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Studies on Effect of Nutrient Media for Clonal Propagation of Superior Phenotypes of *Dalbergia sissoo* Roxb. through Tissue Culture

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Abstract

Micropropagation studies were undertaken using nodal explants collected from two ramets (clone No. 36) belonging to 60-year-old superior tree of *Dalbergia sissoo.* Two nutrient media viz. MS and B5 were used to find out the suitability of the medium. Bud break was achieved in both of the media within 6-8 days under different media combinations supplemented with BAP (0.10–1.0 mg/l) alone as well as in combinations with IAA or NAA (0.10 to 0.50 mg/l). Maximum percentage of bud break (100%) was achieved in both of the media. Maximum number of shoots per explant (8.04) was observed in the MS medium supplemented with 1.0 mg/l BAP + 0.25 mg/l NAA. Out of the two nutrient media tried MS medium was found to be the best, which gave high rate of shoot proliferation as compared to B_5 medium. Maximum number of roots per plantlet (4.47) was observed in $^1/_2$ MS supplemented with (1.0 mg/l) IBA within 18 days. Plantlets so produced were acclimatized and transplanted to pots and 70% survival was achieved.

Key words: Dalbergia sissoo, Micropropagation, *In vitro*, Clonal Propagation.

Abbreviations: MS: MURASHIGE and SKOOG medium; B5: GAMBORG et al. medium; BAP: benzylamino purine; NAA: naphthalene acetic acid; IBA: indole-3-Butyric acid; IAA: indole-3-acetic acid; Kn: kinetin; PGR: Plant Growth Regulator.

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Introduction

Dalbergia sissoo is an indigenous, commercial timber yielding species of northern India. It is found up to 900 m in the sub-Himalayan tract and occasionally ascending to 1500 m between latitude 21.17° N to 32.60° N and longitude 74.8° E to 93.43° E. In the sub-Himalayan tract, it occurs along rivers and streams, gregariously growing on alluvial soil. It is a multipurpose nitrogen fixing tree, but its major end use is as wood, with grower and market appeal as a quality cabinet timber. Any breeding programme to improve wood quality must focus primarily on vigour and form. This is particularly for *D. sissoo*, which is characterized by crooked stem, forking and ramicorn branching. Trees showing straight bole are of rare occurrences. Early trials have shown that crooked stem form of *D. sissoo* is under strong genetic control and has high heritability 42–65% (VIDAKOVIC and AHSAN, 1970). There exists considerable phenotypic variation between trees of different provenances (SEWAL et al., 1988). If such superior phenotypes having desired qualitative and quantitative traits are multiplied vegetatively, tangible gains can be achieved. Conventional vegetative propagation methods using branch cuttings may result in plagiotrophic growth (Personal communication K. White cited by SEWAL et al., 1988). Furthermore, if cuttings are taken from mature trees the success achieved in rooting is not encouraging until and unless the tissue has been rejuvenated following hedging. In this context micropropagation of superior phenotypes may

play an important role in production of planting stock true-to-the parent type on mass scale which will help in establishment of hedge gardens, germplasm bank, clonal seed orchard etc. In the present communication, technique for successful *in vitro* clonal propagation of superior phenotypes of *D. sissoo* has been reported.

Material and Methods

Explant treatment

Young shoots were collected from the ramets (clone No. 36) belonging to 60-year-old superior tree of Gonda source assembled in a clonal seed orchard established at New Forest, Dehradun (Altitude 640 m, latitude 30° N, longitude 78° E, Annual rain fall 216 cm) during March to September for initiation and establishment of cultures. Nodal segments measuring 2.5 to 3.0 cm, were cut and washed in running tap water to remove the dust particles and then washed in liquid detergent (Teepol, 5–8 drops/100 ml) in a vial under gently agitating conditions. Subsequently these were again washed with distilled water to remove traces of detergent. After washing, nodal explants were surface sterilized by soaking in 0.1% $HgCL_2$ for 15 minutes, followed by repeated washing with sterilized water to remove the traces of sterilants.

Culture media

For induction of morphogenesis and proliferation two types of media viz. MS (MURASHIGE and SKOOG, 1962) and B5 (GAMBORG *et al*., 1968) containing 3% and 2% sucrose (as carbohydrate source) were used respectively. For rooting $^1/_2$ strength MS medium containing 2.5% sucrose was used. The concentration of sucrose was reduced to make the plant more autotrophic (DHAWAN and BHOJWANI, 1985). The medium was gelled with 0.7% bacteriological agar (RANBAXY). The pH of medium was adjusted to 5.8 by using 1N NaOH or 1N HCl prior to adding agar. The culture medium was autoclaved at 121° C and 1.0 x 10^5 Pa for 15 minutes.

Table 1. – Effect of MS and B5 media supplemented with different PGR's on percentage of explants showing bud break and number of shoots per explant after 30 days of inoculation.

Sl.No.	PGR Conc. mg/l			Percentage of explant showing bud break after 30 days		Number of shoots per explant after 30 days (Mean ± SE)	
	BAP	IAA	NAA	MS	B5	MS	B5
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.10	–	-	58.34	87.57	1.50 ± 0.24	1.00 ± 0.00
2	0.25	-	-	79.17	91.67	2.00 ± 0.47	1.00 ± 0.00
3	0.50	-	-	62.50	70.84	2.34 ± 0.27	1.00 ± 0.00
4	1.00	-	-	50.00	62.50	2.84 ± 0.36	2.50 ± 0.35
5	0.10	0.10	-	66.67	70.84	1.70 ± 0.29	1.50 ± 0.24
6	0.25	0.10	-	70.84	83.34	2.00 ± 0.47	1.84 ± 0.37
7	0.50	0.10	-	54.17	66.67	2.40 ± 0.57	2.00 ± 0.47
8	1.10	0.10	-	45.84	58.34	2.84 ± 0.83	2.07 ± 0.52
9	0.10	0.25	-	58.34	62.50	2.00 ± 0.47	1.70 ± 0.29
10	0.25	0.25	-	79.17	87.50	2.34 ± 0.55	2.46 ± 0.61
11	0.50	0.25	-	62.50	75.00	2.67 ± 0.72	2.40 ± 0.77
12	1.00	0.25	-	70.84	79.17	3.05 ± 0.55	2.73 ± 0.31
13	0.10	0.50	-	33.34	41.67	1.84 ± 0.36	1.50 ± 0.24
14	0.25	0.50	-	37.50	45.84	2.00 ± 0.47	1.67 ± 0.27
15	0.50	0.50	-	50.00	54.17	2.34 ± 0.72	1.94±0.43
16	1.00	0.50	-	41.67	50.00	3.00 ± 0.82	2.07 ± 0.52
17	0.10	-	0.10	79.84	75.00	2.00 ± 0.47	1.84 ± 0.36
18	0.25	-	0.10	83.34	91.67	2.64 ± 0.70	2.04 ± 0.50
19	0.50	-	0.10	66.67	70.84	2.84 ± 0.83	2.40 ± 0.77
20	1.00	-	0.10	58.34	66.69	3.17 ± 0.59	2.84 ± 0.36
21	0.10	-	0.25	62.50	70.84	2.40 ± 0.57	1.64 ± 0.26
22	0.25	-	0.25	100.00	100.00	2.84 ± 0.36	2.17 ± 0.59
23	0.50	-	0.25	87.50	91.67	3.00 ± 0.47	2.37 ± 0.75
24	1.00	-	0.25	75.00	83.34	3.67 ± 0.98	3.00 ± 0.87
25	0.10	-	0.50	37.50	41.67	2.27 ± 0.67	1.80 ± 0.34
26	0.25	-	0.50	41.67	50.00	2.50 ± 0.41	2.00 ± 0.47
27	0.50	-	0.50	54.17	62.50	2.84 ± 0.36	2.40 ± 0.77
28	1.0	-	0.50	50.00	58.34	3.17 ± 0.59	2.60 ± 0.49

– Denotes absence of particular hormone.

For multiplication of the cultures MS and B5 media supplemented with cytokinin (BAP 0.1 to 1.0 mg/l) and auxins (IAA and NAA 0.1 to 0.5 mg/l) were used (*Table 1*). Multiple shoots obtained from already established cultures were further subcultured in the multiplication medium. For this, a clump of two to three shoots were subcultured on to fresh medium in conical flask (250 ml). *In vitro* grown shoots measuring 2.0 to 2.5 cm in length were excised and kept on $^1/_2$ strength MS medium with different concentrations (0.10 to 1.50 mg/l) of auxins (IAA, IBA and NAA) for rooting (*Table 4*). In all the experiments 24 replicates were maintained on medium for shoot initiation and 26 replicates for each treatment for rooting. Subculturing was carried out at periodic intervals of 30 days.

Culture conditions

All the cultures were incubated in a culture room maintained at 23 ± 1°C for 16 hours in light (illuminated by 40 watt cool white fluorescent tubes, 1200 lux) and for 8 hours in dark.

Acclimatization

Plantlets transferred from culture tubes were hardened *in vitro* by placing them on liquid $^1/_4$ strength MS medium having 2% sucrose, for 7 days, and thereafter, they were transferred to polybags containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags in shade. The polythene bags were periodically withdrawn to acclimatize the plantlets and when new leaves started emerging the polybags were completely removed

Statistical Analysis

The data collected were subjected to statistical analysis to find out the significance of differences observed between the treatments. Wherever data were recorded as percentages, the percentage values were transferred to arc sine prior to carrying out Analysis of Variance (ANOVA). Critical Difference (CD) between the treatments was also calculated.

Results

Establishment and multiplication of cultures:

Bud break was achieved in both the media (MS and B5) within 6-8 days in different media combinations (*Fig. 1, a* and

Table 2. – Results of statistical analyses for percentage of explants showing bud break.

Sl. No.	Source of Variation	Mean values	CD	Significance level
1	Media	B_5 > MS 58.42 48.35	0.158	***
	BAP	B_2 B_1 B_3 B_4 62.85 54.97 51.51 44.21	0.103	***
2	Media	B_5 > MS 49.68 44.34	0.176	***
	IAA	I_2 I_1 I_3 53.68 49.58 37.76	0.269	***
	BAP	B_2 > B_3 > B_4 > B_1 51.04 46.94 46.05 44.01	0.355	***
3	Media	B_5 > MS 54.54 50.22	0.351	***
	NAA	N_2 > N_1 > N_3 63.11 53.98 40.25	0.536	***
	BAP	B_2 > B_3 > B_4 > B_1 59.72 54.32 48.94 46.51	0.707	***

B_1 = BAP (0.10 mg/l)
I_1 = IAA (0.10 mg/l)
N_1 = NAA (0.10 mg/l)
B_2 = BAP (0.25 mg/l)
I_2 = IAA (0.25 mg/l)
N_2 = NAA (0.25 mg/l)
B_3 = BAP (0.50 mg/l)
I_3 = IAA (0.50 mg/l)
N_3 = NAA (0.50 mg/l)
B_4 = BAP (1.0 mg/l)

*** Significant at 0.1% level of probability

b). Maximum percentage of bud break (100%) was achieved both in case of MS and B5 wherein the medium was supplemented with 0.25 mg/1 BAP+ 0.25 mg/l NAA. Callus formation was also observed at the base of the nodal explants. To achieve the maximum rate of proliferation different hormone combinations were used with the two nutrient media (*Table 1*). Only three combinations of MS medium supplemented with PGRs viz. 1.0 mg/l BAP + 0.10 mg/l NAA, 1.0 mg/l BAP + 0.25 mg/l NAA and 1.0 mg/l BAP + 0.50 mg/l NAA were found to be the best based on the observations recorded on number of shoots

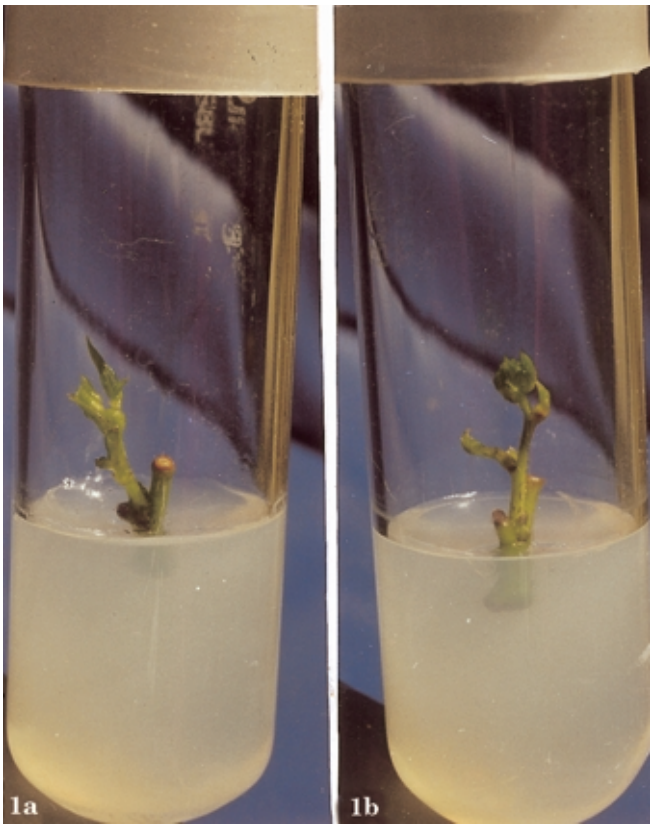

Figure 1. – Bud break in nodal explant, a) MS medium, b) B5 medium.

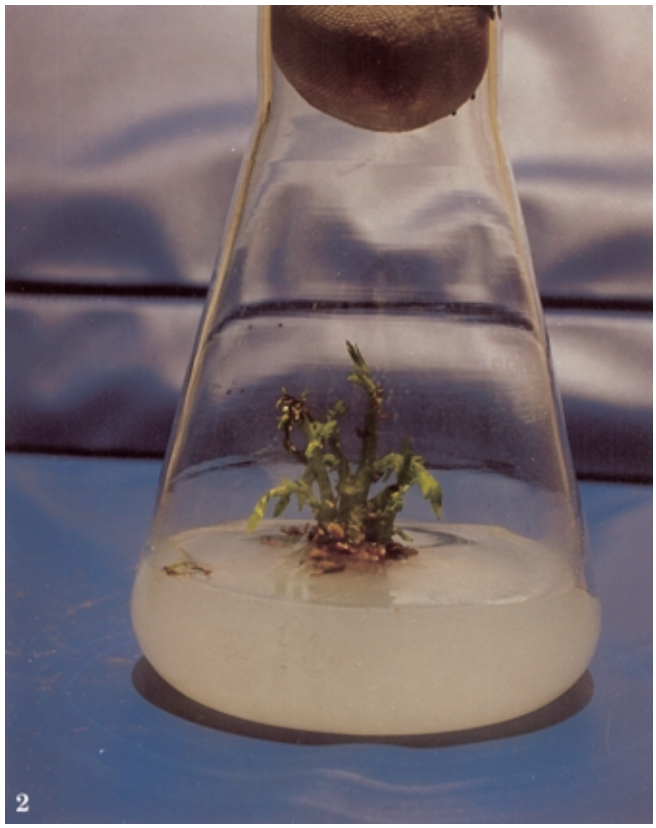

Figure 2. – Multiple shoot formation in MS + BAP (1.0 mg/l) + NAA (0.25 mg/l) medium after 30 days.

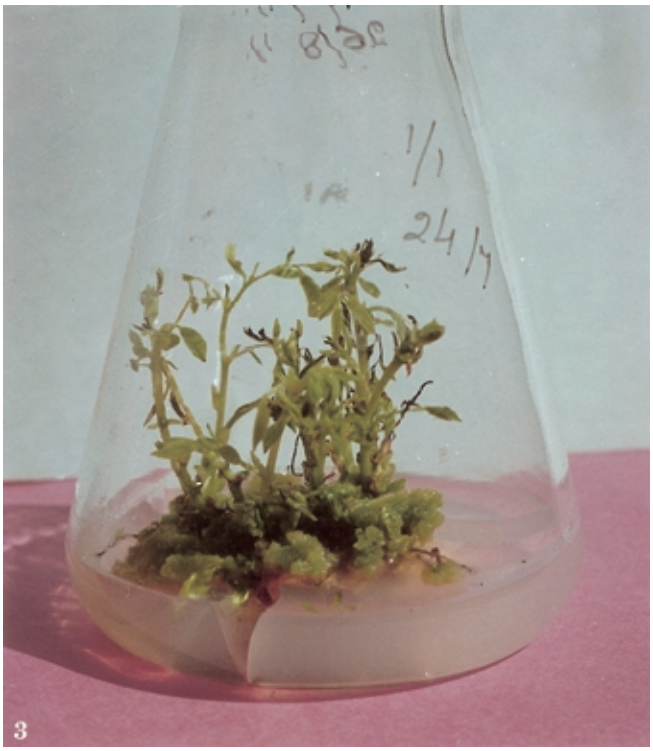

Figure 3. – Multiplication of shoots in MS + BAP (1.0 mg/l) + NAA (0.25 mg/l) medium after 150 days.

Figure 4. – In-vitro rooting in $^{1}/_{2}$ MS + BAP (1.0 mg/l) medium.

Table 3. – Mean ± SE Number of shoots per explant over a five-month subculture period on MS medium.

Sl. No.	Days	BAP (1.0 mg/l) + NAA (0.10 mg/l)	BAP (1.0 mg/l) + NAA (0.25 mg/l)	BAP (1.0 mg/l) + NAA (0.50 mg/l)
1	60	3.50±0.48	4.00±0.59	3.75±0.39
2	90	3.78±0.51	5.81±0.72	4.06±0.52
3	120	4.94±0.47	7.45±1.05	5.56±0.59
4	150	5.15±0.49	8.04±1.05	5.89±0.48
5	180	5.08±0.48	7.92±0.92	5.75±0.39

Table 4. – Effect of different auxins and their concentrations on *in-vitro* rooting.

Sl. No.	PGR Conc. mg/l		Rooting percentage	Mean ± SE Number of roots per shoot	Mean ± SE length of root per shoot (mm)	No. of days taken for rooting
Control	0.00		0.00	0.00	0.00	0.00
1	IBA	0.10	38.46	2.74±0.25	24.84±1.05	20
		0.50	53.85	3.15±0.32	25.17±1.38	20
		1.00	76.92	4.47±0.35	25.50±1.89	18
		1.50	46.15	2.50±0.28	17.88±1.58	20
2	NAA	0.10	23.07	2.47±0.26	21.14±1.68	25
		0.50	38.46	2.57±0.29	20.87±1.71	25
		1.00	69.23	3.04±0.31	22.80±1.89	25
		1.50	30.77	2.27±0.23	17.42±1.68	25
3	IAA	0.10	15.38	1.58±0.27	15.17±1.29	25
		0.50	30.77	2.42±0.29	15.87±1.38	25
		1.00	61.54	2.58±0.31	16.24±1.51	25
		1.50	23.07	1.50±0.19	16.08±1.38	25

developed per explant after 30 days (*Fig. 2*). Rate of multiplication increased up to 150 days in all the treatments.

Maximum number of shoots per explant (8.04) was observed in the MS medium supplemented with 1.0 mg/l BAP + 0.25 mg/l NAA after incubation for 150 days (*Table 3, Fig. 3*). However, corresponding values for number of shoots developed per explant in other two formulations viz MS + 1.0 mg/l BAP + 0.10 mg/l NAA and MS + 1.0 mg/l BAP + 0.50 mg/l NAA were 5.15 and 5.89 respectively. After 4th subculturing (150 days) a slight decrease in regeneration capacity of shoots was observed in all the aforementioned three combinations (*Table 3*).

Rooting

In vitro grown shoots measuring 2.0 to 2.5 cm were excised and put on to 1/2 strength MS medium supplemented with IBA, NAA or IAA individually with different concentrations (0.10, 0.50, 1.0 and 1.50 mg/l). Maximum rooting percentage (76.92 %) was achieved in IBA (1.0 mg/l) without intervening callus phase (*Table 4, Fig. 4*).

Maximum number of roots per plantlet (4.47) was observed wherein 1/2 MS medium was supplemented with 1.0 mg/l IBA. As compared to this auxin none of the other two auxins used were found much effective to induce rooting. The best rooting length was also observed in 1.0 mg/l IBA within 18 days. It was also observed that maximum number of roots per plantlet (4.44) as well as maximum root length was achieved in the same concentration of auxin (IBA 1.0 mg/l). An increase in concentration of auxins beyond 1.0 mg/l decreased the percentage of rooting. Furthermore, induction of callus was observed under higher concentration i.e. 1.50 mg/l of auxins. The differences observed with regard to rooting percentage, number of roots/plantlet and number of days taken for rooting due to different auxins and their concentrations were found to be statistically significant (*Table 5*) except for root length wherein effect of different concentrations was found to be non-significant.

An early induction of rooting was observed in IBA (0.10 to 1.50 mg/l) and the rooting could be achieved within 18 to 20 days. So far as the number of days taken for rooting is concerned the eight various formulations tried with NAA and IAA (0.10 to 1.50 mg/l) gave similar results and rooting took place with in 25 days.

Overall 1.0 mg/l IBA proved to be the best among the three auxins tried for all rooting parameters.

Discussion

In the present study, the percentage of explants showing bud break was higher as well as shoot length was better in B5 medium as compared to MS medium. However, so far as the mean number of shoots per explant under different hormonal combinations is concerned MS medium was found better. Berger and Schaffner 1995 while working on micropropagation of a leguminous tree species *Swartzia madagascariensis* also found more number of shoots per explant on MS and WP nutrient media as compared to B5. This indicates that for culture establishment and shoot length B5 medium is better than MS. Several other workers (Tremblay and Lalonde, 1984; Chalupa, 1992) also observed superiority of MS medium over other media. Tremblay and Lalonde (1984) also observed highest multiplication rate on MS medium in *Alnus crispa* and *A. glutinosa*.

For *in vitro* propagation of *D. sissoo* initially two cytokinins viz. BAP and Kn were used. However, the response of nodal explants to bud break was very poor when media were supplemented with Kn. These findings are in agreement with the results observed by Suwal et. al. (1988) as they reported that Kn used individually or in combination with NAA gave rise to only callus tissue when cotyledon node of *D. sissoo* was used as an explant.

In the present study callusing at the base increased with advancement in subculturing. Dewan et al. (1992) and Ahlawat (1993) also reported formation of callus at the base of explants. During clonal propagation of *Albizia procera* multiple shoots were obtained on MS medium without callus formation after 30 days but callus was also formed after 60 days (Roy and Datta, 1985). In the present study explants raised on MS medium produced larger amount of callus than those raised on B5 medium. However, like for *Acacia auriculiformis* (Mittal et al., 1989) the presence or absence of callus did not seem to have any effect on the multiplication of shoots of *D. sissoo* as this callus is of non-regenerative type.

It was observed that after 4th subculture i.e. after 150 days, multiplication rate decreased slightly. The gradual decline in the frequency of shoot development in *D. sissoo* as also reported earlier for *Acacia nilotica* (Dewan et al., 1992) and *Albizia*

Table 5. – Results of statistical analysis for rooting parameters.

Sl. No.	Character	Source of Variation	Bar Diagram of Mean Values	CD	Significance level
1.	Rooting Percentage	Auxin	IBA > NAA > IAA 47.47 40.45 34.18	0.395	***
		Conc.	T_3 > T_2 > T_4 > T_1 56.15 40.72 34.97 30.96	0.522	***
2.	Number of Roots	Auxin	IBA > NAA > IAA 2.48 2.16 1.66	0.268	***
		Conc.	T_3 > T_2 > T_1 > T_4 2.46 2.13 1.94 1.88	0.354	*
3.	Length of root	Auxin	IBA > NAA > IAA 18.29 16.32 14.28	1.977	***
		Conc.	T_3 > T_2 > T_1 > T_4 17.49 16.63 16.03 15.04	-	NS
4.	Number of days taken for rooting	Auxin	IAA > NAA > IBA 23.25 22.58 18.84	0.275	***
		Conc.	T_1 = T_2 > T_4 > T_3 21.89 21.89 21.67 20.78	0.133	***

Concentration of auxins T_1 = 0.10 mg/l,
T_2 = 0.50 mg/l, T_3 = 1.0 mg/l, T_4 = 1.50 mg/l,
* significant at 5% level of probability,
** significant at 1% level of probability,
*** significant at 0.1 % level of probability
NS not significant.

procera (AHLAWAT, 1993) is indicative of a gradual loss of the morphogenic potential concomitant with advance in culture. This may probably be due to the endogenous levels of certain factor/s, inherited with the explant, which is/are gradually diluted with each passage of subculturing. It has been observed that shoots of *D. sissoo* require subculturing approximately every 25–30 days. If subculturing is delayed beyond the optimum (which was 30 days), leaves abscise, shoot-tips become necrotic, and brown phenolic compounds are released into the medium. MAYNARD *et al.* (1991) faced the same problem of subculturing during *in vitro* propagation of Black Cherry (*Prunus serotina* EHRH.).

Rooting was observed in three auxins viz. IAA, IBA and NAA. Overall IBA proved to be the best as compared to the other auxins with regard to all the rooting parameters. In our experiments 76.92% rooting was observed in $^1/_2$ MS with 1.0 mg/l IBA followed by 69.23% and 61.84% in NAA and IAA respectively (*Table 4*). Documented literature shows that IBA has been found superior for rooting in a number of leguminous tree species like *Bauhinia variegata* and *Parkinsonia aculeata* (MATHUR and MUKUNTHAKUMAR, 1992); *Sesbania sesban* (SUMAN *et al.,* 1990); *Dalbergia lanceolaria* (DWARI and CHAND, 1996) etc. The time taken for rooting in terms of number of days was reduced in IBA as compared to IAA and NAA in *D. sissoo.* PERINET and LALONDE (1983) obtained similar results while working with *Alnus glutinosa.* They observed that presence of IBA in the rooting medium enhanced the number of roots per shoot and reduced the time period required for 100% rooting from 28 days for the auxin free control to 14–21 days for the auxin treatment.

It was observed that higher concentration of auxins i.e. 1.50 mg/l caused reduction in all the rooting parameters. Similar results were also reported by PRABHA *et al.* (1997) in Eucalyptus hybrid (*E. camaldulensis* x *E. tereticornis*), though no numerical data were provided.

Most of the papers relating to in vitro propagation of *D. sissoo* have dealt with explant taken from seedlings (SUWAL *et al.,* 1988) and there exist only few reports on micropropagation wherein explants were taken from mature trees (GILL and GILL, 1993; DATTA and DATTA, 1983; DAS *et al.,* 1997) and from coppice shoots (GULATI and JAIWAL, 1996). Although it is easy to develop a protocol from seedlings but the plants identified for desirable characters cannot be produced, thus the method reported herein for *in vitro* propagation of *D. sissoo* using mature explants will help in mass multiplication of superior phenotypes for desired traits and boosting the productivity per unit area/unit time. Further, this would also help as an aid in tree improvement programmes such as establishment of clonal seed orchards.

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