 ± 0.2

Means followed by the same letter are not significantly different according to Newman-Keuls multiple range test ($P = 0.05$).

Interaction of BAP and auxins: The combination of BAP (4.4×10^{-7} and 4.4×10^{-6} M) with low concentration of auxin (NAA at 5.4×10^{-7} M, IAA at 5.7×10^{-7} M, IBA at 5.0×10^{-7} M or NOA at 4.4×10^{-7} M) improved shoot multiplication as well as the shoot growth (Table 1). In general, the combinations of BAP + NOA, BAP + NAA and BAP + IBA were superior for shoot multiplication than those of BAP or auxins alone or BAP + KIN or BAP + IAA. Earlier workers reported maximum response (60 %) of nodal explants for shoot differentiation on MS medium containing NAA (5.4×10^{-7} M) and KIN (4.4×10^{-6} M) after 30 d, but they have not reported the number of shoots regenerated per explant. The regenerated shoots were also not further multiplied (Datta *et al.* 1983). However, in the present study, BAP in combination with NOA produced a maximum number of 3.4 shoots per explant in 100 % of the cultures (Fig. 3, Table 1). The shoots attained a mean length of 3 - 4 cm after 28 d of culture and provided an average of 2 to 3 culturable nodal cuttings.

These nodal segments were cultured individually on MS medium containing BAP and NOA for further multiplication. Each nodal segment produced 3 - 4 axillary shoots within four weeks. Following this procedure, 18 - 24 shoots were obtained from single nodal segment within 60 d. Growth regulator NOA used in the present study has also been used earlier for regeneration of shoots on nodal segments of another leguminous tree, *Prosopis cineraria* (Kackar *et al.* 1991).

Interaction of BAP, KIN and auxins: The combination of BAP + KIN and auxins (IAA, NAA, IBA and NOA) were not superior for shoot multiplication than those of BAP + auxins (data not shown).

Datta *et al.* (1983) reported browning of the medium due to the release of phenolic compounds from the explants excised from mature tree inhibiting the growth of the callus and shoots. However, in the present study, the explant excised from coppice shoot of mature tree did not produce phenolics, hence responded quickly for shoot formation.

Rooting and transplantation: NAA, IAA or IBA alone or in combination induced roots at the base of the shoots with a good amount of callus (Table 2). This type of rooting proved to be undesirable for successful transplantation of *in vitro* regenerated plantlets to soil. Such a type of undesirable rooting appears to be one of the cause for poor survival rate of the transplants as reported by Kumar *et al.* (1991). The shoots

Table 2. Effect of different auxins added to MS medium on *in vitro* rooting of shoots excised from nodal explants of *Dalbergia sissoo* after 4 weeks. Means of 24 replicates ± SE.

Auxin	Concentration [M]	Rooting [%]	Roots number [shoot^{-1}]	Root length [cm shoot^{-1}]	Callus at the shoot base
NAA	2.7×10^{-6}	60	3.3 ± 0.9 a	2.6 ± 0.3	+
	5.4×10^{-6}	100	5.0 ± 1.0 b	2.6 ± 0.4	+
	1.8×10^{-5}	92	2.7 ± 0.2 a	2.6 ± 0.4	++
IAA	2.8×10^{-6}	–	–	–	–
	5.7×10^{-6}	40	3.0 ± 0.7 a	7.7 ± 0.9	+
	1.1×10^{-5}	60	2.3 ± 0.8 ac	6.8 ± 0.6	+
IBA	2.5×10^{-6}	45	2.1 ± 0.3 c	6.4 ± 0.5	+
	5.0×10^{-6}	62	1.8 ± 0.2 c	6.2 ± 0.3	+
	1.0×10^{-5}	67	4.6 ± 1.2 b	4.0 ± 0.5	+
IBA → charcoal		80	5.0 ± 0.9 b	5.0 ± 0.4	–

Means followed by the same letter are not significantly different according to Newman-Keuls multiple range test ($P = 0.05$).

directly (without intervening callus phase) produced an average of 5 roots (5 cm in length) in 80 % of cultures using three step culture procedure as described in Materials and methods within four weeks (Fig. 4). The rooted shoots (plantlets) were acclimatized by exposing them to a gradually decreasing humidity regime over a period of 15 d by removing the beakers for increasing period per day till they were

finally removed. The plantlets were then transferred to normal garden soil in pots where they are growing very well (Fig. 5). The percent survival of transplants in the pots was 100 %.

The present study demonstrates that nodal explants excised from coppice shoots of mature trees of *D. sissoo* possess a high potential for rapid multiple shoot formation on a simple culture medium.

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