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DEVELOPMENT OF PROTOCOL FOR EFFICIENT MICROPROPAGATION OF A MULTIPURPOSE LOCAL CULTIVAR OF *Dalbergia sissoo* A THREATENED TREE SPECIES

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ABSTRACT

Present study was aimed to develop a protocol for efficient micropropagation of a multipurpose local cultivar of *Dalbergia sissoo*. In nature this species multiply by fragments of roots or seeds which is rare. Stem cuttings are also used but we do not get authentic plantlets for large scale plantation. In the present study nodal explants, taken from healthy and young branches from the plant growing in wild habitat were inoculated in Murashige and Skoog (1962) MS medium, supplemented with different concentrations of 6-Benzuyle amino purine (BAP) either alone or with three different concentrations of NAA (α-Napththalene acetic acid). Similarly, different concentration of Kinetin alone or NAA was also supplemented in MS basal medium along with 3% sucrose and solidified with 0.8% Agar. Among the six different concentrations of BAP alone, MS + 1.5 mg/l BAP was found more suitable for nodal shoot bud initiation. Here the percentage response for shoot bud induction was 88.48, the mean number of shoot buds was 3.72, mean length was 1.75 cm. Buds were initiated after 14.68 days of inoculation. When MS + 1.5 mg/l BAP + 1.0 mg/l NAA was used for inoculation the percentage of response for shoot buds initiation was 92.75, number of shoot buds 4.76, and mean length of 2.18 cm respectively. Time taken for shoot bud initiation was 12 days only. It was further noted that Kinetin alone at the similar concentration induced shoot buds on nodal explants but the percentage of response for shoot bud initiation was 78.62, the number of shoot buds 3.06, the mean length 1.54 cm. Here time taken for shoot bud initiation was 16.56 days. Likewise, MS + 1.5 mg/l KN + 1.0 mg/l NAA when used for inoculation, the percentage of response was 88.72, number of shoot buds 3.54, mean length 1.88 cm respectively. The shoot buds were initiated after 13.58 days. These shoot buds were separated from the explants after 34 days of incubation and were inoculated in the multiplication medium. Here also, the highest percentage of response 96.24, number of shoots developed denovo, 5.84 mean shoot length 3.78 cm and growth of shoots was excellent in MS+ 1.5 mg/l BAP + 1.0 mg/l NAA, in comparison to similar concentration of KN+ NAA, where the percentage response for denovo shoot bud initiation was 89.72, the number of shoots per explant 4.25, the mean length 3.12 and growth was slow than the above. Well developed plantlets were inoculated in rooting medium. Best response was noted n MS+ 1.0 mg/l IBA, where percentage of response was 90.78, number of roots 4.88 and mean length 2.2cm. At similar concentration of NAA, the percentage response was 70.18, number of roots 2.46 and mean length 1.75 cm respectively.

KEYWORDS: Micropropagation, Multipurpose, *Dalbergia sissoo*, Multiplication medium, Rooting medium, Explants

Dalbergia sissoo, commonly called Shisham is most popular timber tree in Bihar. In this area it is noted that Shisham plants grow in wild and they are protected. From this plant several plants develop in that area from the roots and thus large numbers of plants are randomly developed. Here farmers do prune the lateral branches, and thus straight and long tree grows in the field. Lateral branches are annually pruned. They in February to March and new branches develop in April to May. Unfortunately, 95% of adult and young plants of Shisham have been destroyed due to infection by certain soil borne pathogens and farmers have faced a great set back, and severe economic loss. It is due to the scarcity of the adult plants, the timber of Shisham is costly than *Shorea robusta* in the local market. As mentioned above Shisham plants do grow wild from the roots and there is dearth of planting materials, if a farmer is wishing to do plantation of Shisham at large scale. Keeping these ideas in mind the present work was done to develop a protocol for efficient micropropagation of *Dalbergia sissoo* commonly called as Shisham.

SURVEY OF LITERATURE

Micropropagation through tissue culture in general and for the woody trees in particular is the best alternative for the production of large number of uniform planting materials within short periods, which are authentic and free from all pathogen. The possibility of mass propagation of elite, endangered, vulnerable, or difficult to propagate genotypes, in relatively small areas and time period, is the most important characteristic of micropropagation. We get several references related with micropropagation of different tree species. Some of them are being mentioned here. Mascarennas (1989) reported biotechnological application of plant tissue culture to forestry in India. Feswara *et al*; (1998) reported standard procedure for the micropropagation of neem tree (*Azadirachta indica*). Kumar *et al*; (1998) in case of *Ficus carcae* cv-Gular and Mandanha *et al*; (1998) in *Hevea brasiliensis*, reported technique of micropropagation. Clonal propagation techniques for tree species have been reported by Sharma *et al*; (2003) in *Crataeva adansonii*; Vengadesan *et al*; (2003) in *Acacia*

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sinuate (lour); Chand and Singh (2004) in *Pterocarpus marsupium*. Giri *et al*; (2004) reported progress in tissue culture genetic transformation and application of Biotechnology in trees. Chaturvedi *et al*; (2004) reported clonal propagation in *Populus deltoids*. Katherine and Fashey (2004) reported, tissue culture method can be used for micropropagation and rescue of endangered *Moringa* spp. germplasm. Chaturvedi *et al*; (2004) reported micropropagation of *Azadirachta indica* A. jus-via cotyledonary nodes. Pandey *et al*; (2006) reported micropropagation in *Terminalia arjuna* Roxb. Reddy *et al*; (2006) reported micropropagation of *Azadirachta indica*. Emilio *et al*; (2007), reported efficient method of micropropagation and *in vitro* rooting of teak (*Tectona grandis* L.) Rout *et al*; (2008) reported micropropagation in *Acasia chunda* Roxb. Tyagi *et al*; (2010) reported clonal propagation of *Capparis deciduas* throne tissue culture.

Ali *et al*; (2012) observed effect of media type and explant source on micropropagation of *Dalbergia sissoo*, a tree of medicinal importance. Boga *et al*; (2012) reported effect of Benzyle amino purine and Gibberellic Acid on *in vitro* shoot multiplication and elongation of *Dalbergia latifolia* Roxb, an important multipurpose tree. Shukla *et al*; (2012) reported *in vitro* regeneration of multipurpose medicinal tree *Stereospermum suaveolens* and described factors controlling the *in vitro* regeneration. Dangi *et al*; (2014) studied micropropagation of *Terminalia bellerica* from nodal explants of mature tree and assess the genetic fidelity using ISSR and RAPD markers. Kant *et al*; (2014) reported that *in vitro* propagation may be a viable conservation strategy for *Commiphora wightii*, an endangered medicinally important desert tree of India. Kiondo *et al*; (2014) reported micropropagation of *Dalbergia melanoxyllon* Guill & Perr, a threatened tree species. Shtereva *et al*; (2014) reported micropropagation of six *Paulownia* genotypes through tissue culture. Singh *et al*; (2014) reported micropropagation of *Shorea robusta*; an economically important woody plant.

Singh and Mishra (2016) reviewed advances in micropropagation of teak (*Tectona grandis*). Rambabu *et al*; (2016) reported micropropagation of *Gmelina arborea* Roxb through nodal culture. It is an economically important forest tree. Kunwar and Thakur (2017) reported the sterilization protocol for nodal explants of field grown mature tree of *Diploknema butyraceae*. Manikandan *et al*; (2017) reported *in vitro* propagation and mass multiplication of *Dalbergia latifolia* Roxb: a vulnerable tree species from Eastern Ghat, Tamil Nadu, India. Fasola *et al*; (2019) reported micropropagation of baobab, an economically important plant. Teresa (2019) reviewed propagation of Juniper species through plant tissue culture. Yuanyuan *et al*; (2019) reported an efficient micropropagation protocol for an endangered ornamental tree species (*Magnolia sirindhorniae*). They also assess the genetic uniformity of the micropropagated plants through genetic marker. Keeping all these ideas in mind the experiment for micropropagation of *Dalbergia sissoo* was carried out.

MATERIALS AND METHODS

Preparation of Culture Medium

Stock solutions of all the ingredients of MS medium (Murashige and Skoog,1962) were prepared separately with different concentrations. Similarly, stock solutions of plant growth regulators were prepared separately. Desired amounts of all the ingredients were taken in one liter conical flasks and the volume was made 500 ml by adding glass distilled water, 30 gm of sucrose was dissolved properly. The pH was adjusted to 5.8 by adding 1N solution of NaOH. Now 8 gm of Agar powder was dissolved in 500 ml distilled water by heating slowly. This was poured into the above salt solutions. To the above MS medium, immediately desired concentration of BAP, Kinetin and NAA either alone or in combination with each other were added. For rooting different concentrations of auxins such as, NAA and IBA was supplemented. Above prepared medium either with Cytokinin or auxins was dispensed in 250 cc culture flasks. 20-25 ml of medium in each flask. These flasks were plugged properly with cotton plugs covered with muslin cloth. The plugs were wrapped with Aluminum foil to avoid wetting of the plugs during autoclaving. Autoclaving was done at 15 lb pressure and at 121^0C for 18-20 minutes. All these culture flaks were taken out and stored at low temperature for three days. They were used for inoculation. Flasks showing any contamination were discarded after autoclaving.

Preparation of Explants

For explants, healthy and young branches were collected from the *Dalbergia sissoo* plant growing in the campus of University Department of Botany. Both the basal hard portion and apical soft portion of the braches were pruned with sterile knife. Similarly, leaves were removed and then the branches were cut into pieces 3-4 cm long. In a one liter conical flask all the pieces were placed and the mouth was covered with muslin cloth so that materials should not go out along with the water current when placed under running tap water. All the pieces were taken out after 40 minutes of surface washing. They were placed in another flask and treated with detergent for 10 minutes. All the materials were again washed with tap water to remove even the traces of detergent. With the help of pre-sterilized tissue papers, the materials were dried and they were treated with the solution containing 70 mg/l each, ascorbic acid and citric acid for 10 minutes. This was done to reduce the exudation of phenolics by the explants in the culture medium after inoculation. Explants were rinsed thrice with pre-sterilized distilled water to remove traces of the above chemicals. Above explants were treated with 0.1% mercuric chloride for 5 minutes. During this treatment the

explants were shaken vigorously for uniform contact of the surface with the chemical. The materials were taken out and rinsed several times with pre-sterilized distilled water to remove the chemical completely, otherwise it may cause toxic effect to the explants.

Inoculation

Inoculation of the explants containing at least one node in the culture medium as mentioned above was done in the aseptic conditions of the laminar air flow chamber. All precautions were taken to avoid any type of contamination of the cultures. Inoculated cultures were incubated in the plant tissue culture room, where the temperature light and moisture were controlled artificially. Here the temperature was maintained at 26 ± 1^0C, light at 3000 lux, maintained by white fluorescent tube, and moisture at 68-70%. Observation was made on an alternate day. Cultures showing contamination were discarded after autoclaving. Observations were done for percentage of explants showing response for shoot initiation, days after which initiation took place, number of buds per explants, growth rate etc. Mean of the data was tabulated for analysis and discussion. Based on the above, the plant growth regulators, and their concentrations were selected for future experiments. Each experiment was done in triplicate and in each passage 15 cultures were incubated.

RESULTS AND DISCUSSION

In the present experiments attempts were made to initiate axillary shoots in MS basal medium, supplemented with six different concentrations of BAP and Kinetin alone and in other experiments with three different concentrations of NAA. Similarly, the experiments were performed for multiplication of the axillary shoots. Data have been presented in table-2. Well grown plantlets were cultured in the rooting medium which had MS+ NAA and MS+ IBA. Here auxins were used at five different concentrations. The mean of the data for different parameters was taken and presented in table-1 and table-3 respectively.

Table 1: Showing impact of different concentration of BAP and Kinetin alone and with NAA on nodal explants of *D. sissoo*

Medium (mg/l)			% response for shoot bud initiation	Days after shoot bud induced	No. of shoot buds per explant	Length of the shoots in (cm)
MS	BAP					
MS	00		22.85	24.36	1.42	1.02
MS	0.5		46.24	21.38	2.38	1.15
MS	1.0		74.75	18.75	2.86	1.20
MS	1.5		88.48	14.68	3.72	1.75
MS	2.0		86.34	17.46	3.16	1.56
MS	2.5		82.52	17.88	2.56	1.26
MS	BAP	NAA				
MS	1.5	0.25	89.64	13.32	2.84	1.86
MS	1.5	0.5	90.26	13.15	3.16	1.88
MS	1.5	1.0	92.75	12.00	4.76	2.18
MS	1.5	1.5	89.36	13.72	3.58	2.08
MS	1.5	2.0	83.48	13.86	2.82	1.88
MS	KN					
MS	0.5	-	40.56	22.42	2.12	1.06
MS	1.0	-	62.74	20.34	2.74	1.10
MS	1.5	-	78.62	16.56	3.06	1.54
MS	2.0	-	70.18	18.62	2.78	1.38
MS	2.5	-	68.20	18.78	2.35	1.18
MS	KN	NAA				
MS	1.5	0.25	68.28	16.24	2.14	1.46
MS	1.5	0.50	80.54	15.74	2.86	1.72
MS	1.5	1.0	88.72	13.58	3.54	1.88
MS	1.5	1.5	71.38	14.32	2.72	1.64
MS	1.5	2.0	71.54	14.62	2.32	1.55

Table 2: Showing impact of plant growth regulators on the denovo multiple shoots production

Growth regulators mg/l	% of response	No. of denovo shoots	Mean shoot length cm	Growth rate
1.5 mg/l BAP + 1.0 mg/l NAA	96.24	5.84	3.78	+ + + +
1.5 mg/l KN + 1.0 mg/l NAA	89.72	4.25	3.12	+ + +

Table 3: Showing effects auxins on *in vitro* rooting of well grown plantlets, *Dalbergia sissoo* developed through tissue culture

Plant growth regulators mg/l	% of explants showing rooting	No. of roots per plantlet	Root length (cm)
MS+ IBA = 0.1	51.64	1.56	1.66
MS+ IBA = 0.25	72.48	1.78	1.82
MS+ IBA = 0.5	77.32	2.34	1.88
MS+ IBA = 0.75	84.56	3.26	1.94
MS+ IBA = 1.0	90.78	4.88	2.22
MS+ IBA = 1.5	64.24	2.56	1.59
MS+ NAA =0.1	45.36	1.32	1.42
MS+ NAA =0.25	59.62	1.45	1.48
MS+ NAA =0.5	64.72	1.89	1.52
MS+ NAA =0.75	66.85	2.16	1.64
MS+ NAA =1.0	70.18	2.46	1.75
MS+ NAA =1.5	54.81	1.72	1.46
Control	0.0	0.0	0.0

Perusal of table-1 clearly revealed that nodal explants of *Dalbergia sissoo* when inoculated in MS basal medium, supplemented with different concentrations and combinations of plant growth regulators responded for axillary shoot induction but with different percentage of response. From the table-1, it may be noted that highest percentage of response was 88.48 in MS + 1.5 mg/l BAP, followed by 86.34 in MS+ 2.0 mg/l BAP, respectively. Here lowest response for axillary shoot bud initiation was 22.85 in the medium without plant growth regulator. Similarly, the percentage response 46.24 in MS+ 0.5 mg/l BAP alone. It may further be noted that shoot buds were initiated after 14.68 days of inoculation in MS+1.5 mg/l BAP alone followed by 17.46 days in MS+ 2.0 mg/l BAP. Here again maximum period for shoot bud induction was taken by the explants inoculated in hormone free MS medium which was 24.36 days. Highest number of axillary shoots was observed in MS+ 1.5 mg/l BAP which was 3.72 per explant, followed by 3.6 axillary buds on the nodal explants inoculated in MS+ 2.0 mg/l BAP. In the control lowest number of axillary branches was observed which 1.42 was only. Shoot length of the newly formed axillary branches was also measured. The mean length 1.75 cm was found in the branches which were induced in MS+1.5 mg/l BAP. In the control it was only 1.02 cm.

Explants were also inoculated in MS+1.5 mg/l BAP+0.5-2.0 mg/l NAA. Here the highest percentage of response for shoot buds initiation was noted in MS+1.5 mg/l BAP+1.0 mg/l NAA, which was 92.75. This was followed by the explants inoculated in MS+1.5 mg/l BAP+0.5 mg/l NAA, which were 90.25. Here again minimum time 12.0 day was taken by the explants inoculated in MS+1.5 mg/l BAP+1.0 mg/l NAA. This was followed by 13.15 days in MS+1.5 mg/l BAP+0.5 mg/l NAA. It was also noted that the maximum number of axillary shoot 4.76 and maximum mean length of the axillary branches 2.18 cm was noted among the plants developed from the explants inoculated in MS+1.5 mg/l BAP+1.0 mg/l NAA. When the explants were inoculated in MS+1.5 mg/l BAP+1.5 mg/l NAA. The percentage of response for shoot bud initiation was 89.36, tie taken for bud induction 13.72 days, number of shoot buds 3.58 and mean length of the axillary branches 2.08 cm.

Nodal explants were also inoculated in MS+0.5-2.5 mg/l KN. From the table-1, it may be noted that maximum percentage of response 78.62 for induction of axillary shoots, minimum time periods 16.56, maximum number of axillary shoot buds 3.06 and maximum mean length 1.54 of branches were observed among the explants inoculated in MS+1.5 mg/l KN alone. All the above parameters followed, when the explants were inoculated in MS+2.0 mg/l KN. Here maximum

percentage response was 70.18 for axillary shoot induction, minimum periods taken for shoot bud induction, 18.62 days, number of branches, 2.78 and their mean length 1.38 cm were observed among the explants inoculated in MS+0.5 mg/l KN alone.

Similarly, MS+1.5 mg/l KN + four 0.5-2.5 mg/l different concentrations of NAA were also used for inoculation of the explant. Now from the table-1, it may be noted that highest percentage of response 88.72, minimum periods 13.58 days, maximum number of 3.54 of axillary shoot buds and maximum mean length 1.88 cm of the above shoots were observed in MS+1.5 mg/l KN+1.0 mg/l NAA. This was followed by the explants inoculated in MS+1.5 mg/l KN+0.5 mg/l NAA, where percentage of response was 80.54%, time taken for shoot bud induction 14.32 days, maximum number of axillary shoot buds 2.86, and mean length of the branches 1.72 cm. Lowest percentage was observed in MS+1.5 mg/l KN+ 0.25 NAA which was 68.28, response for bud initiation, maximum periods, 16.24, minimum number of axillary branches 2.14 and maximum mean length 1.46 cm only.

Shoot Multiplication

Axillary shoot developed above were taken out form the medium after 34 days of induction, and washed properly to remove the adhering culture medium. Shoots were separated from the nodal explants carefully. They were re-inoculated in the same concentration of growth hormones containing medium, prepared freshly. Mean of the percentage response, number of branches developed denovo, mean shoot length and growth rate etc. were noted after 6-7 weeks of inoculation. Mean of the data was tabulated in table-2. From the table-2, it may be noted that plantlet inoculated in MS+1.5 mg/l BAP + 1.0 mg/l NAA gave better response, where percentage of response was 96.24, number of shoots developed denovo was 5.84, mean length 3.78cm and growth excellent in comparison to the explants inoculated in MS+1.5 mg/l KN+1.0 mg/l NAA, where the percentage of response was 89.22, number of shoots were 4.25 and length 3.12. The growth was slow here.

Rooting in *in vitro* Developed Plantlets

Well grown plantlets were subcultured in rooting medium, MS+ six different concentrations of each IBA and NAA. Mean of the data was tabulated in table-3. From the above table-3, it was observed that best percentage of response for root induction, 90.78, number of roots per explants 4.88, and better growth in length 2.22 cm were among the explants inoculated in MS+1.0 mg/l IBA, followed by MS+0.75 mg/l IBA, where the percentage of response was 84.56, number roots per plant 3.26 and length of the roots 1.94 cm. Both the minimum concentration of IBA, 0.1 mg/l and higher concentration 1.5 mg/l had no promising response for root initiation.

From the table-3, it may further be noted that in the similar concentration of NAA, the percentage of response was 70.18, number of roots 2.46 per plant and growth rate 1.75 cm. It was followed by 66.85 percent of response for rooting number of roots 2.16 per plant and mean length of the roots 1.64 cm, respectively. Here again both the lowest and higher concentrations of IBA had not promising response for rooting among the explants.

DISCUSSION

Dalbergia sissoo a multipurpose timber tree has suffered to, soil borne pathogen in recent past. There is scarcity of large scale planting materials. Through tissue culture this problem can be solved. In the present work, attempt has been made to produce plantlets through tissue culture. It was observed that among the cytokinins, BAP alone or along with NAA may be used for large scale production of planting materials of *Dalbergia sissoo*; within short periods of time. Here multiple shoot form the nodal explants were induced after two to three weeks of culture on shoot induction medium and the highest number of axillary shoots per explant (3.58) was observed on MS + 1.5 mg/l BAP + 1.0 mg/l NAA. However, the number of shoots and their growth were reduced when the concentration of BAP was increased. Induction of multiple shoots in different tree species has been reported by different workers who used BAP and NAA at different concentrations. Some of them may be cited such as: Ali *et al*; (2012) in *Dalbergia sissoo*; Kiondo *et al*; (2012) *Dalbergia melanoxylon*; N'Doye *et al*; (2012) in *Adansomia digitata*; Rambabu *et al*; (2013) in *Gmelina arborea*; Dangi *et al*; (2014) in *Terminalia bellerica*; Rolli *et al*; (2014) in *Adansomia digitata*; Singh *et al*; (2014) in *Shorea robusta*; Manikandan *et al*; (2017) in *D. latifolia*; Cui *et al*; (2019) in *Magnolia serindhorniae*; Taiye *et al*; (2019) in *Adansomia digitata*. All these workers observed that BAP, at an optimal concentration along with NAA induced maximum multiple shoots and beyond or below this the shoot numbers was reduced. However, optimum concentration may vary for different woody tree species. Present finding therefore, corroborate with the above findings because here also the concentration of BAP above the optimum reduced the induction of shoot number and its growth. In the rooting media, IBA and NAA were supplemented separately at six different concentrations. Here IBA at 1.0 mg/l was found more supportive in comparison to NAA at the same concentration. Here IBA at 1.0 mg/l induced roots, where the mean number was 4.8 and mean length 2.22 cm on 16th days of incubation. Present findings was in agreement with the earlier findings that IBA has been very effective auxin for root induction in various species of trees as mentioned above for denovo shoot bud induction.

CONCLUSION

In the present work attempt was made to develop a methods for micropropagation of a multipurpose woody tree, *Dalbergia sissoo* which has faced a critical situation due to soil borne pathogen. Here MS medium supplemented with 1.5 mg/l BAP +1.0 mg/l NAA induced optimum shoot proliferation. Likewise IBA among the auxin at 1.0 mg/l concentration in MS medium was found to be optimum for root induction from the *in vitro* induced plantlets. This method can be exploited for the production of large scale planting material of *D. sissoo*, which are not available.

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