Plant Cell Reports (1998) 18: 122–126

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Propagation of *Dalbergia sissoo* Roxb. through in vitro shoot proliferation from cotyledonary nodes

Received: 1 October 1997 / Revision received: 31 March 1998 / Accepted: 7 April 1998

Abstract A protocol is presented for micropropagation of an economically important timber-yielding forest tree, *Dalbergia sissoo* Roxb. (Sissoo). Multiple shoots were induced from cotyledonary nodes derived from 1-week-old axenic seedlings on Murashige and Skoog's medium containing either N^6-benzyladenine (BA), kinetin (Kn), isopentenyladenine (2iP) or thidiazuron (TDZ), with BA being the most effective growth regulator. High-frequency shoot proliferation (99%) and maximum number of shoots per explant (7.9 shoots) were recorded with BA at an optimum level of 8.9 μ*M*. Concentrations of all cytokinins tested above the optimum level markedly reduced the frequency of shoot proliferation. A proliferating shoot culture was established by repeatedly subculturing the original cotyledonary node on shoot multiplication medium after each harvest of the newly formed shoots. Primary shoots were multiplied as nodal explants, and from each stem node 2 or 3 shoots developed. Thus, 60–70 shoots were obtained in 3 months from a single cotyledonary node. About 91% of the shoots developed roots following transfer to half-strength MS medium containing a combination of 5.7 μ*M* indole-3-acetic acid, 4.9 μ*M* indole-3-butyric acid and 5.3 μ*M* indole-3-propionic acid. Eighty percent of the plantlets were successfully acclimatized and established in soil.

Key words Cotyledonary node · Micropropagation · Woody legume

Abbreviations *BA* N^6-benzyladenine · *IAA* indole-3-acetic acid · *IBA* indole-3-butyric acid · *IPA* indole-3-propionic acid · 2iP N^6-2-isopentenyladenine · *Kn* kinetin · TDZ thidiazuron

Communicated by R. N. Trigiano

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Introduction

Micropropagation is the only aspect of plant tissue culture which has been most convincingly documented with regard to its feasibility for commercial application, and consequently it has been extensively used for rapid and large-scale propagation of a number of plant species. The economical and ecological importance of leguminous forest trees necessitates the application of this technique for their clonal multiplication (Tomar and Gupta 1988). During the past few years, a number of woody legumes have been successfully propagated in vitro using juvenile as well as mature plant parts (Trigiano et al. 1992).

Dalbergia sissoo Roxb. (Sissoo) is one of the most important timber-yielding forest tree legume species of India. Because of its great strength, durability and elasticity, the wood is used for making furniture, cabinets, musical instruments, ornamental veneers and high-quality commercial plywood. Carefully selected and manufactured Sissoo plywood also meets the specifications of aircrafts (Anonymous 1970). In order to meet the high market demand, many of the trees have been indiscriminately logged, resulting in a significant decline in their population. Sissoo is conventionally propagated through seeds or root suckers. However, these methods are not very efficient in producing sufficient numbers of planting sources because the germination frequency of the seeds is low and the production of root suckers depends on the age of the trees.

Micropropagation of *D. sissoo* via nodal segments (Datta and Datta 1983; Datta et al. 1983) or axillary buds (Dwara et al. 1984) derived from mature trees has been reported. These reports, however, were mostly preliminary in nature, and the data provided were inadequate. Information on shoot number, shoot length, rate of shoot proliferation, percentage of rooting and number of roots per shoot was missing. Moreover in two of these three cases (Datta and Datta 1983; Datta et al. 1983), root and shoot development was reported to have been accompanied by an intervening callus formation at the basal end of the explants. This formation is an undesirable feature in a micro-

propagation protocol because it hinders the proper establishment of a vasular connection between shoot and root and often poses a problem during the transfer of plants to soil (Dwara et.al. 1984).

Plant regeneration from cell suspension cultures has also been reported for *D. sissoo* (Kumar et al. 1991), but this method is unsuitable for a rapid propagation programme as it is time-consuming. On the other hand, seedling explants are, in general, more responsive than explants derived from mature trees, and many tree species have been successfully propagated in vitro through axillary proliferation from cotyledonary nodes of seedlings (Perinet and Lalonde 1983; RavishankarRai and Jagadishchandra 1987; Nandwani and Ramawat 1993; Purohit and Kukda 1994; Purohit and Dave 1996; Das et al. 1996; Bhuyan et al. 1997). To date, however, reports on micropropagation of *D. sissoo* using cotyledonary nodes is absent. The present paper describes a simple protocol for the rapid and large-scale propagation of *D. sissoo* in vitro through high-frequency axillary shoot proliferation from cotyledonary nodes derived from axenic seedlings. As most of the woody species segregate for phenotypic traits when propagated by seeds, the plants regenerated in vitro from seedling explants would display inherent genetic variation. This could, however, be controlled using an elite seed population as was selected for in the study presented here.

Materials and methods

Seed germination and establishment of shoot cultures

A 50- to 60-year-old elite tree of *D. sissoo* was selected from which the dried and mature pods were collected. Healthy seeds were removed from the pods, washed with running tap water for 15 min, immersed in an aqueous solution of 5% liquid detergent, Laboline (Qualigens, India), for 5 min and rinsed 5–6 times with distilled water. The seeds were surface-disinfected with an aqueous solution of 0.1% $HgCl_2$ for 8 min followed by five or six rinses in sterile distilled water. The surface-disinfected seeds were transferred to screw-capped glass tubes (30 ml, Borosil, India) containing growth regulator-free half-strength MS medium (Murashige and Skoog 1962) with 1.5% sucrose, 50 mg/l myo-inositol and 0.6% agar (Bacteriological grade, Hi-media, India). The pH of the medium was adjusted to 5.8. The culture tubes were incubated at 25 °C±1 °C,under lighting at a 35 μmol m^{-2} s^{-1} irradiance level provided by white-fluorescent tubes and 60% relative humidity. Seven-day-old axenic seedlings served as the source of explants. After removal of the radicle and primary shoot from the seedlings, cotyledonary nodes were inserted vertically into 200-ml screw-capped glass jars containing MS medium with 3% (w/v) sucrose, 100 mg/l (w/v) myo-inositol and either 2.2–22.2 μ*M* benzyladenine (BA), 2.3–23.2 μ*M* kinetin (Kn) or 2.5–24.6 μ*M* isopentenyladenine (2iP), or 0.046–4.6 μ*M* thidiazuron (TDZ). The pH of the medium was adjusted to 5.8 before 0.8% agar was added and it was sterilized. The cultures were maintained under conditions similar to those described earlier for seed germination.

Each treatment consisted of seven replicates (culture vessels), and the experimental unit was two explants per vessel. Each experiment was conducted twice. Data on percentage of shoot development, shoot number and shoot length were recorded after 30 days. Data were analysed using analysis of variance (ANOVA) for a completely randomised design (CRD). Percentage data were subjected to arcsine transformation prior to analysis. Fisher's least significant difference (LSD) was used to compare the means.

Multiplication of shoot cultures

The original cotyledonary nodes were repeatedly subcultured on fresh shoot multiplication medium [MS+BA (8.9 μ*M*)] after each harvesting of the newly formed shoots. The primary shoots obtained from each harvest at an interval of 30 days were cut into single node pieces (2–3 nodes/shoot) and transferred to MS medium containing 4.4 μ*M* BA for axillary shoot proliferation.

Rooting of shoots and transfer of plantlets to soil

Shoots 3–4 cm in height with two or three leaflets derived from secondary cultures were transferred to half-strength MS medium containing 50 mg/l myo- inositol, 2% sucrose and 0.7% (w/v) agar. The medium was augmented with 2.8–11.5 μ*M* indole-3-acetic acid (IAA), 2.5–9.9 μ*M* indole-3-butyric acid (IBA) and 2.6–10.6 μ*M* indole-3-propionic acid (IPA) either each individually or in combinations. The rooted shoots were transferred to half-strength MS for further elongation of the roots. Each treatment consisted of five replicates (culture vessels) and two or three explants per experimental unit. Each experiment was conducted three times. The experimental design was the same (CRD) as that used for shoot proliferation and variance of the means was expressed as standard error (SE).

Well-rooted shoots were removed from the culture medium, and the roots were washed gently with water to remove agar. Plantlets were then transferred to small plastic pots (7.5 cm in diameter) containing autoclaved vermi-compost (Ranjan's Agrotech, Bhubaneswar) and kept in a plant growth chamber at 26 °C with lighting at a 50 μmol m^{-2} s^{-1} irradiance level provided by white fluorescent tubes and at 85% relative humidity. The relative humidity was reduced gradually, and the plantlets were removed from the growth chamber after 3 weeks. Plantlets were then transferred to larger earthenware pots (18 cm in diameter) containing soil : compost (1:1) and kept in the shade for another 2 weeks before being transferred outdoors under full sun.

Results and discussion

Establishment of shoot cultures

Ninety-eight percent of *D. sissoo* seeds germinated within 3–4 days of inoculation on a growth regulator-free half-strength MS medium. Cotyledonary nodes excised from 7-day-old axenic seedlings were used as explants in the present studies, and the morphogenic response of these explants to various cytokinins was documented (Table 1). Cotyledonary nodes failed to respond morphogenically to a growth regulator-free MS medium. The addition of a cytokinin to the medium was essential to induce multiple shoots from the explants. The frequency of axillary shoot proliferation and the number of shoots per explant increased with increasing concentration of cytokinins up to an optimum level. BA at a concentration of 8.9 μ*M* induced multiple shoot buds in virtually all explants within 4–6 days. The buds appeared as small green protuberances in the cotyledonary nodes that elongated into slender shoots possessing diminutive leaves (Fig. 1). The number of shoots per explant was recorded to be 7.9, and they had an average length of 4.3 cm after 30 days. Multiple shoots were also induced in the cotyledonary nodes on medium containing Kn or 2iP within 10–12 days. The optimum concentrations of Kn and 2iP at which a maximum number of explants exhibited shoot development were 9.3 μ*M* and 9.9

Table 1 Influence of different cytokinins on in vitro shoot proliferation from cotyledonary nodes of *D. sissoo*

Cytokinins (μ*M*)	Percentage shoot proliferation	Number of shoots per experimental unit	Shoot length (cm)
MS basal			
BA			
2.2	45	1.5	3.1
4.4	83	2.8	3.5
8.9	99	7.9	4.3
22.2	38	2.9	1.2
F value*	128	187.3	35.7
LSD ($P<0.05$)**	5.8	0.59	0.65
Kn			
2.3	–	–	–
4.6	59	1.0	2.9
9.3	82	2.1	3.4
23.2	45	1.6	1.5
F value*	50.9	16.4	19.6
LSD ($P<0.05$)**	4.8	0.42	0.63
2iP			
2.5	–	–	–
4.9	54	1.2	3.2
9.9	73	2.8	3.6
24.6	43	1.3	1.7
F value*	46.4	37.0	28.0
LSD ($P<0.05$)**	4.06	0.46	0.56
TDZ			
0.046, 0.23	–	–	–
2.3	39	2.5	1.3
4.6	–	–	–

* Significant at $P<0.01$
** Significant at $P<0.05$

Table 2 Influence of different auxins on rooting of the in vitro-formed shoots of *D. sissoo*[a]

Auxin (μ*M*)	Percentage rooting	Number of roots per experimental unit	Root length (cm)
Half-strength MS basal	–	–	–
IAA			
2.8, 5.7, 11.5	–	–	–
IBA			
2.5, 4,9	–	–	–
9.9	45 ± 1.3	2.8 ± 0.2	4.2 ± 0.1
IPA			
2.6, 5.3	–	–	–
10.6	29 ± 1.3	2.1 ± 0.2	3.4 ± 0.2
IAA + IBA + IPA			
2.8 + 2.5 + 2.6	43 ± 1.3	3.2 ± 0.2	4.5 ± 0.2
5.7 + 4.9 + 5.3	91 ± 1.3	5.8 ± 0.1	5.3 ± 0.1

[a] Each value represents the mean ±SE of three independent experiments with five replicates in each

μ*M* respectively. However, the frequency of shoot proliferation was relatively low and there were fewer shoots per explant with Kn and 2iP than with BA (Table 1). Unlike BA-treated shoots, the ones that developed on a Kn- or 2iP-supplemented medium had normal dark-green, trifoliate imparipinate leaves (Fig. 2). BA-induced axillary shoot proliferation from the cotyledonary nodes of seedlings have been reported in several plant species such as *Alnus glutinosa* (L.) Gaertn. (Perinet and Lalonde 1983), *Phaseolus* species (Mallik and Saxena 1992), *Arachis hypogaea* L. (Saxena et al. 1992), *Wrightia tinctoria* R.Br. (Purohit and Kunda 1994), *Melissa officinalis* L. (Tavares et al. 1996), *Sterculia urens* Roxb. (Purohit and Dave 1996) and *Murraya koenigii* (L.) Spreng. (Bhuyan et al. 1997). The frequency of shoot proliferation of *D. sissoo* declined markedly at higher concentrations of cytokinins (Table 1). Moreover, the shoots that developed on these media failed to elongate.

Thidiazuron has been reported to induce axillary as well as adventitious shoots in many tree species especially recalcitrant ones (Huetteman and Preece 1993). TDZ at 0.046–4.6 μ*M* was used in the present study to induce axillary shoots in the cotyledonary nodes of *D. sissoo*. However, it was the least effective of all the cytokinins tested for inducing shoot proliferation (Table 1). Although multiple shoots were induced on a TDZ-containing medium, the shoots failed to elongate and were often fasciated (Fig. 3). The formation of stunted shoots, or the fasciation of the shoots on TDZ-supplemented medium has been reported for several plant species such as *Malus* spp (apple; van Nieuwkerk et al. 1986) and *Rhododendron* spp. (Preece and Imel 1991). Inhibition of shoot elongation may be due to the high cytokinin activity of TDZ, whereas the presence of a phenyl group in TDZ may be the possible cause of shoot-bud fasciation (Huetteman and Preece 1993). Unlike the adenine-type cytokinins (BA, Kn, 2iP), TDZ is more effective at lower concentrations and therefore direct comparison of TDZ with each of these cytokinins at the same concentration is not possible.

Multiplication of shoot cultures

The shoot cultures were multiplied by repeatedly subculturing the original cotyledonary nodes on the shoot multiplication medium [MS+BA (8.9 μ*M*)] after each harvesting of the newly formed shoots. These cotyledonary nodes could be subcultured twice without affecting the shoot forming potential (data not shown). In 30 days, 6–8 shoots from each explant were obtained. The primary shoots obtained after each harvest were multiplied as stem nodal segments on MS containing 4.4 μ*M* BA, and 80% of these nodes produced multiple shoots (2 or 3 shoots/nodes) in 25–30 days (Fig. 4). Therefore, in 90 days about 60–70 shoots were obtained from a single cotyledonary node, which is several times higher than any earlier reported results. For example, only 1–3 shoots were reported to have developed from a single axillary bud of *D. sissoo* (Dwara et al. 1984).

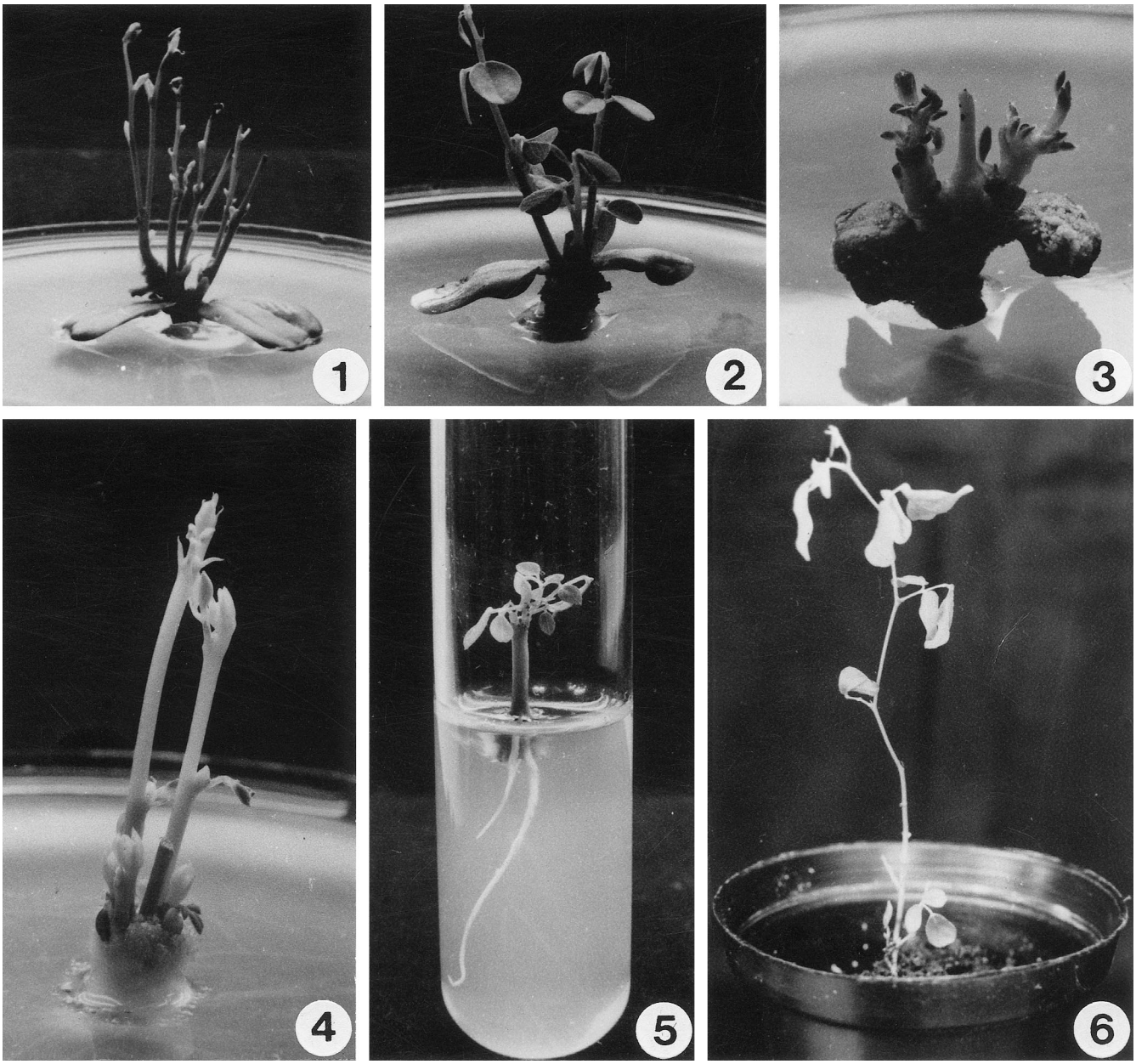

Fig. 1 Multiple shoots developed from a cotyledonary node of *D. sissoo* on MS+BA (8.9 μ*M*)

Fig. 2 Shoot development from a cotyledonary node of *D. sissoo* on MS+2iP (9.9 μ*M*)

Fig. 3 Fasciated shoots developed from a cotyledonary node of *D. sissoo* on MS + TDZ (2.3 μ*M*)

Fig. 4 Multiple shoots developed from a nodal segment of *D. sissoo* on MS+BA (4.4 μ*M*)

Fig. 5 A rooted shoot of *D. sissoo* on half-strength MS+IAA (5.7 μ*M*) +IBA (4.9 μ*M*) +IPA (5.3 μ*M*)

Fig. 6 A plantlet of *D. sissoo* 2 weeks after transfer to vermi-compost

Rooting of shoots and establishment of plants in soil

Half-strength MS without any growth regulators failed to induce root formation in the regenerated shoots even after 60 days. Of the three auxin supplements used to induce root formation IBA was most effective. Half-strength MS containing 9.9 μ*M* IBA induced rooting in 45% of the shoots within 18–20 days; in 30 days, about three roots had formed per shoot, and roots averaged 4.2 cm in length. The percentage of rooting was markedly enhanced by supplementing the medium with a combination of 5.7 μ*M* IAA, 4.9 μ*M* IBA and 5.3 μ*M* IPA. 91% of the shoots rooted in this medium within 6–8 days (Fig. 5). The combined presence of these three auxins favoured root induction in several tree species such as *Cinnamomum zeylanicum* Breyn. (RavishankarRai and Jagadishchandra 1987) and *Morus*

species (Pattnaik and Chand 1997). The roots elongated along with the development of a few new roots following transfer to a medium without growth regulators. In 30 days the number of roots per shoot was 5.8 and they averaged 5.3 cm in length (Table 2). The rooted plantlets were successfully acclimatised in a plant growth chamber for 3 weeks followed by another 2 weeks in the shade (Fig. 6) and eventually established outdoors under full sun. Of the plants transfered to vermi-compost 68% survived. Eighty percent of the plants transferred to soil survived. The regenerated plants did not show any detectable phenotypic variation.

The in vitro protocol described herein for regenerating a large number of plantlets of *D. sissoo* using cotyledonary nodes of axenic seedlings is an improved method as compared to that reported earlier and thus could be useful for a large scale plantation programme of this timber-yielding tree legume.

Acknowledgements The financial assistance from the Department of Biotechnology, Ministry of Science and Technology, Government of India, through a major R & D project is gratefully acknowledged.

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