



# Rapid multiplication of *Dalbergia sissoo* Roxb.: a timber yielding tree legume through axillary shoot proliferation and ex vitro rooting

J. B. Vibha · N. S. Shekhawat · Pooja Mehandru ·  
Rachana Dinesh

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**Abstract** An efficient and improved method for in vitro propagation of mature tree of *Dalbergia sissoo*, an ecologically and commercially important timber yielding species, has been developed through axillary shoot proliferation. Bud breaking occurred from nodal shoot segments derived from rejuvenated shoots produced during early spring from a 20–25-year-old lopped tree, on MS medium containing 8.88  $\mu\text{M}$  benzylaminopurine (BAP). Multiple shoots differentiated (20–21 shoots/node) on re-culture of explants on half-strength agar gelled amended MS medium with a combination of 2.22  $\mu\text{M}$  of BAP and 0.002  $\mu\text{M}$  of thidiazuron (TDZ) with 1.0 mM each of  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{K}_2\text{SO}_4$ , KCl, and  $\text{NH}_4(\text{SO}_4)_2$ . The maximum shoot multiplication (29–30 shoots/node) was achieved on subculturing in the above mentioned but liquid medium. Furthermore, the problem of shoot tip necrosis and defoliation observed on solid medium were overcome by the use of liquid medium. Ex vitro rooting was achieved on soilrite after basal treatment of microshoots with 984  $\mu\text{M}$  of indole-3-butyric acid (IBA) for 2 min. About 90 % microshoots were rooted on soilrite within 2–3 weeks under the greenhouse conditions. From 20 nodal shoot segments, about 435 hardened plants were acclimatized and transplanted. This is the first report for rapid in vitro propagation of mature trees of *D. sissoo* on liquid medium followed by ex vitro rooting.

**Keywords** Ex vitro rooting · Regeneration · *Dalbergia sissoo* · Plant growth regulators · Liquid culture

## Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
Kn	Kinetin
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NOA	2-Naphthoxy acetic acid
PFD	Photon flux density
PGRs	Plant growth regulators
RH	Relative humidity
TDZ	Thidiazuron

## Introduction

*Dalbergia sissoo* (shisham) is a multipurpose tree and one of the seven most valuable timber species of India, thus making it an important tree legume for large-scale industrial plantation (Bakshi and Sharma 2011). The bark and wood of *D. sissoo* are used as an expectorant, antihelminthic, and antipyretic; roots are used as astringent, and leaves are used for eye infection and gonorrhoea (Kritikar and Basu 1975). It enriches the soils through nitrogen fixation and with fast decomposing leaves (Bakshi and Sharma 2011). This species also plays an important role in environmental conservation and ecosystem balance (Bari et al. 2008). Therefore, there is a need for rapid propagation of desired genotypes of this forest tree selected for yield and quality of timber.

*D. sissoo* is conventionally propagated through seeds/planting sucker/stem cutting (Bari et al. 2008). This tree species is open-pollinated and the seed raised plants show wide genetic variability and are genetically not similar to mother plant. This method of propagation does not carry the optimum genetic gain of cloning of selected/mature tree

J. B. Vibha · N. S. Shekhawat (✉) · P. Mehandru · R. Dinesh  
Biotechnology Center, Department of Botany, Jai Narain Vyas  
University, Jodhpur 342033, Rajasthan, India  
e-mail: biotechunit@gmail.com

(Bonga and Von Aderkas 1992). Methods of vegetative propagation are laborious, time consuming, and constrained by low multiplication rate. Propagation of woody trees through tissue culture has many advantages over conventional vegetative propagation method like fast multiplication of the elite genotypes, quick release of improved cultivars, production of disease-free plants, season-independent production of plants, germplasm conservation, and facilitating their easy exchange (Pena and Seguin 2001; Asthana et al. 2011). In vitro approach to clone selected and mature trees(s) of *D. sissoo* can be applied as an efficient tool for micropropagation.

Earlier, micropropagation of *D. sissoo* using seedling-derived explants has been reported by a number of workers (Das et al. 1997; Pattnaik et al. 2000; Singh et al. 2002; Singh and Chand 2003; Chand and Singh 2004, 2005; Bari et al. 2008). The disadvantage of using juvenile rather than adult/selected specimen for propagation is that the full genetic developmental potential of the former is less known than that of adult/mature plant (Bonga and Von Aderkas 1992; Pijut et al. 2012). Till date, in vitro plant regeneration of *D. sissoo* from explants of mature tree has also been reported, but these explants produced callus (Datta and Datta 1983) and less number of shoots (Joshi et al. 2003; Thirunavoukkarasu et al. 2010). Datta et al. (1982) and Gulati and Jaiwal (1996) rooted the microshoots of *D. sissoo* under in vitro condition by two-step method. Generally, woody plant species are recalcitrant to adventitious regeneration during their maturation stage as the vigor for shoot production and competence for rooting declines (Singh et al. 2002; Bonga et al. 2010). *D. sissoo* reaches maturity after 20–25 years in the arid regions. Selection of desired genotype for quality and quantity of timber yield is possible at this age. We report here an improved micropropagation protocol from mature tree of *D. sissoo* through axillary bud proliferation from nodal shoot segment followed by successful transplantation of ex vitro rooted plantlets. Ex vitro rooting of shoots is advantageous as compared to in vitro rooting of plantlets as it results in better root system, requires less time, and allows acclimatization with ease. The cost of tissue culture raised plants can be reduced by such amendment in production process (Yan et al. 2010; Phulwaria et al. 2012a).

## Materials and methods

### Explants preparation and surface sterilization

About 20–25-year-old tree of *D. sissoo* was selected for establishment of cultures. The selected tree was lopped during winter (December–January). The flushed/rejuvenated shoots produced during subsequent spring (March–April) were harvested and used as explant. Fresh shoot sprouts (4–5-cm long with 2–4 nodes) were washed thoroughly and pretreated with

0.1 % (w/v) bavistin (a systemic fungicide; BASF India Ltd., Mumbai, India) for 15 min followed by surface sterilization with 0.1 % (w/v) HgCl<sub>2</sub> (Hi-Media, India) for 3–5 min under aseptic conditions. Each treatment was followed by rinsing with sterile water 4–5 times. The nodal shoot explants were treated with chilled sterile antioxidant solution (0.1 % each of ascorbic acid and citric acid) for 15 min.

### Nutrient media

Murashige and Skoog (1962) medium with sucrose (3 %) and 0.8 % (w/v) agar–agar (Bacteriological grade, Qualigens Fine Chemicals, Mumbai, India) was used for culture. The pH of the medium was adjusted to 5.8±0.02 prior to autoclaving for 15 min at 121 °C.

### Bud breaking and multiple shoot initiation

Surface-sterilized explants were inoculated vertically on the culture medium with various concentrations (0.0, 4.44, 8.88, 13.32, 17.76 µM) of BAP and (4.65, 9.28, 13.92, 18.56 µM) of Kn. The cultures were incubated in culture room under controlled conditions of temperature (26±2 °C), 12 h d<sup>-1</sup> illuminations of PFD 30–40 µ mol m<sup>-2</sup> s<sup>-1</sup> and 60 % relative humidity (RH).

### Multiplication of shoots

The explants with regenerated shoots were repeatedly transferred to fresh nutrient medium with different concentrations (1.11, 2.22, 4.44 µM) of BAP or (1.16, 2.32, 4.65 µM) Kn or in combination of 2.22 µM of BAP with 0.46, 2.32, 4.65 µM of Kn and 0.002, 0.004 µM of TDZ. The effect of different media, namely WP, (Lloyd and McCown 1981), MMS (Shekhawat et al. 1998), and MS (Murashige and Skoog 1962), with different strengths (full, half, and one fourth) on shoot multiplication were also evaluated. Each medium was supplemented with 2.22 µM of BAP, 0.002 µM of TDZ, and 1 mM each of calcium nitrate, ammonium sulfate, potassium chloride, and potassium sulfate.

In another set of experiments, the in vitro regenerated shoots were transferred to half-strength MS liquid medium with the abovementioned salts and Plant growth regulators (PGRs). The shoots were cultured in 10 to 20 ml liquid medium in 150-ml flask or 10, 20, or 30 ml liquid medium/250-ml flask. These were kept on rotatory shaker, maintained at 80 rpm under high light intensity (PFD 45–50 µmol m<sup>-2</sup> s<sup>-1</sup>). The cultures were further multiplied by (1) the transfer of mother explants, after harvesting newly formed shoots, to optimized shoot multiplication medium (liquid MS medium) for four passages and (2) subculturing of excised newly formed shoots on fresh medium. Repeated transfer was done after every 15–17 days.

## Ex vitro rooting of microshoots and hardening of plantlets

About 3–4 cm long microshoots were harvested and treated with indole-3-butyric acid (IBA) (0, 492, 984, 1,476  $\mu\text{M}$ ), NAA (537, 1074, 1611  $\mu\text{M}$ ), NOA (495, 989, 1484  $\mu\text{M}$ ), and a combination of IBA (246, 492, 738  $\mu\text{M}$ ) with NAA (247, 495, 742  $\mu\text{M}$ ) for 2-min. The treated shoots were transferred to autoclaved soilrite (a mixture of horticulture grade perlite with Irish peat moss and exfoliated vermiculite supplied by Kel Perlite, Bangalore, India). Shoots were moistened with one-fourth strength of MS salt solution. The culture bottles were capped and placed in the greenhouse near the pad section (RH 80–90 % and  $28\pm 2$  °C). After initiation of the root(s), the plantlets were gradually shifted from the pad section towards the fan section (temperature  $32\pm 2$  °C; 60–70 % RH) and the caps of bottles gradually loosened and finally removed after 15–20 days. Finally, the plants were transferred into polybags containing a mixture of organic manure, garden soil, and sand (1:1:1) and kept under greenhouse conditions at 25–30 °C, 80–90 % RH. Rooting was recorded after 2–3 weeks.

## Experimental design and data analysis

The experiments were set up in completely randomized block design and repeated thrice. All the experiments were conducted with a minimum of 20 replicates per treatment. To confirm

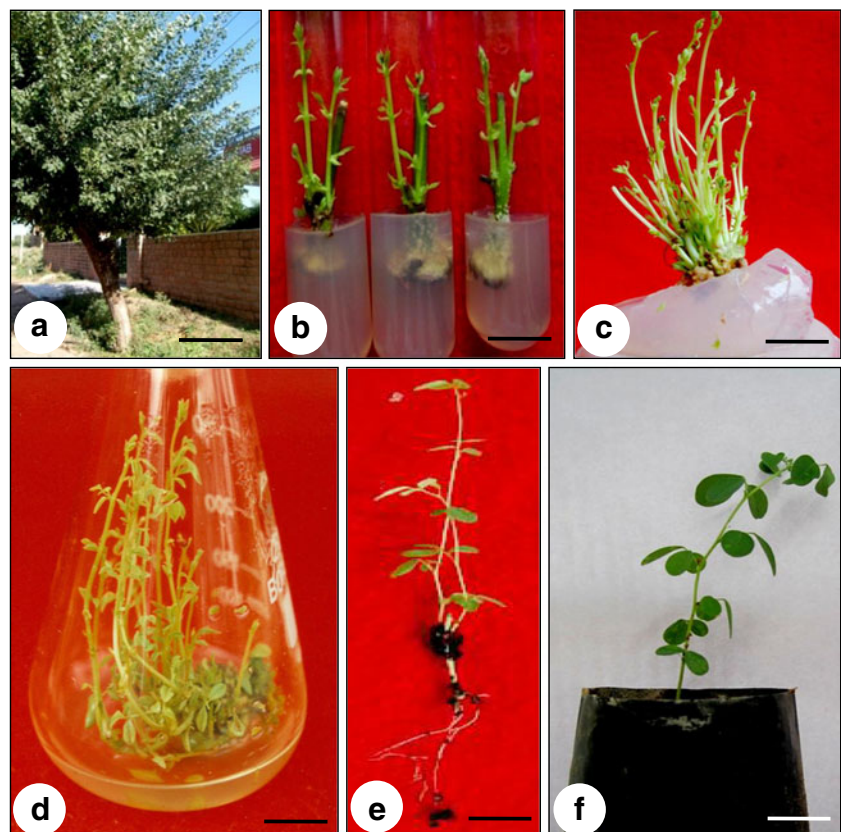
the reliability of the method, the experiments were repeated with four other local accession of *D. sissoo*. The number and length of shoots as well as roots were scored after regular intervals. The data was analyzed statistically using SPSS v. 17 (SPSS, Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range tests at  $P < 0.05$ . The results are expressed as means  $\pm$  SD of three experiments.

## Results and discussion

### Establishment of cultures

The cultures could be established from the nodal shoots of explants derived from fresh flushed/rejuvenated shoot of spring harvested from mature tree (Fig. 1a) and lopped during the preceding winter. The ability to clone woody plants is greatly influenced by the maturation stage and by the physiological stage of the initial explants (Montenius 1987). In the present study, nodal segments with 1–2 nodes, (4–5 cm in length) derived from rejuvenated (reinvigorated) shoots exhibited bud breaking. The old/perennial shoots of unlopped tree, if cultured, did not respond in vitro. It is suggested that the perennial shoots/tissues experience types of stresses like summer heat, severe winter, and extreme drought during the

**Fig. 1** In vitro propagation of *Dalbergia sissoo*. **a** Mature lopped tree (20–25 year old) of *Dalbergia sissoo* used as source of explant (scale bar 1.5 m). **b** Shoot bud induction from nodal explants on MS medium + 8.88  $\mu\text{M}$  of BAP after 8–10 days (scale bar 1 cm). **c** Multiplication of shoots on MS medium containing 2.22  $\mu\text{M}$  BAP and 0.002  $\mu\text{M}$  TDZ after 20–25 days (scale bar 1 cm). **d** Shoot elongation and amplification in liquid MS medium with 2.22  $\mu\text{M}$  BAP after 10–15 days (scale bar 1.4 cm). **e** Ex vitro rooted shoots treated with 984  $\mu\text{M}$  of IBA for 5 min (scale bar 2 cm). **f** Acclimatized plant of *Dalbergia sissoo* in greenhouse (scale bar 1.7 cm)



**Table 1** Effect of cytokinins (BAP or Kn) on bud breaking and shoot growth from nodal explants of *Dalbergia sissoo*

BAP ( $\mu\text{M}$ )	Kn ( $\mu\text{M}$ )	Response (%)	Shoot no. (mean $\pm$ SD)	Shoot length (cm) (mean $\pm$ SD)
0.0	–	00	0.0 $\pm$ 0.0 <sup>e</sup>	0.0 $\pm$ 0.0 <sup>f</sup>
4.44	–	50	1.5 $\pm$ 0.52 <sup>cd</sup>	1.1 $\pm$ 0.06 <sup>d</sup>
8.88	–	100	3.7 $\pm$ 0.42 <sup>a</sup>	2.2 $\pm$ 0.07 <sup>a</sup>
13.32	–	80	2.8 $\pm$ 0.72 <sup>b</sup>	1.5 $\pm$ 0.09 <sup>e</sup>
17.36	–	60	1.8 $\pm$ 0.63 <sup>c</sup>	0.5 $\pm$ 0.17 <sup>e</sup>
–	4.65	0	0.0 $\pm$ 0.0 <sup>e</sup>	0.0 $\pm$ 0.0 <sup>f</sup>
–	9.28	20	1.2 $\pm$ 0.42 <sup>d</sup>	0.6 $\pm$ 0.08 <sup>c</sup>
–	13.92	40	1.6 $\pm$ 0.51 <sup>cd</sup>	1.6 $\pm$ 0.08 <sup>b</sup>
–	18.56	30	1.5 $\pm$ 0.53 <sup>cd</sup>	1.4 $\pm$ 0.07 <sup>c</sup>

Means followed by the same letter within columns are not significantly different ( $P < 0.05$ ) using Duncan's multiple range test. Data were recorded after 15 days of culture

year, and accumulate anti-metabolic substances like phenols and ABA, which inhibit growth in culture (Shekhawat et al. 1993). Pruning/lopping/pollarding of mature tree and elimination/removal of aged tissues resulted in the production of reinvigorated shoots during subsequent spring. The lopping of tree is important for obtaining explants that respond in culture. Such types of pretreatments to mature trees have also been suggested for reinvigoration and rejuvenation (Bonga and Von Aderkas 1993) of woody plants. Explants harvested from woody plants of the desert area are very sensitive to injuries and sterilization process. Their cut ends show excessive browning and darkening. The treatment of explants with chilled, sterile antioxidant solution checked excessive phenolic exudation and browning of tissue and cultures (Phulwaria et al. 2012a, b). It has been observed in woody plants that antioxidant solutions may promote growth and development of the shoots particularly at the stage of culture establishment (Shekhawat et al. 1993; Sanjaya et al. 2006; Rai et al. 2010). Explants were cultured on 0.8 % agar-gelled MS medium containing BAP or Kn for shoot bud induction. Axillary bud proliferation occurred after 8–10 days of inoculation with 2–3

shoots per node (Fig. 1b). BAP was found to be more effective than Kn in terms of percent response and number of shoots per explants. MS medium supplemented with 8.88  $\mu\text{M}$  BAP was most suitable for multiple shoot induction and it induced 100 % bud break (Table 1). The effectiveness of BAP on multiple shoot induction has also been reported in a number of plants (Rai et al. 2010; Phulwaria et al. 2012a, b).

#### Multiplication of shoots

##### *Effect of plant growth regulators*

The shoots regenerated from the nodal explant were repeatedly transferred to the fresh medium with lower concentration of cytokinin alone (BAP or Kn) or a combination of BAP with Kn and TDZ for shoot multiplication (Table 2). The number of shoots increased on every transfer, reaching the maximum on fourth passage. Tripathi and Kumari (2010) also suggested that the repeated transfer of mother explants suppresses apical dominance allowing induction of basal meristematic cells to form multiple shoots. MS medium supplemented with the

**Table 2** Effect of concentrations and combinations of cytokinin (BAP, Kn, and TDZ) on multiplication of shoots of *D. sissoo* on MS medium

BAP ( $\mu\text{M}$ )	Kn ( $\mu\text{M}$ )	TDZ ( $\mu\text{M}$ )	Shoot no. (mean $\pm$ SD)	Shoot length (cm) (mean $\pm$ SD)
0.0	–	–	00 $\pm$ 0.0 <sup>i</sup>	00 $\pm$ 0.0 <sup>h</sup>
1.11	–	–	5.5 $\pm$ 0.52 <sup>f</sup>	1.4 $\pm$ 0.52 <sup>f</sup>
2.22	–	–	12.8 $\pm$ 0.72 <sup>b</sup>	2.3 $\pm$ 0.76 <sup>bc</sup>
4.44	–	–	3.5 $\pm$ 0.54 <sup>h</sup>	1.3 $\pm$ 0.08 <sup>d</sup>
–	1.16	–	3.6 $\pm$ 0.62 <sup>h</sup>	1.8 $\pm$ 0.07 <sup>g</sup>
–	2.32	–	3.8 $\pm$ 0.72 <sup>h</sup>	1.6 $\pm$ 0.60 <sup>e</sup>
–	4.65	–	4.6 $\pm$ 0.62 <sup>g</sup>	2.4 $\pm$ 0.19 <sup>b</sup>
2.22	0.46	–	7.9 $\pm$ 0.73 <sup>e</sup>	1.8 $\pm$ 0.92 <sup>d</sup>
2.22	2.32	–	10.5 $\pm$ 0.95 <sup>d</sup>	1.5 $\pm$ 0.90 <sup>f</sup>
2.22	4.65	–	7.7 $\pm$ 0.64 <sup>e</sup>	1.6 $\pm$ 0.08 <sup>e</sup>
2.22	–	0.002	20.9 $\pm$ 0.72 <sup>a</sup>	3.9 $\pm$ 0.14 <sup>a</sup>
2.22	–	0.004	11.9 $\pm$ 0.76 <sup>c</sup>	2.3 $\pm$ 0.14 <sup>c</sup>

Means followed by the same letter within columns are not significantly different ( $P < 0.05$ ) using Duncan's multiple range test. Data were recorded after 25 days of culture

**Table 3** Effect of culture media on multiplication of shoots of *D. sissoo* (each supplemented with BAP (2.22 μM)+TDZ (0.002) and 1 mM each of calcium nitrate, ammonium sulfate, potassium chloride, and potassium sulfate)

Types of media	Shoot no. (mean±SD)	Shoot length (cm) (mean±SD)
MS	6.20±0.91 <sup>e</sup>	2.4±0.20 <sup>g</sup>
MMS	6.90±0.87 <sup>e</sup>	3.0±0.27 <sup>f</sup>
½ MS agar gelled	27.9±0.99 <sup>b</sup>	3.9±0.30 <sup>c</sup>
¼ MS agar gelled	10.4±0.73 <sup>c</sup>	3.5±0.29 <sup>e</sup>
WP	7.90±0.99 <sup>d</sup>	3.5±0.15 <sup>e</sup>
MS liquid	7.20±0.78 <sup>d</sup>	5.2±0.47 <sup>b</sup>
½ MS liquid	29.6±0.70 <sup>a</sup>	6.7±0.46 <sup>a</sup>
¼ MS liquid	6.50±1.50 <sup>e</sup>	3.6±0.27 <sup>d</sup>

Means followed by the same letter within columns are not significantly different ( $P < 0.05$ ) using Duncan’s multiple range test. Data were recorded after 25 days of culture

combination of 2.22 μM BAP and 0.002 μM TDZ was found best for the multiplication of shoots. A maximum number of 20.9±0.72 shoots (3.9±0.14 cm shoot length) were produced within 15–20 days (Fig. 1c) on this combination. TDZ is among the most active cytokinin-like substances and usually induces greater in vitro shoot proliferation than many other cytokinins, particularly in woody plant species when used in very low concentration (Huetteman and Preece 1993). The amplified cultures of *D. sissoo* exhibited shoot tip necrosis and defoliation.

*Effect of different types of culture media*

Various culture media with few amendments, i.e., NH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub>, K<sub>2</sub>SO<sub>4</sub>, KCl, and Ca(NO<sub>3</sub>)<sub>2</sub> were evaluated to check the shoot tip necrosis, defoliation, and callus formation. In the present

study, half-strength agar-gelled MS medium supplemented with 2.22 μM BAP, 0.002 μM of TDZ, along with 1 mM each of Ca(NO<sub>3</sub>)<sub>2</sub>, K<sub>2</sub>SO<sub>4</sub>, KCl, and NH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub> was the most suitable for multiplication (Table 3). Incorporation of ammonium sulfate in medium reduced hyperhydricity, shoots tip necrosis, and promoted growth of shoots. Effect of ammonium ions and cytokinins on hyperhydricity and rate of multiplication was reported by Ivanova and Staden (2008) in *Aloe polyphylla*. Sulfate ions promote absorption of nitrate and buffered the culture medium (Ivanova and Staden 2008; Kopriva et al. 2009). Calcium has an important role in cell signaling, acting as a secondary messenger and thus directly influencing the absorption of other nutrients (Peres et al. 2009). The addition of KCl and Ca(NO<sub>3</sub>)<sub>2</sub> help in improving the growth of culture of woody legume. These findings are further supported by earlier reports (Rathore et al. 2004; Husain and Anis 2009). According to Preece (1995), in vitro performance of explants can be improved by balancing the nutrient level in the medium.

*Shoot multiplication in liquid medium*

The maintenance of sustainable and high rate of multiplication of healthy shoots was achieved in half-strength liquid medium containing abovementioned salts and PGRs. The shoots multiplied with higher rate (29.6±0.70) and length (6.7±0.46 cm) and without tip necrosis and defoliation (Fig. 1d; Table 3). The rate of shoot amplification achieved for *D. sissoo* in the liquid medium was higher than reported earlier (Datta and Datta 1983; Gulati and Jaiwal 1996; Joshi et al. 2003; Thirunavoukkarasu et al. 2010). Liquid culture offers many potential advantages over solid cultures like faster growth rates, rapid uptake of nutrients by tissues, and dilution of exuded growth inhibitors, i.e., phenolics released by explants,

**Table 4** Effect of auxins concentrations (pulse treatment) on ex vitro root induction from shoots of *D. sissoo*

	IBA (μM)	NAA (μM)	NOA (μM)	Response (%)	Roots no. (mean±SD)	Root length (cm±SD)
	00	–	–	0	0	0
	492	–	–	78	1.5±0.72 <sup>cd</sup>	1.2±0.52 <sup>f</sup>
	984	–	–	90	3.7±0.82 <sup>a</sup>	2.8±0.12 <sup>a</sup>
	1,476	–	–	63	1.9±0.56 <sup>bc</sup>	2.6±0.15 <sup>b</sup>
	–	537	–	75	1.3±0.48 <sup>d</sup>	1.2±0.10 <sup>f</sup>
	–	1,074	–	85	1.1±0.56 <sup>d</sup>	1.8±0.08 <sup>de</sup>
	–	1,611	–	79	1.0±0.66 <sup>d</sup>	1.6±0.12 <sup>e</sup>
	–	–	495	60	1.1±0.61 <sup>d</sup>	0.5±0.09 <sup>h</sup>
	–	–	989	55	1.1±0.59 <sup>d</sup>	0.8±0.08 <sup>g</sup>
	–	–	1,484	65	1.0±0.66 <sup>d</sup>	1.2±0.10 <sup>f</sup>
	246	–	247	60	1.5±0.73 <sup>cd</sup>	1.8±0.08 <sup>de</sup>
	492	–	495	63	2.0±0.66 <sup>bc</sup>	2.0±0.06 <sup>d</sup>
	738	–	742	70	2.2±0.52 <sup>ab</sup>	2.2±0.09 <sup>c</sup>

Means followed by the same letter within columns are not significantly different ( $P < 0.05$ ) using Duncan’s multiple range tests. Data were recorded after 25 days

thus minimizing negative effect on growth (Kim et al. 2003; Gupta and Timmis 2005; Preece 2010). Many factors such as the type, size, and volume of liquid in vessels, time period of repeated transfer, light, and temperature significantly influence the growth of tissue in culture. About 10–15 ml of amended liquid medium in 250-ml flask was found to be optimum for multiplication of cultures. On lesser volume of medium, the growth of cultures was slow and the medium dried up within a week; a volume of medium more than mentioned above caused hyperhydration of shoots, which affected shoot growth and multiplication. Reduction in the number and growth of shoots was due to hyperhydration which is a result of lower oxygen availability and submergence of shoots (Pati et al. 2011). Time period of subculturing was found to be an important factor for the maintenance and multiplication of cultures. Repeated transfer was done after every 15–17 days, as senescence (leaf fall) and browning of media occurred with delay.

#### Ex vitro rooting and hardening of shoots

In many woody plant species which are difficult to root, the in vitro generated shoots rooted successfully under ex vitro condition and survived (Benmahioul et al. 2012). The in vitro regenerated shoots treated for 2 min with 984  $\mu\text{M}$  of IBA exhibited the highest (90 %) rooting. About  $3.7 \pm 0.82$  number of roots per shoot with  $2.8 \pm 0.12$  cm root length was produced within 3–4 weeks (Fig. 1e; Table 4). IBA has been observed to induce strong rooting response and has been extensively used to promote rooting in a wide range of woody tree species (Eeswara et al. 1998; Husain et al. 2008). Ex vitro rooted plants were successfully acclimatized with  $96.6 \pm 2.8$  % of transplantation survival rate. The ex vitro rooting is not only helpful in reducing resources, time, and labor costs but also simplifies the protocol by eliminating the rooting step under sterile conditions (Preece and Shutter 1991; Dinkel-Meier et al. 1993; Pospisilova et al. 1999; Yan et al. 2010; Phulwaria et al. 2012b). Ex vitro rooting of shoots is advantageous in terms of better root system, time reduction, and ease of acclimatization (Yan et al. 2010; Benmahioul et al. 2012).

In conclusion, this paper describes a simple and improved protocol for propagation of *D. sissoo* using liquid medium followed by ex vitro rooting. Following this protocol, about 435 hardened plants could be produced starting with 20 nodal shoot segments. The method described here overcomes constraints like tip necrosis, defoliation, and callus at base by amendment in medium using salts like  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{K}_2\text{SO}_4$ , KCl, and  $\text{NH}_4(\text{SO}_4)_2$  followed by the use of liquid medium for elongation of shoots. The use of ex vitro rooting technique for root development in the large-scale commercial production of plantlets seems to be a more appropriate method compared to in vitro rooting. It is an economical method in terms of cost, labor, and time and gives higher yields and profits.

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