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RESEARCH ARTICLE

IN VITRO REGENERATION OF *DALBERGIA SISSOO* ROXB. FROM CALLUS OF COTYLEDONARY EXPLANTS

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ABSTRACT

The *in vitro* studies with *Dalbergia sissoo* showed that the callus raised from cotyledonary explants had only the potential for shoot regeneration rather than the callus raised from nodal explants in provided medium. Best callus induction was observed on MS supplemented with 4.0 mg/l BAP, 0.1 mg/l NAA and 3% sucrose through nodal explants, but callus induction in cotyledonary explants was showed on 2.0 mg/l 2, 4-D, 2.0 mg/l 2, 4-D and 0.05 mg/l Kn. Callus of both explants were subcultured 3-4 time on 4.0 mg/l BAP and 0.1 NAA for multiplication and to increase the compactness of callus. Further callus of both the explants were transferred on different combination of BAP and Kn with and without adenine sulphate and casein hydrolysate for shoot emergence. Shoot regeneration was observed on MS with 2% sucrose supplemented with 4.0 mg/l Kn, 2.0 mg/l BAP, 10.0 mg/l Adenine sulphate with casein hydrolysate only in case of the callus of cotyledonary explants. For rooting 3-4 cm long shoots were inoculated on ½ MS medium augmented with IBA (0.5, 1.0 and 2mg/l). All rooted plantlets were hardened and successfully transferred to the soil.

INTRODUCTION

Shisham (*Dalbergia sissoo* Roxb.), a deciduous tree of family Paplionaceae, is an important plant of great economic importance. It is cultivated in forest plantations as well as along the canals, roadsides, railway lines, water channels and boarders of the agricultural fields. Shisham wood is used in furniture, construction work, agriculture implements plywood industries and fuel purposes. Trees will always be attacked by a range of organisms that cause decline. It is well established fact that Shisham is facing severe threat of Die back, due to the unidentified causes. In this situation top leaves turn light yellow, bark and collar regions turns brown by giving rot like appearance so can be easily peeled off. Withering of individual branches is also observed from top to down (Khan, 1961). Dieback disease has caused wide-spread mortality of *D. sissoo* throughout South Asia, and trees of all ages from saplings to mature trees are affected. Due to high caloric content the wood is an excellent source of fuelwood and charcoal. It is valued for its ability to increase soil fertility through fixation of atmospheric nitrogen and is commonly planted as an ornamental, windbreak and shade tree (Thirunavoukkarasu, 2010). The propagation of the *D. sissoo* through seed is unreliable due to poor germination and death of the young seedlings under natural environmental condition (Anonymous, 1989).

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Micropropagation of *D. sissoo* via different plant parts has been reported for example, nodal segments (Iyer *et al*., 2009), axillary buds (Dawara *et al*., 1984) derived from mature trees, a cotyledonary node from seven-day axenic seedling (Lal and Singh, 2012), zygotic embryo (Husaini *et al*., 2008) and callus derived from semi-mature zygotic embryos (Singh and Chand, 2003). Due to high timber-yielding values and many medicinal properties, the plant is being over-exploited in recent years. The conventional method for the propagation of *D. sissoo* through seed is unreliable due to poor germination and death of the young seedlings under natural environmental condition. The efficiency of reproduction is also found to be less seed viability and lack of vegetative propagation methods (Ali *et al*., 2012). In present study regeneration was achieved through callus derived from immature cotyledonary explants.

MATERIALS AND METHODS

For semi-mature cotyledons, healthy green pods were collected from a 30-year old elite tree of *D. sissoo.* Nodal parts and semi-mature cotyledons were used as explants. Both the explants were washed under running water for 30 minute, treated with an aqueous solution of Tween 20 (HIMEDIA, Mumbai, active ingredient-Polysorbitol 20) for 5 min and then rinsed with distilled water 3-4 times. The explants then disinfected with 0.1 % (w/v) aqueous mercuric chloride for 8-10 min and further rinsed with sterile distilled water 3-4 times in laminar air flow cabinet. After removing the seed coat, the immature cotyledons

were excised and cultured on semi-solid medium containing MS salts and vitamins (Murashige and Skoog, 1962) with 3% (w/v) sucrose with different concentration of auxin and kinetin. The pH of the medium was adjusted to 5.7 with 1 N NaOH or 1N HCl prior to adding agar 0.8% w/v as a basal medium was dispensed in to culture flask plugged with non-absorbant cotton and sterilized at 121º C at 1.06 Kg/cm^2 pressure for 15 min. The culture maintainence by regular subculturing at 3-week intervals to fresh medium with the same hormone supplemented medium. Cotyledonary explants of *D. sissoo* were inoculated on 2.0 mg/l 2, 4-D with and without 0.05 mg/l Kn for callus formation, callus from nodal explants were induced on 4.0 mg/l BAP with 0.08, 0.1, 0.8 and 2.0 mg/l NAA and for subculturing of callus of cotyledonary and nodal explants, 4.0 mg/l BAP with 0.1 mg/l NAA was used, every fourth week subcultures was done. For shoot regeneration, callus of both the explants transferred on MS medium supplemented with 4.0 mg/l Kn and 2.0 mg/l BAP with 2.0% sucrose, with and without adenine sulphate (10.0 mg/l) and casein hydrolysate (5.0, 10.0 mg/l).

RESULTS AND DISCUSSION

The callus growth response from cotyledonary explants was carried out on 2.0mg/l 2, 4-D with and without 0.05 mg/l Kn suppemeneted and from nodal explants on 1.0-4.0 mg/l BAP with 0.08 to 2.0 mg/l NAA supplementation. In all the sets, callus induction was noted to occur in 10-15 days, however callus was fragile and pale yellow, moderate in amount with 68-70% frequency in case of cotyledonary explants and callus derived from nodal explants was green with 76-96 % frequency.

With increasing concentration of BAP from 1.0 to 4.0 mg/l along with 0.08 to 2.0 mg/l NAA callus turned to compact from fragile and also increased in amount. Modification in concentration of NAA from 0.08 to 0.1 mg/l maintained the same type and degree of response of callus but further increase in NAA concentration by 10 times or 20 times led to lesser amount of callus growth, though the type remained the same, compact green (Table-1).

Shoot differentiation was recorded on MS (2% sucrose) supplemented with 4.0 mg/l Kn, 2.0 mg/l BAP, with addition of 10.0 mg/l adenine sulphate and also with 5.0 mg/l casein hydrolysate, after 4-month of culture. Shoot foundation was noted to be about 2.51±0.78 without adenine sulphate or 2.40±0.89 in sets supplemented with 10.0 mg/l adenine sulphate along with 5.0 mg/l casein hydrolysate. Further increase in casein hydrolysate to 10 mg/l reduced the number of shoots to 1.49±0.85. In 1.0 mg/l BAP with 2.0 mg/l Kn and 3% sucrose supplemented sets, 1.98±0.66 were observed which reduced to nil if BAP increased to double concentration with 1.0 mg/l Kn (Fig:-1). However, on MS (3% sucrose) augmented with 1.0 mg/l Kn, 2.0 mg/l BAP showed 1.78±0.54 number of shoots (Table-2). For elongation of shoots 2.0mg/l BAP supplemented with 10 mg/l Kn and 0.1 % PVP was used. The differentiation of shoot was noticed only in callus generated by the cotyledonary explants, while no shoot differentiation was observed in callus generated by nodal explants. The differential response of differentiation could be due to the varying concentration of the growth regulators used in the medium and the explants types (Rout and Das, 1997).The callus attached with regenerated shoots shows browning and shoot also showed necrosis with time. Frequent subculturing and addition of 0.1% PVP (antioxidant) is

Table 1. Effect of different concentrations of auxin and cytokinins on callus formation from cotyledonary and nodal explants of *D. sissoo*

S. No.	Explant type	MS +Phytohormones	Day of response	Morphology of callus	Degree of Response	Callus Frequency (%)
1.	Immature cotyledons	2 2,4-D	10	Fragile and Pale Yellow	++	68
2.	Immature cotyledons	2 2,4-D+0.05 Kn	10	Fragile and Pale Yellow	++	70
3.	Nodal part	1.0 BAP+0.1 NAA	15	Compact and Green	++	79
4.	Nodal part	2.0 BAP+0.1NAA	15	Compact and Green	++	86
5.	Nodal part	3.0 BAP+0.1 NAA	15	Compact and Green	+++	88
6.	Nodal part	4.0 BAP+0.1 NAA	12	Compact and Green	+++	96
7.	Nodal part	4.0 BAP+0.08 NAA	12	Compact and Green	+++	86
8.	Nodal part	4.0 BAP+0.8 NAA	12	Compact and Green	++	88
9.	Nodal part	4.0 BAP+2.0 NAA	15	Compact and Green	++ ++	76

Table 2. Effect of different concentrations of cytokinins with and without ADS and CH on shoot regeneration using callus of cotyledonary explants of *D. sissoo*

S. No.	Medium (MS) + Phytohormones + AS (mg/l) + CH (mg/l)	Mean No. of shoots ± SD	Mean Shoots length (cm)± SD
1.	4.0 Kn+2.0 BAP+ 2% Sucrose	2.51±0.78	2.24±0.77
2.	4.0 Kn+2.0 BAP+ 2% Sucrose +10 AS	1.53±0.67	2.02±0.92
3.	4.0 Kn+2.0 BAP+ 2% Sucrose +10 AS + 5.0 CH	2.40±0.89	2.23±0.84
4.	4.0 Kn+2.0 BAP+ 2% Sucrose +10 AS + 10.0 CH	1.49±0.85	1.61±0.72
5.	2.0 Kn+1.0 BAP + 3% Sucrose	1.98±0.66	1.23±0.86
6.	2.0 Kn+2.0 BAP + 3% Sucrose	-	-
7.	1.0 Kn+2.0 BAP + 3% Sucrose	-	-
8.	1.0 Kn+2.0 BAP + 3% Sucrose	1.78±0.54	1.41±0.58

Table 3. Effect of Auxins on *in vitro* rooting from regenerated shoots of *D. sissoo*.

S. No.	½ MS medium with PGR concentration (mg/l)	Root induction time (days)	Rooting percentage	Mean root length (cm) ± SD
1.	0.5 IBA	12	52	2.11±0.71
2.	1.0 IBA	12	68	2.53±0.67
3.	2.0 IBA	12	80	2.97±0.98

required to avoid shoot necrosis. ADS had no effect on leaf retention, and at higher concentration of these compounds the growth of the shoots was poor (Raghawa swamy, 1992). Calcium deficiency is said to cause shoot necrosis which usually results in a weak shoot with distorted or poorly developed leaves. Increased calcium levels in the medium reduced shoot necrosis in several temperate trees (Sha *et al*., 1985).

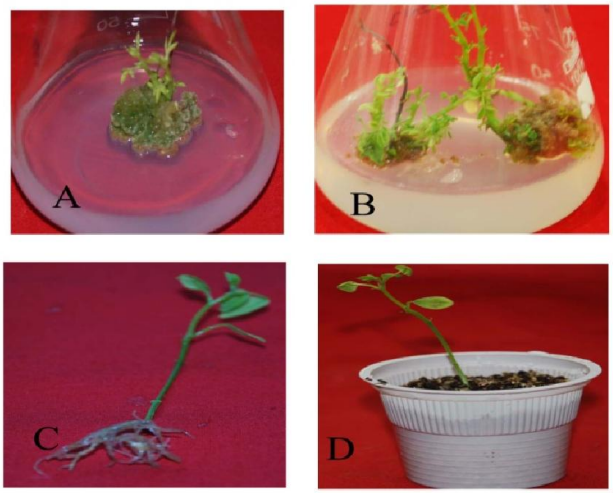

Figure 1. *in vitro* micropropagation and hardening of *D. sissoo*
A. shoot regeneration from callus;
B. Elongation of shoot;
C. Root induction and ;
D. plastic cup containing regenrated plantlets

In vitro regenerated shoots (4-5 cm long) from callus of cotyledonary explants of *D. sissoo*, separated from callus and transferred to the rooting medium. Half strength semi solid MS medium supplemented with IBA (0.5, 1.0, 2.0 mg/l), 3% sucrose were used for rooting. Maximum response was shown on 2.0 mg/l IBA compared to the 1.0 mg/l IBA and 0.5 mg/l (Table-3). Roots on 2.0 mg/l IBA were healthy and long, number of roots was also high. High frequency of rooting was achieved by IBA in *Aristolochia indica* (Manjula *et al*, 1997), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Avicinnia marina* (Al-Bahrany and Al-Khayri, 2003) and *Eclipta alba* (Baskaram and Jayabalan, 2005) also. The rooted plantlets were acclimatized and transferred in plastic pots containing sterile vermiculite, soil and compost in 1:1:1 ratio and covered with transparent polythene bags to ensure high humidity condition.

Intermittently the plantlets were watered with half strength MS-salt solution free of sucrose at alternate days. The plants were maintained in culture room for about 15 days. The polythene bags were removed and then plants were transferred to pots containing soil and compost in 1:1 ratio, further watered with tap water and brought to room temperature and shady climate (greenhouse) for about 15 days. Finally they were transferred and maintained in direct sunlight under field conditions.

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