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## Development of Source Independent Micropropagation System in *Dalbergia sissoo* Roxb, as a Basis for Germplasm Conservation and Disease Free Plants Production

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**Abstract** Plant micropropagation has becoming an imperative strategy at commercial level that provides large number of plants in less time and space. In woody trees, micropropagation is more tedious and cumbersome process than other crop plants. Less success rate in tree micropropagation has been reported in literature. Therefore in this study, expedite, reproducible and source independent micropropagation protocol was developed in *Dalbergia sissoo* (Shisham) that is an economically important timer yielding tree with medicinal values. For this purpose, eight micropropagation media were studied with various combination of BAP (benzylaminopurine) and IAA (indole acetic acid). Auxially and epicormic shoots of *Dalbergia sissoo* having at least one nodal bud were used as explants. We studied the impact of these media combination and explant types on micropropagation. We found MPMS5 is the best medium combination that gave maximum number of shoots (2.78 shoots per explant) followed by MPMS7. This selected media combination can be used for *Dalbergia sissoo* micropropagation as a routine work. Similarly rooting media combinations were also studied for the best root induction in micropropagated shoots of *Dalbergia sissoo* but little success had been achieved.

**Keywords** *Dalbergia sissoo*; Micropropagation; Shisham; Tissue culture; Woody tree

## Introduction

Urbanization and deforestation are the pivotal forces those bring negative stress to natural resources. Evolution of problems relating to these forces is basically due to rapid increase in human population. Ecosystem in general and habitats of endangered species in particular are affected by these situations. Many industries depend upon the continuous supply of wood, can not totally rely on natural sources, therefore efforts should be done to find new ways to fulfill their demand for timber (Jain, 1997 and Tzfira et al., 1998). Conventional propagation through cuttings and suckers do not provide the required plant material to support the increasing timber demand due to various factors (Ali et al., 2005). Therefore the focuses should be on replacing these conventional means with modern in-vitro techniques. Biotic and abiotic factors also affect trees that result in declined tree populations (Rehman et al., 2012). Development of resistant material against various natural calamities

will reduce supply-demand strain and will be more beneficial for farmers as well for industries (Minocha and Jain, 2000). Different conventional and non conventional strategies have been employed to multiply the resistant plants in huge number (Pradhan et al., 1998). Micropropagation facilitates multiplication from limited mother stock, keep product uniformity, not dependent upon season and many other agronomic advantages (Kunisaki et al., 2003). Micro-propagation in economic tree species is also now becoming an emerging tool and requirement of this modern era.

*Dalbergia sissoo* Roxb is a very important timber yielding perennial tree found throughout IndoPak subcontinent. It has been used for forestation in most parts of the subcontinent for years (Priyanka et al., 2012). It has great economic importance as its wood is used in making furniture, agricultural tools, used as fuel and also has some medicinal value. At least half a

dozen fungal diseases are reported in IndoPak. But in last few years this tree has been badly affected by a disease called; Shisham decline or dieback, that was announced as an epidemic for Shisham all over the Pakistan in 1998 (Naz, 2002; Bajwa et al., 2003). Die-back disease of Shisham was first time observed and reported in Nepal. This disease has now been reached epidemic proportion in Bangladesh and other countries of South Asia. This disease is characterized by wilting and subsequent loss of side branches, leading to so called stagheadness.

To combat with these problems, tissue culture and biotechnological approaches have been employing for many decades. Micropropagation of woody trees has been attempted many times but still is not emerged as routine. In *D. sissoo*, during the last couple of years, several attempts have been done to develop efficient micropropagation. Impact of various explants and media types on micropropagation have been studied which resulted in small success but a breakthrough is still to come which will make *D. sissoo* micropropagation a routine work. Therefore in this study, an effective and source independent micropropagation protocol was established that would be a milestone for all in vitro studies in *D. sissoo*.

## 1 Results

Tissue culture has key role in providing rapid and healthy plant multiplication techniques that are crucial for plant species facing certain biotic and abiotic stresses in nature. Various diseases, especially dieback, are badly affecting our natural Shisham resources; hence establishment of an efficient micropropagation protocol is necessary. This study addresses the development of source independent micropropagation protocol for *D. sissoo*. For this purpose two explant sources i.e. Axillary and epicormic shoots, were selected and micropropagation response was evaluated on different media combinations.

### 1.1 Micropropagation Response of Shisham (*Dalbergia sissoo*)

For establishing micropropagation in *D. sissoo* eight different micropropagation media (MPM) combinations supplemented with varying con-

centrations of BAP and IAA were studied. The data was collected in the form of number of shoots obtained per explant and ANOVA table was constructed.

The analysis of variance table (Table 1) depicts the considerable significant variation among media combinations. MPMS 5 gave maximum number of 2.78 shoots per explant (Figure 1) followed by MPMS 7 and MPMS 2 on which 2.72 and 2.68 average shoots per explant were obtained respectively. The other five combinations showed a comparatively low response for shoot induction. Among which MPMS 3 showed the lowest response with 2.15 mean numbers of shoots per explant (Table 2 and Figure 1).

Both explant sources responded well for micropropagation, epicormic shoots giving 2.53 shoots per explant and Axillary shoots giving 2.46 shoots per explant. On these micropropagation media, single shoots as well as bunch of shoots/multiple shoots were observed (Fig 2, Fig 3, Fig 4, & Fig5). But the media to explant interaction represented non-significant results, which means the micropropagation response does not depend upon the explant source (Response of different micropropagation media is shown in Fig6, Fig 7, Fig 8, Fig 9, Fig 10, Fig 11, Fig 12 & Fig 13). A common thing that had been seen was the formation of calli from explants at media-explant junction. The callus formation was seen to be increasing with the increase in BAP concentration, so MPMS 4 & MPMS 8 giving maximum callus. Different shoot development stages are shown in Fig 14 & Fig 15.

### 1.2 Root Induction from Micropropagated Shoots of *D. sissoo*

For root induction three rooting media were studied. Well developed micropropagated shoots were shifted to root induction media. Root initiation from few micropropagated shoots were started after 20-25 days of shifting on root induction media (Fig 16 & Fig 17). Of these, one rooting medium; RM2 (Rooting medium 2) was already reported by Thirunavoukkarasu et al., 2010 and we used it as control. In Shisham root induction of in vitro regenerated shoots is very poor and in our study we attempted this challenge but very little success was achieved in case of rooting.

Table 1 Analysis of variance table for micropropagation of *D. sissoo* on different media combinations.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Explant	1	0.6750	0.6750	1.56 <sup>NS</sup>
MPMS	7	21.4917	3.0702	7.08 <sup>**</sup>
MPMS x Explant	7	2.6250	0.3750	0.86 <sup>NS</sup>
Error	464	201.2000	0.4336	
Total	479	225.9917		

NS = Non-significant ( $P > 0.05$ ); \*\* = Highly significant ( $P < 0.01$ )

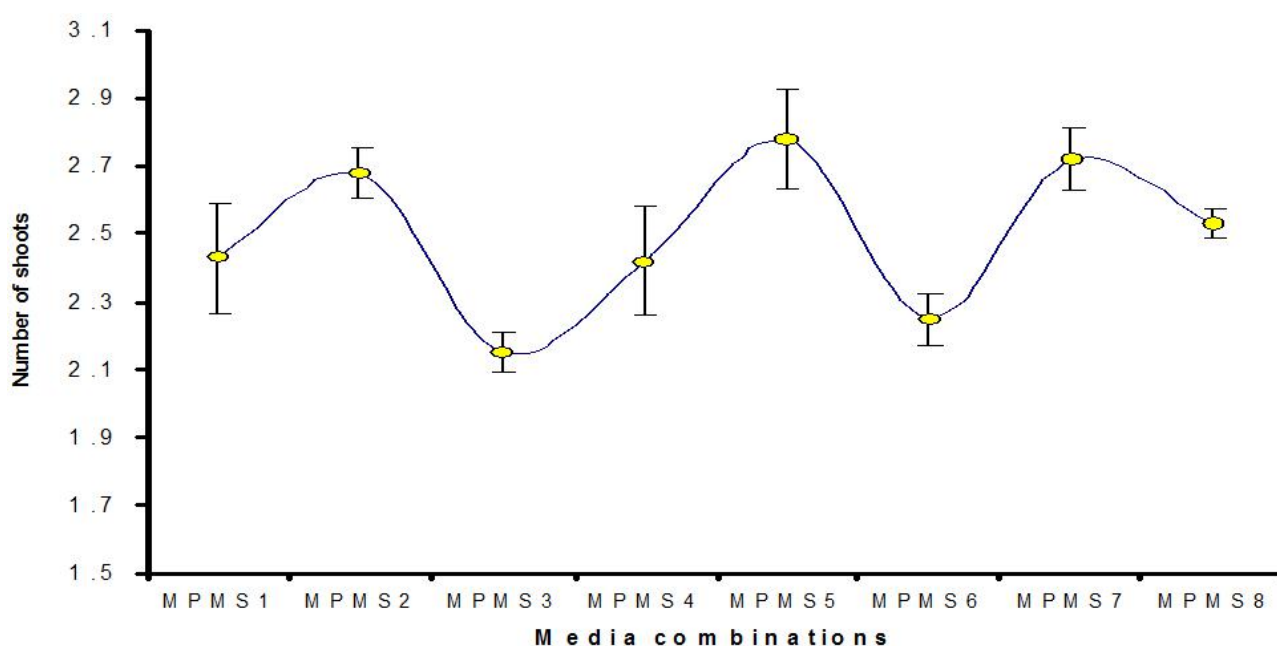


Figure 1 Mean number of shoots per each media combination. MPMS 5 showing maximum micropropagation response followed by MPMS 7 & MPMS 2. Lowest response of MPMS 3 is also shown.

Table 2 Number of Shoots per explant on Eight MPMS  $\pm$  SE. Means sharing similar letters are statistically non-significant ( $P > 0.05$ ).

MPMS	Mean $\pm$ SE	
MPMS 1	2.43 $\pm$ 0.159	AB
MPMS 2	2.68 $\pm$ 0.073	AB
MPMS 3	2.15 $\pm$ 0.058	B
MPMS 4	2.42 $\pm$ 0.159	AB
MPMS 5	2.78 $\pm$ 0.148	A
MPMS 6	2.25 $\pm$ 0.076	AB
MPMS 7	2.72 $\pm$ 0.093	A
MPMS 8	2.53 $\pm$ 0.044	AB



Figure 2 Bud sprouting



Figure 3 Shoot development started



Figure 5 Multiple micropropagated shoots



Figure 4 Single micropropagated shoot



Figure 6 MPMS 1



Figure 7 MPMS 2



Figure 9 MPMS 4



Figure 8 MPMS 3



Figure 11 MPMS 6



Figure 10 Maximum number of shoots developing on MPMS 5



Figure 12 MPMS 7

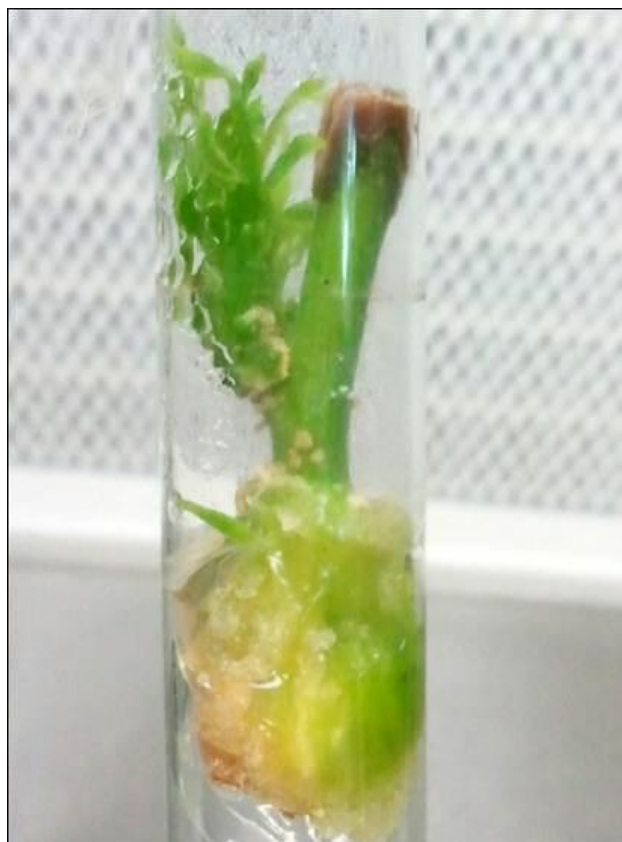


Figure 13 MPMS 8



Figure 14 Micropropagated shoots of *D. sissoo* growing as bunches



Figure 15 *D. sissoo* shoot development at different stages



Figure 16 Root initiation



Figure 17 Root development

## 2 Discussion

Shisham is a deciduous tree of great economic importance but currently it has badly been affected by certain diseases in which Shisham dieback is on top. So there was a need of an efficient micropropagation protocol that could be a milestone in further tissue culture studies of Shisham. Due to the strong heterozygosity of trees, their production using seeds or other conventional means is not appropriate, if one wants to conserve the characteristics of a desired tree species (Das and Mitra, 1990). So in almost all vegetatively propagated species, the establishment of efficient tissue culture system has been the foundation of mass scale planting material and production of disease free and high quality plants. It is essential for successful reforestation programs and affluent forest management as well.

In current study, two types of explants viz epicormic and axillary shoots were used to identify appropriate explants source for optimal response. We observed that micropropagation was independent to explant source. The present investigation also suggested that nodal explants with greenish active buds showed faster multiplication with more number of shoots as compared to nodal explants with brownish buds. Same response was reported in mulberry species as well

(Chitra and Padmaja, 1999; Oka and Ohyama, 1975).

The sterilization process has been, more or less as hard to standardize, as was to optimize the adequate media requirements for micropropagation itself. In the sterilization process extra chloroxylonol treatment was done for efficient bacterial and fungal contamination control. Seventy percent ethanol was used pre-treatment to mercuric chloride, again to free the cuttings from any kind of bacterial and fungal contamination. A waxy residue was observed in the rinse water, after treating the explant with ethanol. The elimination of the waxy covering from explant cuttings may cause the explants to become more permeable to the mercuric chloride, causing elevated phenolic exudation.

The micropropagation response was severely affected by phenolic compounds' exudation from the sterilized explants. This may occur due to the ooze of anthocyanins and other plant fluids in the culture media from the surface of explant as a result of injury, which explant faced due to the ethanol treatment. The anthocyanins were visible as a browning around the bottom of the cuttings, observed even more easily in comparatively clear media. This was decreased by addition of some auxin, which owing to its cell

division property, quickly heals phloem and stops exudation. For this purpose IAA was added as an auxin in every micropropagation medium in addition to BAP. Moreover this problem was overcome by subsequent subculturing of explants that indicated browning. Two-three subcultures were enough to lessen the exudation enough, so it could not affect the shoot formation.

Induction and multiplication of *in vitro* shoots of almost all woody tree species is easier if the explants are obtained from developing plantlets, generally said to be at juvenile stage and they give superior results than mature explants (George, 1993). The harder the explant cutting became, the longer the sterilization time required and yet harder to control contamination. The bacteria survive longer on hard explants and grow faster under favorable conditions. Soft cuttings grew faster and illustrated less contamination upon initiation, which is why young juvenile explant cuttings with fresh green buds were selected for micropropagation.

Micropropagation is done via different concentrations of various growth regulators, either alone or in combination. Inclusion of auxins and cytokinins in the micropropagation medium greatly influences the shoot formation and development. Generally response and the quality of *in vitro* cultures are highly influenced by the concentrations and type of nutrients present in the culture media (Niedz and Evens, 2007). Outcome of different micro & macronutrients on growth and number of *in vitro* culture and micropropagation was investigated by many researchers (Cohen, 1995; Rout *et al.*, 1998; Thirunavoukkarasu and Debata, 2002). *In vitro* shoot induction in most of the plant species is done using various cytokinins, among which BAP is reported to be the most responsive and efficient (Arumugam and Rao, 1996; Behera *et al.*, 2008; Nayak *et al.*, 2007; Pradhan *et al.*, 1998; Purohit and Dave, 1996). Carelli and Echeverrigary, (2002) showed that BAP produces more shoots as compared to Kinetin and 2iP in micropropagation systems. BAP has also been reported the superlative growth regulator for shoot development and multiplication in Shisham by several researchers. Bari *et al.*, (2008) reported an average 2.2 shoots form callus of Shisham, via combination of 1.5 mg/L BAP + 0.5 mg/L IAA in MS media. Current study also indicated that the

addition of BAP, among all cytokinins, proved to be substantially useful for *in vitro* shoot formation from juvenile buds of Shisham. We obtained a maximum 2.78 number of shoots using MS medium supplemented with 6.6  $\mu$ M BAP + 1.71  $\mu$ M IAA. However deformity and abnormal shoot induction was reported as a result of high BAP concentration by some researchers (Carelli and Echeverrigary, 2002).

### 3 Materials and Methods

#### 3.1 Explants to be used

Material for Shisham micropropagation was collected from the territory of University of Agriculture Faisalabad. Axillary and epicormic shoots were used as explants in this study. Cuttings of 1.5-2.5 cm, each containing at least one nodal bud were used. To the best of our information, Shisham trees that were used as source for getting explants (axillary and epicormic shoots), were wild genotypes to the Faisalabad area, and thus the potential value as clonal material was unidentified.

#### 3.2 Explant sterilization

Explants were thoroughly washed and treated with tween- 20 (7drops/100ml) followed by the treatment with 0.7% Chloroxyleneol solution. These explants were dipped in 70% ethanol for 10 seconds and then treated with 0.1 % Mercuric Chloride ( $\text{HgCl}_2$ ) solution for 5 minutes. All these operations were done under high axenic conditions in laminar air flow cabinet. Ultra pure water was used for the preparation of all these solutions to be used in sterilization of explants.

#### 3.3 Media preparation and culture conditions

Micropropagation media were prepared by using growth regulators of varying concentrations in addition to MS Salts and sucrose in common. For micropropagation eight media combination with varying levels of IAA and BAP were studied (Table 3). For root induction, half MS media with and without IAA and IBA were used (Table 4). Gellan gum powder was used as solidifying agent and pH was adjusted to 5.8 using NaOH and HCl. Micropropagation media were poured into test tubes that were wrapped by using polypropylene sheets and then autoclaved at 121°C, 15 *psi* for 20 minutes. Culturing of explants on micropropagation media were done under aseptic conditions in laminar air flow hood. These cultures were incubated and maintained in controlled conditions at  $25 \pm 2^\circ\text{C}$  for 16/8 hrs light/dark period.

Table 3 Micropropagation Media (MPM) for *Dalbergia sissoo* Roxb.

Micropropagation Media for Shisham (MPMS)	M.S Salt	Sucrose	BAP	IAA	Gellan Gum Powder
MPMS 1	4.33 g/L	30 g/L	6.66µM	1.43 µM	2.66 g/L
MPMS 2	4.33 g/L	30 g/L	8.8 µM	1.43 µM	2.66 g/L
MPMS 3	4.33 g/L	30 g/L	11.1 µM	1.43 µM	2.66 g/L
MPMS 4	4.33 g/L	30 g/L	13.32µM	1.43 µM	2.66 g/L
MPMS 5	4.33 g/L	30 g/L	6.6 µM	1.71 µM	2.66 g/L
MPMS 6	4.33 g/L	30 g/L	8.8 µM	1.71 µM	2.66 g/L
MPMS 7	4.33 g/L	30 g/L	11.1 µM	1.71 µM	2.66 g/L
MPMS 8	4.33 g/L	30 g/L	13.32µM	1.71 µM	2.66 g/L

Table 4 Root induction Media (RIM) for *Dalbergia sissoo* Roxb.

Root Induction Media for Shisham (RIMS)	M.S Salt	Sucrose	IBA	IAA	Myoinositol	Gellan Gum Powder
RM 1	2.165 g/L	30 g/L	4.9 µM	5.7 µM	100 mg/L	2.66 g/L
RM 2 (reported) used as control	2.165 g/L	30 g/L	7.35 µM	0	0	2.66 g/L
RM 3	2.165 g/L	30 g/L	11.0 µM	0	0	2.66 g/L

### 3.4 Data collection and Statistical analysis

Data of micropropagation were collected in the form of number of shoots per explant. Analysis of Variance table was made among different media combinations and explant sources. Experiment was designed under CRD in factorial design and data was collected and analyzed (Steel and Torrey, 1982).

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