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Micropropagation of *Dalbergia sissoo* Roxb. Through Tissue Culture Technique

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Abstract: Multiple shoots of *Dalbergia sissoo* Roxb. (Sissoo) were incited from seeds through indirect somatic embryogenesis method. Seeds were inoculated in Murashige and Skoog's medium without any growth hormone. Than cotyledonary leaves were struck and used for callus induction on MS medium amplified with 2, 4-dichlorophenoxyacetic acid (0.5 to 4 mg mL^{-1}). After 3 to 4 weeks the embryogenic callus clumps was transferred to medium supplemented with cytokinin (BAP 1 to 5 mg L^{-1}, kinetin 1-5.0 mg L^{-1}) for embryo maturation and germination. The high-frequency shoot proliferation (82%) and maximum number of shoots per explants were recorded in MS medium containing NAA (0.5)+BAP (0.5). The findings of recent investigations have shown that, it is possible to induce indirect somatic embryogenesis in *Dalbergia sissoo* and plant regeneration from callus cultures derived from cotyledonary leaves as explants.

Key words: *Dalbergia sissoo*, micropropagation, explants, seeds

INTRODUCTION

The solicitation of modern biotechnology techniques like tissue culture has verified to be a powerful tool augmenting forest. Basically, tissue culture is a technique by which small pieces of a plant regenerate to a complete plant in a nutritional media under complete aseptic conditions. These explants segregate and progressively develop either into an unorganised mass of cells called callus and subsequently discriminate to form plants or directly bestowed rise to shoots or embryos. Due to the increase in economical importance of leguminous forest trees impels the application of *in vitro* micropropagation technique for multiplication of these trees (Parveen *et al*., 2010). During the last few years, many leguminous woody trees have been successfully *in vitro* micropropagated using different young as well as mature plant parts (Borthakur *et al*., 2012).

Dalbergia sissoo is one of the most influential tree legumes of the Indian subcontinent and is esteemed mainly for its wood. *Dalbergia sissoo* prevalently confessed as sheesham or Indian rosewood (family Fabaceae or Legumenosae), is a passably fast-growing multipurpose deciduous tree (Thirunavoukkarasu *et al*., 2010). It is an important timber-yielding tree. Because of its great strength, durability and elasticity, the wood is used for making furniture, cabinets, musical instruments, ornamental veneers and high-quality commercial plywood (Lal and Singh, 2012). Bulk of this species is a good source of foreign exchange to the Country. *Dalbergia sissoo* is commonly planted as an ornamental, windbreak, shade tree. The wood of *D. sissoo* is an excellent source of fuel wood and charcoal due to the presence of high caloric content. It is also useful in increasing soil fertility through fixation of atmospheric nitrogen (Thirunavoukkarasu *et al*., 2010).

D. sissoo also have many medicinal properties. It is used as an aphrodisiac, abortifacient, expectorant, anthelmintic and antipyretic. It is also useful in conditions like emesis, ulcers, leucoderma, dysentery, stomach troubles, skin diseases, blood diseases, syphilis, stomach problems, dysentery, nausea, nose disorders and expectorant. Different parts such as roots, bark, wood, leaves and seeds are being used as the remedy in many diseases. *D. sissoo* is antidiarrhoeal as it affects bacterial virulence. *D. sissoo* is a folk remedy, especially for excoriations, gonorrhoea and skin ailments (Shah *et al*., 2010).

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 semi mature cotyledons

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(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. sisso* 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).

Hence, the present study has been intended to improve a stable and consistent procedure of this arrogant plant. That could be used for mass propagation of this plant species to contest the increasing demand from the timber industry as well as for the conservation of germplasm.

MATERIALS AND METHODS

Explants preparation: Immature green seeds of *Dalbergia sissoo* were collected from mature tree at (6-9 year old) 120 days after flowering. Seeds were washed under running tap water to remove dust particles for 15 min and treated with liquid detergent for 5 min and rinsed three times with distilled water. Further, sterilization treatments were done under a laminar-flow chamber. The explants were then disinfected with 0.1% (w/v) mercuric chloride for 8 min under aseptic conditions. After this these explants were then thoroughly washed 3-4 times with sterilized double distilled water to remove the traces of mercuric chloride now the explants is ready for inoculation on required medium.

Inoculation in culture medium and condition: These sterilized seeds were inoculated on MS medium without any growth hormone for seed germination. From 8 to 10 days old *in vitro* seedling the cotyledonary leaves were excised and transfer to medium containing 0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1} 2, 4-D for callus induction. The pH of the medium was adjusted to 5.75 with 0.1 N NaOH or 0.1 N HCl solution prior to adding 0.8% (w/v) agar. The cultures were incubated at a temperature of 25±2°C and a photoperiod of 16 h light (intensity of 2000-3000 lux). The cultures were observed after one week. Embryogenic callus clumps were formed after 2 to 3 weeks. These clumps were transferred to MS zero media for somatic embryo development.

Subculturing for germination and multiplication: After 3 to 4 week the initiated somatic embryos was transferred to the medium supplemented with cytokinins 1.0, 3.0 and 5.0 mg L^{-1} BAP, 1.0, 3.0 and 5.0 mg L^{-1} Kn and combination of both 0.5 mg L^{-1} BAP+0.5 mg L^{-1} Kn and 0.5 mg L^{-1} BAP+1.0 mg L^{-1} Kn for embryo maturation and initiation of shoots. The initiated shoot was again transfer for further multiplication in the medium containing different concentration of Auxin 1.0 mg L^{-1} IAA, 1.0 mg L^{-1} IBA and cytokinin+auxin combination 1.0 mg L^{-1} 2,4-D+1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA+0.5 mg L^{-1} BAP, 1.0 mg L^{-1} NAA+1.0 mg L^{-1} BAP.

Rooting of shoots and transfer of plantlets to soil: Shoots of 2-3 cm in height with two or three leaflets derived from cultures were transferred to MS medium containing 1 mg L^{-1} NAA+20 g sucrose or IAA or IBA or 200 mg activated charcoal separately for rooting. The rooted shoots were transferred to MS medium for further elongation of the roots. The rooted plants were washed with water to remove media then transferred to pots containing autoclaved vermiculite soil and sand (1:2:1) and covered with polyethylene bags for one week to maintain high humidity and subsequently exposed to low air humidity for increasing period and finally polyethylene bags were removed. These hardened plants then transferred to the greenhouse (Das, 2011; Chinnaraj and Malimuthu, 2011).

RESULTS

In the current investigation, indirect somatic embryogenesis induced from *in vitro* raised seedlings by which cotyledonary leaves were used as explant. The seed germinated within 6-7 days of inoculation on MS medium (without any growth hormone or zero media).

It was perceived to affect the number of somatic embryos provoked per explant as well as the number of days taken for induction. The cotyledonary leaves show the production of callus from the Peripheral surface with in 22-25 days. First the callus is initiated and multiplied in auxin rich media called Proliferation Medium (PM). The maximum percentage of callus initiation was reported in the medium containing 2, 4-D (2.0-3.0 mg L^{-1}) within 3-4 weeks. The fresh white and fragile callus was obtained shown in Table 1.

The embryogenic callus clumps were transferred from high 2, 4-D containing medium MS medium supplement with cytokinins for development of embryos embryogenic clumps developed into embryo when it transferred into medium with zero or low level of auxin this medium called Embryo Development Medium (EDM). The globular

Table 1: Effect of growth hormone on callus induction of *D. sissoo*

Growth hormones 2,4-D (mg L^{-1})	Callus morphology	Callus scoring
0.5	Friable white creamy	+
1	Friable white creamy	+
2	Friable white creamy	+++
3	Friable white creamy	+++
4	No friable	+

Table 2: Response of multiplication from somatic embryos to various cytokinins effect

MS Medium+ Auxins/Cytokinins	Culture with germinated somatic embryos (%)	No. of shoots Culture	Approx average length of longest shoot
BAP (1.0)	93	10.9±1.7	63±8.9
BAP (3.0)	97	9.8±1.5	61±9.0
BAP (5.0)	93	9.4±1.8	65±6.3
KN (1.0)	77	5.1±1.4	48±5.5
KN (3.0)	83	4.8±1.4	51±6.6
KN (5.0)	83	4.0±1.6	58±7.0
BAP (0.5)+KN (0.5)	93	8.9±2.5	66±7.5
BAP (0.5)+KN (1.0)	90	7.1±1.4	62±5.3

Table 3: Effect of auxin and cytokinin on multiple shoot formation

MS Medium+ Growth hormone	Culture with multiple shoot (%)	No of shoots/ culture
IAA (1.0)	36	2.1±0.6
IBA (1.0)	36	2.3±0.5
2,4-D (1.0)+BAP (1.0)	Callus formation	-
NAA (0.5)+BAP (0.5)	47	3.2±0.6
NAA (1.0)+BAP (1.0)	20	2.9±0.9

glossy white embryos started appearing from the surface of cotyledons with in 15 days. After sub-culturing which further develop into dark green embryos, there was also development of leafy structures. The high frequency somatic embryo development was found to be dependent not only on initial induction phase but also on medium containing growth hormone. The somatic embryo's was significantly high when it was transferred to the medium containing 1.0, 3.0 and 5.0 mg L^{-1} BAP and in combination of BAP with kinetin i.e 0.5 mg L^{-1} BAP+0.5 mg L^{-1} Kn and 0.5 mg L^{-1} BAP+1.0 mg L^{-1} Kn. From Table 2 it has been revealed that the medium containing 3.0 mg L^{-1} BAP produces highest level of somatic embryos i.e., 97% comparison to other media.

For further development of these embryos into complete plantlets, embryos with well-developed cotyledons were transferred to the growth regulator free medium with full, half strength MS major salts and other. However, there was no further development of these embryos into complete plantlets. For maturation and germination of somatic embryos, these were transferred to MS medium supplemented with Auxin 1.0 mg L^{-1} IAA, 1.0 mg L^{-1} IBA and cytokinin+auxin combination 1.0 mg L^{-1} 2,4-D+1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA+0.5 mg L^{-1} BAP, 1.0 mg L^{-1} NAA+1.0 mg L^{-1} BAP.

Table 3 depicted that the 47% of multiple shoots were found in 0.5 mg L^{-1} NAA+0.5 mg L^{-1} BAP while callus is formed in 1.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} BAP. When somatic embryos cultured in MS media supplemented with Auxin 1.0 mg L^{-1} IAA and 1.0 mg L^{-1} IBA, 36% somatic embryos germinated.

DISCUSSION

The age of the explants was found to play an important role in the induction of indirect somatic embryogenesis. The immature seeds germinate easily compare to old or mature.

The 2, 4-D is known to play an important role in the induction of embryogenic cultures. For indirect somatic embryogenesis tissues generally were transferred from high to low concentration of auxin (2, 4-D), it leads to increase in somatic embryogenesis. Additionally, it was reported that the higher concentration of auxin results in the high production of ethanol and ethylene which cause the repressive effect of auxin on somatic embryogenesis (Singh *et al*., 2002; Rastogi *et al*., 2008). This study confirmed the production of ethanol and ethylenes by auxin are responsible to inhibit the somatic embryogenesis.

For further development of embryos into complete plantlets, embryos with well-developed cotyledons were transferred to the growth regulator free MS medium but there was no further development of these embryos. From these preliminary studies, it suggested that preconditioning or further maturation of this somatic embryo, cytokinin may be necessary for the successful germination into plantlets. The addition of a cytokinin and auxin to the medium was essential to induce multiple shoots from the explants (Bari *et al*., 2008) after the addition of different concentration of cytokinin and auxin in MS medium multiple shoots were found. When somatic embryos cultured in MS media supplemented with auxin, alone somatic embryos were germinated.

CONCLUSION

The increasing demand of *D. sisso* in timber industries, the plant is being over-exploited. In addition, the vegetative propagation methods are less efficient. So, the plant has been developed through indirect somatic embryogenesis using MS media supplemented with different auxin (2, 4-D) and cytokinins (BAP, Kn) and alone. The advantage of somatic embryogenesis in conservation of depleting flora and rapid and large scale clonal propagation of elite varieties. Somatic embryogenesis is very useful for genetic transformation and might be helpful in producing synthetic seeds. The findings of recent investigations have shown that, it is possible to induce indirect somatic embryogenesis in *D. sissoo* and plant regeneration from callus cultures derived from cotyledonary leaves as explants.

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ABBREVIATIONS

BAP = 6-benylaminopurine
2,4-D = 2,4-dichlorophenoxyacetic acid
Kn = Kinetin
IAA = Indole-3-acetic acid
IBA = Lndole-3-butyric acid
NAA = Naphthaleneacetic acid

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