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Full Length Research Paper

Tissue culture studies on clonal variations in micropropagation of *Dalbergia sissoo*

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The present study was undertaken to study the *in vitro* responses of different clones of *Dalbergia sissoo*. For this study, three clones: one from Uttarakhand (clone No.09), Rajasthan (clone No.59) and Haryana (clone No.79) were investigated. These clones were selected on the phenotypic characters of candidate plus trees namely: higher volume of wood, clean bole length and stem form, etc. These clones were established and maintained at the Vegetative Multiplication Garden of Forest Research Institute, Dehradun. In this study, a standard procedure was developed for rapid multiplication of *Dalbergia sissoo* through axillary bud proliferation. Nodal explants were collected from different clones and were sterilized with 0.1% mercuric chloride for 15 min. Explants were collected for a year and the effect of season was adjudged. In all the three clones, explants collected from March to May respond at higher frequency and induced more shoots at optimum requirement of each clone. In clone No.9, maximum response of 92.35% was recorded, while in clone No. 59 and clone No.79, it was 89.31 and 91.63% respectively. For *in vitro* shoot multiplication in clone No.9 BAP at 2.5 µM was found superior with multiplication rate of 2.41, while in clone No.59 and clone No.79 BAP at 5 µM was found superior with 2.35 and 2.37 multiplication rates respectively. *In vitro* roots were induced on IBA supplemented medium. Effective IBA concentration was found to be 5 µM in clones No.9 and No.79 where 65.12 and 62.74% of root induction was obtained, while 7.5 µM IBA gave maximum of 60.50% of root induction in clone No.59. Plants were hardened and acclimatized before field transfer in all the three clones.

Key words: Tissue culture, micropropagation, clones, *Dalbergia sissoo*.

INTRODUCTION

Dalbergia sissoo Roxb. (Fabaceae, subfamily papilionoideae) is a medium to large deciduous tree with a light crown, commonly known as Shisham. Shisham is native to the foothills of the Himalayas of India, Pakistan and Nepal, which is its centre of origin. It is also grown in the sewage-irrigated greenbelt around Khartoum, Sudan. It is primarily found and grows along river banks below 900 m elevation, but ranges naturally up to 1500 m. *Dalbergia sissoo* is best known internationally as a premier timber species of the rosewood genus. However, Shisham is also an important fuel wood, shade, shelter and fodder tree. With its multiple products, tolerance of light frosts and long dry seasons, this species deserves greater consideration for agro forestry applications.

For plantation forestry, it is desired that the plantlets of timber-yielding trees should be of good quality, pest and disease resistant as well as of straight bole with wider girth and fewer branches. Cloning superior material, through conventional vegetative cutting method, result in uneven growth, as only tip cutting give rise to straight growing plants, where as branch cuttings produce trees which retain their characteristics as branches (Suwal et al., 1988). Micropropagation of plants is an invaluable aid in the multiplication of elite clones for development of high quality plantations. Present studies were undertaken to increase productivity both quantitatively and

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qualitatively as well as to meet the conservation aspect which is the need of the hour.

MATERIALS AND METHODS

Plant material

In the present study, nodal segments collected from different superior clones were used as explants for axillary bud proliferation. These clones were established at the Vegetative Multiplication Garden (V.M.G.) of Forest Research Institute, Dehradun, where assemblage of clones raised by means of mature cuttings and cuttings from root suckers of selected plus trees were maintained as shoot hedges. Three clones were selected from this garden based on their performance in clonal trials at three different regions of India as well as their performance in V.M.G Garden. This has generated the curiosity to investigate the *in vitro* proliferation pattern, multiplication rate and rooting response of different clones growing in similar and diverse geographic locations in the country. Among all the clones planted, one clone each from Uttarakhand (Clone No. 09), Haryana (Clone No. 59) and Rajasthan (Clone No. 79) was chosen to study the variation in the above characters. The clones under study were chosen on the basis of their performance in Vegetative Multiplication Garden of F.R.I, Dehradun as well as on the phenotypic scores of candidate plus trees namely: higher volume of wood, clean bole length and straight stem form, etc. In the present study, clone number was given by Plant Physiology Division, Forest Research Institute, Dehradun. Details of the geographical identity of these clones are given subsequently:

CLONE	LOCATION AND STATE
09	Pathri, Uttarakhand
59	Chichrauli, Haryana
79	Hanumangarh, Rajasthan

The present study dealt with the tissue culture protocol for micropropagation of *D. sissoo* for the three selected superior clones to distinguish the physico-chemical parameters and behavior within the clones.

Methods

Sterilization of explants

Nodal segments measuring 2-3 cm containing axillary buds were first surface sterilized using 0.1% $HgCl_2$ for 15 min, after which they were washed with sterile distilled water 3-4 times for further inoculation in medium.

Effect of cytokinins: To study the effect of cytokinins on axillary bud proliferation on nodal segments (collected from the selected three superior clones), BAP and Kn (Kinetin) were added in full strength MS medium at concentrations of 2.5, 5.0, 7.5, 10.0 and 12.5 µM separately.

Effect of cytokinin-auxin interaction: Nodal segments collected from the three superior clones were inoculated on full strength MS medium supplemented with combinations of BAP (2.5-10.0 µM) and auxin NAA at varying concentrations (0.5 to 1.5 µM) to observe the effect of cytokinin-auxin combination on axillary bud proliferation.

Effect of season: Influence of season of explants collection on its establishment and axillary shoot proliferation was investigated. For this purpose, nodal segments collected from all the three superior clones were collected from March to February and cultured on full strength MS media supplemented with BAP (0.0-12.5 µM) separately or in combination with NAA (0.5-1.5 µM). All media were supplemented with 3% sucrose and gelled with 0.7% agar-agar.

Effect of basal medium and medium strength: In order to assess the effect of basal media on axillary shoot proliferation, nodal segments were cultured on different media namely: MS, B_5 and WPM supplemented with optimal BAP requirement of each clone.

To study the effect of basal medium strength on axillary shoot proliferation on nodal segments collected from different superior clones, the basal medium was used at ½×, 1×, and 2× strength of its major salts along with cytokinin.

Multiplication of in vitro raised shoots

In vitro proliferated axillary shoots, 1.5 to 2.0 cm in length were subcultured on the defined medium to increase their numbers. Effects of various physico-chemical parameters on *in vitro* multiplication of these *in vitro* raised axillary shoots were studied.

Effect of cytokinins: Effect of cytokinins namely BAP and kinetin were studied on multiplication of *in vitro* raised axillary shoots. These cytokinins were incorporated in full strength MS medium at concentrations of 2.5, 5, 7.5, 10 and 12.5 µM separately. Propagules of the three shoots each were cultured.

Effect of cytokinin-auxin interaction: To study the effect of cytokinin-auxin interaction on *in vitro* shoot multiplication, axillary shoots were cultured on a medium supplemented with BAP (2.5-10.0 µM) and in combination with NAA (0.5-1.5 µM). Propagules of the three shoots each were cultured.

Rooting of shoots

Effect of auxins: Auxins namely: NAA, IBA or IAA were

supplemented in MS (1/2 ×) medium at 2.5, 5.0, 7.5 and 10 µM concentration to investigate their effect on rooting of *in vitro* raised shoots.

Effect of medium strength: To investigate the effect of medium concentration, major salts of the MS medium were varied from 1/4×-1× keeping the minor and organic salts constant. IBA at optimal hormonal requirement of each clone was uniformly added to all medium strengths.

Hardening and acclimatization of plantlets

Generally, tissue culture raised plants are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization. Rooted shoots from four week old cultures were transferred to soil under shade house either directly (in rainy season) or after hardening. For hardening, the plantlets were taken out from the flasks, washed to remove adhered agar and then transferred to autoclaved 250 ml screw cap glass bottle containing 1/3 volume of vermiculite. These plantlets were nurtured with half strength MS medium (without organics) twice a week for two weeks and were then kept in tissue culture room. After two weeks, these bottles were shifted to a mist chamber having relative humidity of 80-90% with a temperature of 30 ± 2°C. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 3-4 days prior transfer to polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1. In the mist chamber, the plants were kept for three weeks and irrigated with half strength MS medium. Later, these polybags were shifted to high-density double deck agronet open shade house for acclimatization. After one month in shade house, the plants were transferred to bigger polybags/pots containing same soil composition and were irrigated with tap water. Finally, plants were further kept in shade house for two months.

RESULTS

Axillary bud proliferation

Effect of cytokinins

In the present study, effect of cytokinins, that is, BAP and Kn (Kinetin) on axillary shoots on the cultured nodal segment collected from different clones of *D. sissoo* was investigated. BAP proved its superiority which induced multiple buds. In clone No. 09, BAP at 2.5 µM level induced an average of 3.47 axillary shoots per responding explants, which was higher than the number of buds induced at other concentrations tried. Increase in BAP concentration beyond 2.5 µM resulted in decline in number of axillary shoots induced per explants and only 2.55 axillary shoots per explants were induced at 12.5 µM BAP concentration. In clone No. 59, BAP at 10.0 µM level induced an average of 2.86 axillary shoots per responding explants, which was higher than number of buds induced at other concentrations tried. Increase in BAP concentration beyond 10.0 µM resulted in decline in number of axillary shoots induced per explants and only 2.61 axillary shoots per explants were induced at 12.5 µM BAP concentration. In clone No. 79, BAP at 7.5 µM level induced an average of 3.15 axillary shoots per responding explants, which was higher than number of buds induced at other concentrations tried. Increase in BAP concentration beyond 7.5 µM resulted in decline in number of axillary shoots induced per explants and only 2.44 axillary shoots per explants were induced at 12.5 µM BAP concentrations (Figure 1; Table 1).

On the medium supplemented with kinetin, in clone No. 09, the number of axillary shoots induced per explant was 2.14 at 2.5 µM, which increased to about 2.22 axillary shoots per explant at 5.0 µM concentration. Increase in kinetin concentration beyond 5.0 µM resulted in decline in average number of axillary shoots regenerated and only 1.66 axillary shoots were induced at 12.5 µM concentration. In clone No. 59, number of axillary shoots induced per explant was 1.34 at 2.5 µM, which increased to 2.18 per explant at 10.0 µM concentration. Increase in kinetin concentration beyond 10.0 µM resulted in decline in average number of axillary shoots regenerated and only 2.10 axillary shoots induced at 12.5 µM concentration. In clone No. 79, number of axillary shoots induced per explant was 1.59 at 2.5 µM, which increased to 2.21 per explant at 10.0 µM concentration. Increase in kinetin concentration beyond 10.0 µM resulted in decline in average number of axillary shoots regenerated and only 2.18 axillary shoots were induced at 12.5 µM concentration (Figure 2).

Percentage of responding explants on 2.5 µM BAP in clone No. 09, was 93.95% which steadily declined with increased concentration and only 74.75% explants responded at 12.5 µM concentration, while in clone No.59, it was 91.44% at 10.0 µM which steadily declined with increased concentration and only 77.92% explants responded at 12.5 µM concentration, and in clone No. 79, it was 93.15% at 7.5 µM which steadily declined with increased concentration and only 70.82% explants responded at 12.5 µM concentration.

On kinetin, percentage of responding explants in clone No. 09 was recorded as 90.23% at 5.0 µM concentrations, but the percentage response declined to 75.72% at 12.5 µM concentrations. In clone No. 59, number of responding explants was recorded as 90.29% at 10.0 µM concentrations, but the percentage response declined to 88.94% at 12.5 µM concentrations, while percentage response in clone No. 79 was recorded as 90.08% at 10.0 µM concentration, but the percentage response declined to 86.49% at 12.5 µM concentration (Table 2).

Overall, the axillary bud break response was the best in clone No. 09 as compared to the other two clones

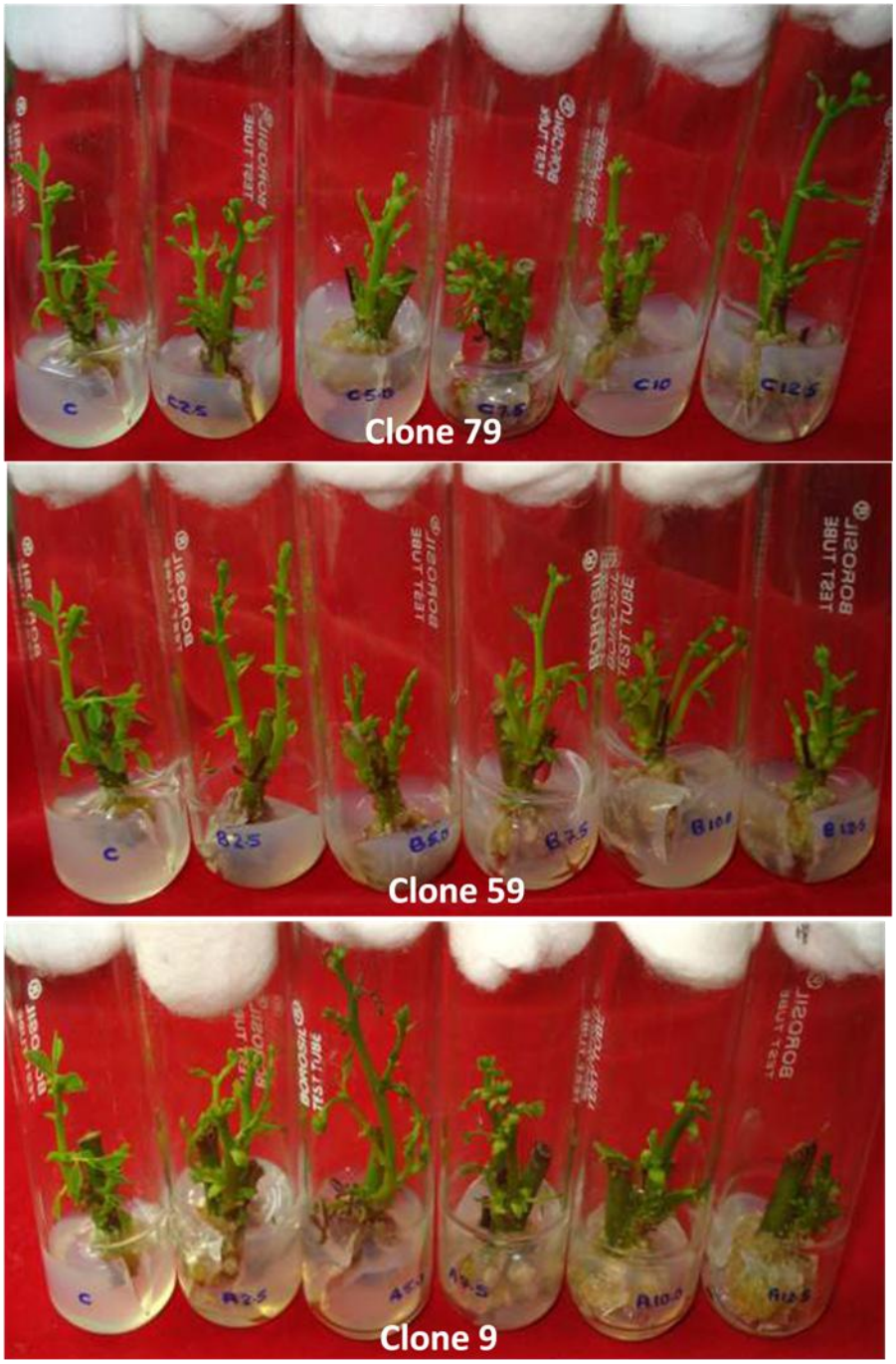

Figure 1. Axillary bud indication in three clones on MS medium supplemented with control and BAP (2.5, 5.0, 7.5, 10.0, 12.5 µM)

studied, that is, clones 59 and 79.

Effect of cytokinin-auxin interaction

In order to study the effect of cytokinin-auxin interaction on bud induction collected from different clones of *D. sissoo,* cytokinin BAP (2.5 to 10 µM) was supplemented with NAA (0.5 to 1.5 µM). It was observed that MS medium supplemented with BAP (2.5-10 µM) and NAA (0.5 to 1.5 µM) declined the axillary bud break response.

Among the combinations of BAP and NAA tried, a combination of 2.5 µM BAP and 1.0 µM NAA supported the optimal response of 87.17% in clone No. 09, while in clones no. 59 and 79, the combination of 10.0 µM BAP +

Table 1. Effect of cytokinin (BAP) in MS medium on axillary bud induction using nodal segments of selected clones of *Dalbergia sissoo*.

BAP (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length
0	82.77±1.09	2.50±0.09	1.28±0.03	70.66±1.27	2.27±0.03	1.22±0.03	75.85±1.58	2.44±0.02	1.23±0.01
2.5	93.95±1.34	3.47±0.08	1.53±0.02	73.76±0.63	2.43±0.04	1.19±0.02	83.76±1.64	2.83±0.03	1.36±0.02
5.0	90.55±0.59	3.26±0.08	1.41±0.02	72.30±1.47	2.61±0.03	1.22±0.03	91.55±1.66	2.98±0.05	1.38±0.03
7.5	81.45±1.56	3.10±0.06	1.24±0.02	83.47±1.30	2.77±0.03	1.26±0.03	93.15±1.63	3.15±0.02	1.45±0.02
10.0	77.41±1.01	2.74±0.05	1.17±0.02	91.44±1.48	2.86±0.07	1.33±0.03	83.24±1.36	2.64±0.03	1.30±0.03
12.5	74.75±0.42	2.55±0.04	0.93±0.02	77.92±1.13	2.61±0.03	1.20±0.03	70.82±1.43	2.44±0.02	1.13±0.03
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	1.57	0.10	0.03	1.81	0.06	0.04	1.70	0.04	0.04

Data were recorded after 3 weeks.

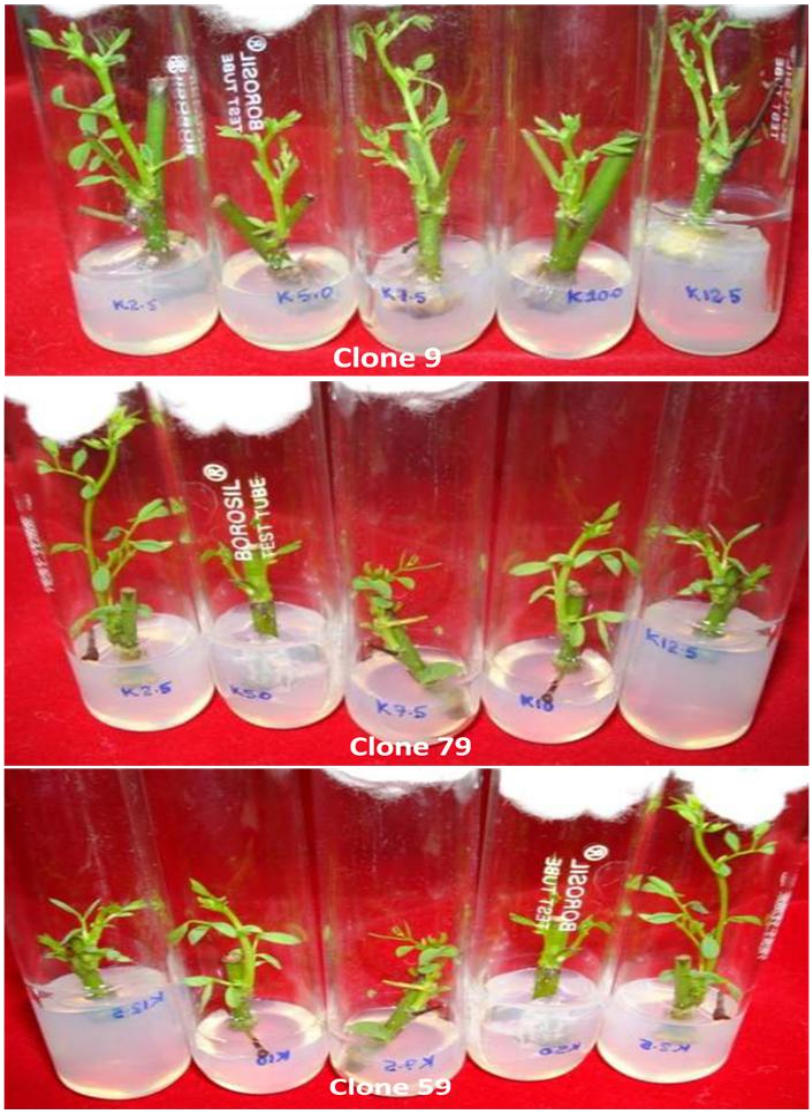

Figure 2. Axillary bud induction in three clones on MS medium supplemented with control and Kn (2.5, 5.0, 7.5, 10.0 µM).

Table 2. Effect of cytokinin (Kn) in MS medium on axillary bud induction using nodal segments of selected clones of *Dalbergia sissoo*.

Kn (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length
0	86.80±1.12	1.80±0.06	1.16±0.02	70.66±1.27	1.15±0.06	0.87±0.03	78.63±1.68	1.46±0.03	0.95±0.04
2.5	86.47±0.55	2.14±0.03	1.23±0.02	84.48±2.03	1.34±0.02	0.91±0.05	80.06±1.87	1.59±0.03	1.00±0.02
5.0	90.23±0.88	2.22±0.05	1.24±0.01	85.48±1.19	1.50±0.05	1.12±0.02	85.92±1.61	1.69±0.04	1.17±0.02
7.5	83.75±0.76	1.88±0.06	1.16±0.03	86.99±2.38	1.63±0.03	1.12±0.03	85.97±1.40	1.81±0.02	1.19±0.02
10.0	79.43±0.45	1.66±0.02	1.02±0.02	90.29±0.85	2.18±0.04	1.10±0.03	90.08±0.72	2.21±0.03	1.22±0.03
12.5	75.72±0.54	1.66±0.02	1.03±0.03	88.94±1.88	2.10±0.03	1.11±0.04	86.49±1.66	2.18±0.05	1.08±0.05
Significance	***	***	***	***	***	***	**	***	***
CD at 5%	1.10	0.06	0.03	1.84	0.06	0.05	2.24	0.05	0.04

Data were recorded after 3 weeks.

Table 3. Effect of different seasons on axillary bud induction supplemented with optimum BAP requirement of each clone of *Dalbergia sissoo.*

Variable	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length	Response (%)	Mean shoot no.	Mean shoot length
Jan	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Feb	7.95±0.73	0.18±0.03	0.33±0.09	5.72±0.94	0.18±0.03	0.13±0.02	8.47±0.37	0.15±0.02	0.14±0.02
Mar	86.59±0.59	2.64±0.08	1.36±0.04	85.16±1.23	2.56±0.02	1.37±0.20	85.53±1.25	2.61±0.02	1.41±0.03
Apr	94.83±0.56	3.36±0.08	1.61±0.04	89.98±1.27	3.28±0.05	1.60±0.03	93.32±1.28	3.35±0.02	1.64±0.03
May	93.09±0.44	3.20±0.04	1.48±0.05	89.33±1.45	3.09±0.05	1.51±0.03	90.20±1.35	3.20±0.02	1.55±0.03
Jun	86.60±0.59	2.73±0.03	1.37±0.05	81.10±1.17	2.63±0.04	1.41±0.02	84.08±1.55	2.71±0.04	1.44±0.02
July	44.20±1.00	2.11±0.02	1.23±0.06	37.17±2.13	2.01±0.04	1.28±0.02	40.68±1.62	2.09±0.04	1.31±0.02
Aug	78.04±0.89	2.45±0.04	1.21±0.03	71.86±1.60	2.33±0.03	1.21±0.02	75.60±1.91	2.40±0.04	1.24±0.03
Sep	62.69±0.30	2.77±0.05	0.93±0.04	63.02±1.97	2.66±0.03	0.99±0.02	60.98±1.87	2.75±0.03	1.01±0.02
Oct	41.42±0.57	2.25±0.04	0.87±0.04	42.48±1.75	2.17±0.02	0.88±0.02	45.82±1.58	2.20±0.04	1.00±0.01
Nov	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Dec	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	0.80	0.06	0.06	1.87	0.05	0.09	1.80	0.04	0.09

Data were recorded after 3 weeks.

1.0 µM NAA and 7.5 µM BAP + 1.5 µM NAA supported the optimal response of 84.81 and 87.25% respectively.

Effect of season

Nodal explants were collected from different clones during different seasons. These explants exhibited variation with season of their collection in respect to percentage of responding explants, number of induced axillary shoots, length of induced axillary shoots as well as growth regulator requirements. Explants collected from all the three clones during April showed maximum response in terms of all three parameters at optimum hormonal requirement of each clone. Percentage of explants exhibiting response increased during months of March to April. Response declined after these months. Length of regenerating axillary shoots also exhibited variation and increased till April and showed a decline thereafter (Table 3).

Effect of basal media and its concentration

Effect of basal media namely: MS, B_5 and WPM was studied on axillary bud induction on nodal explants from different clones.

In all the three clones, percentage of responding explant was more on full strength MS medium supplemented with optimum requirement of each clone

Table 4. Effect of different basal media and their strength supplemented with optimum BAP requirement of each clone for axillary bud proliferation.

Media	Conc.	Clone no. 09			Clone no. 59			Clone no. 79		
		Response (%)	Mean shoot number	Mean shoot length (cm)	Response (%)	Mean shoot Number	Mean shoot length (cm)	Response (%)	Mean shoot number	Mean shoot length (cm)
MS	2×	89.57±1.59	2.98±0.25	1.41±0.12	85.32±1.48	2.71±0.54	1.25±0.13	86.69±2.45	2.99±0.23	1.32±0.07
	1×	92.35±2.11	3.39±0.23	1.50±0.21	89.31±1.78	2.99±0.24	1.32±0.22	91.63±2.65	3.11±0.21	1.42±0.05
	1/2×	90.69±1.45	3.16±0.36	1.45±0.09	86.78±2.05	2.83±0.21	1.29±0.09	88.32±1.89	3.03±0.32	1.35±0.06
B5	2×	81.69±1.22	2.65±0.35	1.32±0.11	77.39±2.35	2.43±0.35	1.11±0.15	78.87±1.85	2.52±0.34	1.18±0.11
	1×	85.31±1.35	3.05±0.26	1.39±0.14	79.65±1.63	2.68±0.29	1.22±0.14	82.31±1.95	2.83±0.36	1.29±0.07
	1/2×	83.12±1.41	2.92±0.35	1.34±0.15	76.33±1.45	2.27±0.11	1.20±0.11	79.63±2.34	2.35±0.41	1.25±0.08
WPM	2×	85.42±1.36	2.72±0.45	1.35±0.14	81.98±2.64	2.37±0.15	1.16±0.08	82.29±2.78	2.41±0.15	1.22±0.06
	1×	90.63±1.74	3.11±0.41	1.41±0.14	83.89±2.56	2.82±0.44	1.24±0.11	84.11±2.36	2.91±0.16	1.31±0.9
	1/2×	88.21±1.77	2.99±0.29	1.36±0.12	81.67±2.41	2.54±0.36	1.19±0.16	83.65±2.11	2.76±0.19	1.25±0.11
Significance		***	**	***	***	***	***	***	***	***
CD at 5%		0.85	0.05	0.04	1.54	0.07	0.06	2.05	0.06	0.04

Data were recorded after 3 weeks.

as compared to other strength tried. In clone No.09, explants cultured on full strength MS, B_5 and WPM, responded at a frequency of 92.35, 85.31 and 90.63% respectively (Figure 1) and in clones No. 59 and 79, explants cultured on MS, B_5 and WPM media responded at a frequency of 89.31, 79.65 and 83.89%, and 91.63, 82.31 and 84.11% respectively (Table 4).

Multiplication of *in vitro* raised shoots

Effect of cytokinins

In order to investigate the effect of cytokinins on multiplication of *in vitro* proliferated axillary shoots, different cytokinins were tested. In clone No. 09, BAP at 2.5 µM was found optimal and an average of 7.22 shoots was recovered per culture propagule of three shoots in a period of three weeks. Increasing BAP concentration beyond 2.5 µM resulted in decline in multiplication potential. In clones No. 59 and No. 79, BAP concentration of 5.0 µM was found optimal and an average of 7.04 and 7.10 *in vitro* shoots were recovered per culture propagule of three shoots in a period of three weeks respectively (Figure 3), and increasing BAP concentration beyond 5.0 µM resulted in decline in multiplication potential in both clones (Table 5).

On kinetin supplemented medium, 2.5 µM kinetin gave response of 4.73 shoots developed from a propagule of three shoots in clone No. 09, but the number of shoots developed decreased to 3.59 at 12.5 µM kinetin, whereas in clones No. 59 and No.79, 5.0 µM kinetin gave response of 4.66 and 4.69 shoots developed from a propagule of three shoots respectively, for *in vitro* shoot multiplication. Similarly, at levels higher than optimal, multiplication rate decreased and only 3.41 and 3.44 shoots per propagule were developed at 12.5 µM concentration respectively (Table 6).

Effect of cytokinin-auxin interaction

In order to investigate the effect of cytokinin-auxin interaction, BAP (2.5 to 10.0 µM) was used in combination with NAA (0.5 to 1.5 µM). In clone No. 09, an average number of 7.11 shoots were recovered at 2.5 µM BAP + 0.5 µM NAA with 2.37 multiplication rate, while in clones No. 59 and 79, average number of shoots were 6.55 and 7.06 with the multiplication rate of 2.18 and 2.35 respectively at 5.0 µM BAP + 0.5 µM NAA. In clone No. 09, average shoot length was 2.06 cm at 2.5 µM BAP + 0.5 µM, while in clones No. 59 and 79 it was 1.97 and 2.05 cm respectively at 5.0 µM BAP + 0.5 µM NAA. Results reveal that combination of NAA with BAP in the medium did not enhance shoot multiplication rate as compared to BAP alone. 2.5 µM BAP in clone No. 09 and 5 µM BAP in clones No. 59 and 79 was found to be best for multiplication.

Rooting of *in-vitro* raised shoots

Effect of auxin

Well developed healthy shoots of 2.0 to 3.5 cm in length

Figure 3. Comparative *in vitro* shoot multiplication in each clone.

Table 5. Effect of cytokinin (BAP) in MS medium on shoot multiplication.

BAP (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Mean shoot Number	Mean shoot length (cm)	Multiplication rate	Mean shoot number	Mean shoot length (cm)	Multiplication rate	Mean shoot number	Mean shoot length (cm)	Multiplication rate
0	7.01±0.02	1.93±0.03	2.34±0.02	6.17±0.02	1.83±0.02	2.06±0.02	6.22±0.16	1.86±0.03	2.07±0.05
2.5	7.22±0.03	2.11±0.01	2.41±0.03	6.38±0.02	1.87±0.02	2.13±0.03	6.47±0.05	2.02±0.02	2.16±0.02
5.0	6.20±0.02	1.95±0.02	2.07±0.02	7.04±0.02	2.00±0.02	2.35±0.02	7.10±0.05	2.06±0.04	2.37±0.02
7.5	5.95±0.06	1.83±0.02	1.98±0.04	6.85±0.02	1.73±0.03	2.28±0.03	6.92±0.04	1.82±0.05	2.31±0.01
10.0	5.88±0.03	1.76±0.04	1.96±0.04	6.65±0.02	1.67±0.02	2.22±0.02	6.85±0.04	1.71±0.04	2.28±0.01
12.5	5.59±0.03	1.53±0.03	1.86±0.03	6.45±0.03	1.50±0.03	2.15±0.03	6.63±0.04	1.65±0.05	2.21±0.01
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	0.05	0.04	0.05	0.03	0.03	0.04	0.11	0.06	0.04

Data were recorded after 3 weeks.

were used for various *in vitro* rooting experiments. *In vitro* rooting was obtained when *in vitro* grown shoots were transferred on MS medium supplemented with auxins. A varied effect on *in vitro* rooting was observed by auxin IBA, NAA and IAA when incorporated in MS medium at different concentrations (2.5-10.0 µM). IBA supplemented MS medium produced maximum number of roots as compared to NAA.

In clone No. 09, MS medium supplemented with 5.0 µM IBA gave the maximum rooting response of 65.12% with 6-7 roots on an average per shoot. At decreased concentration of 2.5 µM, IBA rooting percentage was drastically decreased to 43.71% whereas higher concentration of IBA (10 µM) showed decreased number of *in vitro* root production. Thus 5.0 µM IBA supplemented MS medium was found to be the optimal medium for *in vitro* rooting (Table 7).

IAA and NAA (2.5-10.0 µM) when supplemented in the MS medium also induced *in vitro* roots. The rooting percentage was found less as compared to that of IBA supplemented MS medium. Best rooting response of 43.32 and 53.82% was obtained on 5.0 µM IAA and 7.5

Table 6. Effect of cytokinin (Kn) in MS medium on shoot multiplication.

Kn (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Mean shoot number	Mean shoot length (cm)	Multiplication rate	Mean shoot number	Mean shoot length (cm)	Multiplication rate	Mean shoot number	Mean shoot length (cm)	Multiplication rate
0	4.36±0.02	2.32±0.02	1.45±0.04	4.25±0.02	2.29±0.02	1.42±0.02	4.36±0.03	2.32±0.02	1.45±0.02
2.5	4.73±0.03	2.41±0.02	1.58±0.03	4.42±0.03	2.42±0.03	1.47±0.02	4.49±0.03	1.48±0.02	1.50±0.01
5.0	4.54±0.04	2.47±0.03	1.51±0.03	4.66±0.03	2.38±0.02	1.55±0.02	4.69±0.02	1.44±0.02	1.56±0.05
7.5	4.37±0.05	2.35±0.02	1.46±0.03	4.34±0.03	2.33±0.02	1.45±0.03	4.35±0.02	1.44±0.02	1.45±0.01
10.0	4.23±0.04	2.11±0.04	1.41±0.02	4.16±0.02	2.14±0.02	1.39±0.02	4.18±0.03	1.39±0.03	1.39±0.01
12.5	3.59±0.02	1.91±0.03	1.20±0.03	3.41±0.03	1.91±0.02	1.14±0.03	3.44±0.02	1.15±0.03	1.15±0.01
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	0.05	0.04	0.04	0.04	0.03	0.03	0.04	0.04	0.03

Data were recorded after 3 weeks.

Table 7. Effect of IBA in ½ MS medium on *in vitro* rooting.

IBA (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)
Control									
2.5	43.71±1.87	4.76±0.09	3.43±0.1	42.71±2.15	4.32±0.05	3.20±0.05	44.06±0.75	4.40±0.14	3.24±0.05
5.0	65.12±1.41	6.65±0.05	3.37±0.06	58.16±1.23	5.53±0.05	3.25±0.07	62.74±1.21	6.47±0.03	3.31±0.06
7.5	57.69±1.46	5.99±0.13	3.23±0.08	60.50±1.90	5.92±0.06	3.29±0.05	51.64±1.71	5.74±0.05	3.21±0.07
10.0	51.38±2.08	4.71±0.07	2.83±0.06	52.07±1.52	5.01±0.10	2.76±0.05	48.61±0.83	4.33±0.06	2.70±0.10
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	13.84	1.45	0.41	13.43	1.02	0.44	10.70	1.59	0.45

Data were recorded after 3 weeks.

µM NAA supplemented MS medium with 4-5 roots on an average per shoot respectively.

In clone No. 59, MS medium supplemented with 7.5 µM IBA gave the maximum rooting response of 60.50% with 5-6 roots on an average per shoot. At decreased concentration of 2.5 and 5.0 µM IBA, rooting percentage decreased to 42.71 and 58.16% respectively whereas higher concentration of IBA (10 µM) showed decreased number of *in vitro* root production. Thus 7.5 µM IBA supplemented MS medium was found to be the optimal medium for *in vitro* rooting.

IAA and NAA (2.5-10.0 µM) when supplemented in the MS medium also induced *in vitro* roots. The rooting percentage was found less as compared to that of IBA supplemented MS medium. Best rooting response of 42.28 and 38.53% was obtained on 7.5 and 7.5 µM IAA supplemented MS medium with 4-5 roots on an average per shoot respectively.

In clone No. 79, MS medium supplemented with 5 µM IBA gave the maximum *in vitro* rooting response of 62.74% with 6-7 roots on an average per shoot. At decreased concentration of 2.5 µM IBA, rooting percentage was decreased to 44.06% whereas higher concentration of IBA 7.5-10 µM also showed decreased number of *in vitro* root production. Thus 5 µM IBA supplemented MS medium was found to be the optimal medium for *in vitro* rooting.

IAA and NAA (2.5-10.0 µM) when supplemented in the MS medium also induced *in vitro* roots. The rooting percentage was found less as compared to that of IBA supplemented MS medium. Best rooting response of 43.35 and 42.62% was obtained on 7.5 and 7.5 µM IAA supplemented MS medium with 4-5 roots on an average per shoot respectively (Tables 8 and 9).

Effect of medium strength

MS medium strength was varied from 1/2×-2×, to study

Table 8. Effect of IAA in ½ MS medium on *in vitro* rooting.

IAA (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)
Control									
2.5	38.17±1.69	3.73±0.12	2.91±0.03	33.85±0.87	3.24±0.02	2.77±0.03	34.39±1.84	3.53±0.08	2.78±0.04
5.0	43.32±1.25	4.14±0.06	3.41±0.03	41.04±0.80	3.78±0.02	2.91±0.02	41.43±1.78	3.94±0.07	3.13±0.06
7.5	42.95±2.55	4.29±0.04	2.53±0.03	42.28±0.62	4.03±0.03	2.22±0.02	43.35±2.17	4.02±0.07	2.32±0.04
10.0	40.89±1.40	4.16±0.04	2.05±0.02	38.72±0.84	3.88±0.03	1.68±0.03	38.67±1.73	3.98±0.06	1.85±0.06
Significance	NS	**	***	***	***	***	*	**	***
CD at 5%	3.59	0.37	0.87	5.65	0.53	0.85	5.90	0.34	0.85

Data were recorded after 3 weeks.

Table 9. Effect of NAA in ½ MS medium on *in vitro* rooting.

NAA (µM)	Clone no. 09			Clone no. 59			Clone no. 79		
	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)
Control									
2.5	27.66±2.02	3.97±0.08	1.84±0.04	22.94±0.85	3.29±0.03	1.58±0.02	23.23±1.39	3.78±0.05	1.62±0.04
5.0	52.24±2.52	4.17±0.05	2.42±0.07	33.26±0.61	3.79±0.02	2.09±0.02	29.77±1.70	3.98±0.08	2.13±0.04
7.5	53.82±1.53	4.92±0.07	3.73±0.10	38.53±0.45	4.08±0.04	3.23±0.02	42.62±1.46	4.33±0.03	3.45±0.05
10.0	47.92±2.63	4.08±0.09	3.14±0.03	34.08±0.99	3.68±0.05	2.67±0.02	39.49±2.40	3.84±0.05	2.94±0.07
Significance	***	***	***	***	***	***	***	***	***
CD at 5%	18.37	0.66	1.26	10.01	0.49	1.08	13.53	0.38	1.24

Data were recorded after 3 weeks.

Table 10. Effect of MS salt concentration supplemented with optimum IBA requirement of each clone for *in vitro* rooting of shoots of *Dalbergia sissoo*.

Media	Conc.	Clone no. 09			Clone no. 59			Clone no. 79		
		Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)	Response (%)	Average root number	Average root length (cm)
MS	2×	42.45±1.93	5.84±0.05	2.46±0.08	39.07±1.01	5.18±0.06	2.28±0.04	40.69±1.92	5.18±0.09	2.35±0.01
	1×	49.16±1.31	6.21±0.08	3.09±0.06	42.04±1.72	5.73±0.07	3.01±0.05	45.10±1.22	5.63±0.16	3.11±0.01
	1/2×	62.49±1.53	6.49±0.03	3.25±0.04	58.21±1.56	6.12±0.12	3.10±0.04	60.02±2.29	6.21±0.08	3.21±0.01
Significance		***	***	***	***	***	***	***	***	***
CD at 5%		12.96	0.78	0.57	12.95	0.79	0.62	12.94	0.78	0.50

Data were recorded after 3 weeks.

its effect on rooting of *in vitro* raised shoots of *Dalbergia sissoo*. In clone No. 09, best rooting response was 62.49% while in clones No. 59 and 79, it was 58.21 and 60.02% respectively, and percentage response declined when the salt concentration of the medium increased beyond 1× in all the clones (Table 10).

Figure 4. Hardening of tissue culture plants of three clones.

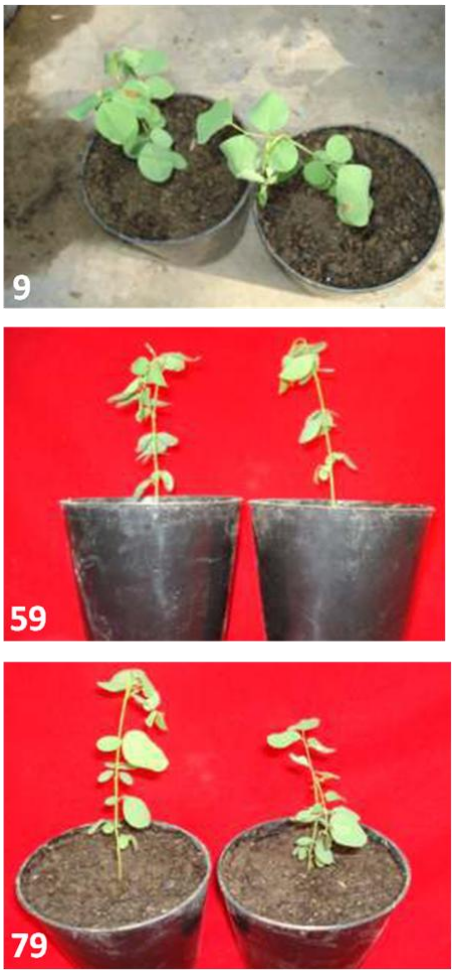

Figure 5. Micropropagated plants of clones 09, 59 and 79.

Hardening and acclimatization of plantlets

In vitro rooting was completed in 4-5 weeks. During this period, healthy plantlets with good roots and shoot system were developed. *In vitro* rooted shoots (plantlets) were hardened and acclimatized prior to field transfer. The five weeks old tissue culture raised plantlets which were directly transferred to polybags containing soil: sand: FYM, without hardening and acclimatization showed 20-25% survival rate similar to the plantlets that were also transplanted in the rainy season. In contrast, 60-65% survival was obtained when plantlets were hardened and acclimatized prior to field transfer. For hardening, the *in vitro* rooted plantlets were first washed with water so as to remove adhered agar/medium and then transferred to autoclaved culture bottles containing vermiculite. These plantlets were supplied with half strength MS medium without organics twice a week and later they were transferred to mist chamber at relative humidity of 80-90% and temperature of 30±2°C (Figure 4). Plants were shifted to polybags containing sand: soil: FYM in 1:1:1 proportion and placed in the mist chamber for four to five weeks. After mist chamber stage, the plants became hardened and were shifted to open shade house conditions for acclimatization to outer environmental conditions. During hardening, the shoots elongated, and the leaves turned green and expanded. In shade house, the plants were further transferred to bigger polybags or earthen pots and were irrigated with water (Figure 5).

DISCUSSION

In Shisham, although a number of workers have

attempted tissue culture related work, limited success to micropropagate this important timber species had been recorded. In the present study, making use of earlier works done and other related research investigations, success has been achieved in developing micropropagation protocol through axillary bud proliferation. A simultaneous study was carried out taking three superior clones of Shisham to find out if conditions varied within the clones.

In the present study, the role of season on response of cultured nodes of the selected clones of *D. sissoo* was investigated in respect of percentage explants responding, number of shoots induced and length developed. In all the three clones, it was observed that explants collected during the months of March to June established readily with higher mean number of axillary shoots. Axillary bud break response of explants declined in successive months and explants collected during the winter months (November to January) did not respond as buds turned hardy. This was also observed by Arya et al. (2005) in mature trees of *D. sissoo* where significant differences were found in morphogenic response of nodal explants when they were collected in different months. The success of *in vitro* differentiation of shoots/adventitious buds on the shoot or nodal explants is affected by season as also reported by many workers (Narayanswamy, 1977; Yang, 1977).

During investigation, the effect of two cytokinins namely: BAP and Kinetin were also studied on axillary bud proliferation from nodal segments and multiplication of *in vitro* raised shoots of three selected clones of *D. sissoo*. Axillary buds of these clones were proliferated readily on BAP supplemented medium than medium supplemented with other cytokinins. Superiority of BAP over other cytokinins in axillary bud break as well as during *in vitro* shoot multiplication has been well documented in earlier reports on cultured nodes of *Dalbergia latifolia* (Raghavaswamy et al., 1992).

It was also observed that BAP alone was found suitable for shoot induction and multiplication in all the three clones of *D. sissoo* when compared with Kn or in combination with NAA. These findings are in agreement with the findings of Kanwar et al. (1995) for seedling as well as mature explants of *Robinia pseudoacacia*.

During the present investigations, it was also observed that MS medium was better for axillary bud proliferation as well as for *in vitro* shoot multiplication in all the three clones when compared with B_5 and WPM medium. The suitability of MS medium was also observed in the reports on axillary bud proliferation using nodal segments from mature trees of *Acacia nilotica* (Mathur and Chandra, 1983; Singh et al., 1993), *Prosopis cineraria* (Shekhawat et al., 1993).

The role of auxins in root development is well established (Scott, 1972). The determination of shoot/root formation is generally dependent on the cytokinin/auxin ratio in the nutrient medium (Skoog and Miller, 1957). Our observation on root induction in shoots of selected clones of *D. sissoo* reveal that IBA was more effective than any other auxin (IAA, NAA) in inducing roots. Efficacy of IBA on root induction is well documented. Report of Rout and Das (1993) on rooting of shoots of *Madhuca longifolia* mentions effectiveness of IBA over NAA and IAA for root induction. Similar were the observations of Rahman and Blake (1988) where shoots of jackfruit (*Artocarpus heterophyllus*) from juvenile tissues can be readily rooted on medium supplemented with IBA than other auxins (NAA, 2, 4-D).

In the present investigation of selected clones of *D. sissoo*, efficiency of *in vitro* rooting was found to be highly variable amongst three clones. Similar to our observations were those of Kalia et al. (2004) in *D. sissoo*. The role of genotypic variations in rooting has been encountered in studies of different research groups (Horgan and Holland, 1989). They recorded variation in the *in vitro* rooting of *in vitro* shoots and number of roots per shoot. In the present study, similar variation was observed in the *in vitro* performances of the three clones. 5 µM IBA proved to be effective for rooting in the clone from Uttarakhand and Haryana while 7.5 µM IBA proved to be effective for Rajasthan clone. Similar efficacy of IBA on root induction is reported in many genera. Effective concentration of IBA for *in vitro* rooting varied between 0.25-25 µM for leguminous species like *Acacia auriculiformis* (Ranga Rao and Prasad, 1991) and *Prosopis tamurago* (Nandwani and Ramawat, 1992a). David (1982) found that effectiveness of auxin on *in vitro* rooting percentage is proportional to the concentration used in the medium.

During rhizogenesis, a diluted mineral fraction (50 to 75%) is generally used. This practice is applied for herbaceous plants, woody ornamentals, fruit trees as well as forest trees (Mohammed and Vidaver, 1988). A similar study conducted in the selected three clones of *D. sissoo* showed that of the different strengths of MS medium tested (full, half and quarter) for *in vitro* rooting, maximum rooting with highest average number of roots and root length was observed at half strength. Successful rooting using media of lower strength has been reported in other leguminous tree species namely: *Dalbergia latifolia* (Ravishankar Rai and Jagdishchandra, 1989) and *Delonix regia* (Rahman et al., 1992). Effectiveness of lower media strengths for rooting has also been seen in *Piper longhum* L. (Bhat et al., 1992) and *Campanula isophylla* (Brandt, 1992).

Murashige (1974) was the first to promote *in vitro* hardening of plants and recommended reduction of medium nutrients. Hardening in low-carbohydrate medium and exposure to higher levels of light intensity is recommended. This forces the regenerants to rely on their photosynthetic apparatus for nutrition (Kozai et al., 1988). Such hardened plantlets when transferred to *ex vitro* conditions gave better results as compared to non-hardened ones, which could not survive on field transfer.

In this study, the hardened plantlets were acclimatized by transferring them into polybags containing soil: sand: FYM in 1:1:1 ratio and covering them with perforated polybags to maintain high humidity. These plantlets were initially maintained in shade. The perforated polybags were removed after fifteen days and the plants were shifted to agronet shade house for one month.

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