MICROPROPAGATION OF DALBERGIA SISSO ROXB

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Abstract

During current research work a comparatively innovative technique was followed for the clonal propagation of Shisham (*Dalbergia sissoo* Roxb.). Effect of different greenhouse mediums consisting of sawdust, sand and cocopeat was investigated in order to compel epicormic (latent buds) from the huge stem (branch) portions separated from juvenile sections of trees (older). In different environmental conditions, softwood shoots were forced during the dormant season. The current study showed that, while comparing natural and laboratory conditions, the maximum production (1.33) of softwood shoot was favored by natural environment. However, cocopeat medium produced maximum (2.91) number of softwood shoots under laboratory settings. Sterilized cocopeat in spring season produced the largest (2.91) mean number of softwood shoot suggesting that media had substantial impact. Under *in vitro* as well as greenhouse conditions, rooting experiment (with these softwood shoots) was not successful as the major obstacle is contamination.

Keywords: Clonal propagation, Cocopeat, Shisham, Latent buds, Soft wood shoots.

Introduction

Dalbergia sissoo Roxb. is member of Family Fabaceae (sub-family Papilionaceae) and reffered as "Shisham", *sissoo*, tahli, and sisu. It is a medium to large sized, leguminous tree having a small crown. It belongs to Genus *Dalbergia* which consist of 25 species that are widely distributed in tropical and subtropical region, the generic name was named after Swedish botanist Nils and Carl Dalberg (Das *et al*., 1997). In Pakistan, India, Bangladesh, Afghanistan, Nepal and Bhutan these species are present (Kanwar and Kumar, 2009). In Punjab, (Pakistan) for production of coal for running steam engines, shisham was introduced in Changa Manga plantation in 1886. It also provides financial support to farmers as it is a cash plant. *D. Sissoo* is used as fuel and lumber wood in addition to providing shelter and shade (Shah *et al*., 2021; Shah *et al*., 2002). However, it is recognized as most valued timber-producing tree in India (Chand and Singh, 2004).

All utilizes this plant for making furniture and agricultural instruments and also for construction work. It is a great source of tannins, resins, alkaloids, and fibers (Ali *et al*., 2019). It is also utilized to treat several diseases like stomach and skin problems, dysentery, emesis, leukoderma and ulcer (Sharma *et al*., 2001; Ishtiaq *et al*., 2006; Ahmad, 2007). *D. sissoo* reduces bacterial pathogenicity and having anti diahorrhoeal potential (Brijesh *et al*., 2006). As stimulant and astringent, leaves, roots and barks of shisham are beneficial (Hussain *et al*., 2008). For better hair growth and for treating dandruff leaves extract of shisham has been found effective (Sultana *et al*., 2006). Shisham has recently been over exploited due to its numerous health benefits and high timber yield. It is a leguminous plant and do atmospheric nitrogen fixation which in return enhance the fertility of soil (Orwa *et al*., 2009; Thirunavoukkarasu *et al*., 2010).

It is also called as nodulated tree because it contains globose to elongated nodules of different sizes. Shisham is often reproduced through planting suckers or by seeds, both these methods are not reliable as these have poor germination rate and results in the mortality of young seedling in their native habitat. Seed-based plant propagation also exhibit variety. Therefore, clonal propagation methods are more advantageous as it reduces variability and assures enhanced production (Husen, 2004). In early years of 1900, it was noted that shisham has experienced deterioration or dieback but it has never reached up to a dangerous level. In Pakistan, Shisham is under serious threat as a result of rise in dieback disease cases, which were lower initially (Bajwa *et al.*, 2003). Deforestation is a critical global concern, and Pakistan is suffering from it due to depletion of large forest area. From 1990-2005, Pakistan has lost 6,25,000ha of forest area which is about 24.7%.

This large-scale extinction is undoubtedly severe and costly. The issue is of greater concern while considering that Pakistan has just 4.2 million ha of forest area or less than 0.037 ha per capita which is not up to global average of 1 ha. Micropropagation is only aspect of plant tissue culture. In recent years, in *vitro* propagation of several woody legumes has been successfully done by utilizing both young and mature plant components (Trigiano *et al*., 1992). During dormancy, the use of shoot tips separated from trees and shrub for shoot forcing was the main objective (Read and Yang, 1991). To stimulate softwood shooting, large stem sections can be separated from juvenile parts of trees and shrubs and for *in vitro* studies it can be utilized as source of explant (Cameron and Sani, 1994). In contrast to other woody trees (temperate species) shisham has never been studied for forcing large stem segments (shoot forcing).

Aims and Objectives

The aim of present study is to propagate *D. sissoo* on massive scale by using some novel technique that can be a future technique to increase its cultivated area and can also help in reducing the chances of dieback disease and other similar problems. It also included the checking of various forcing or propagation media *in vitro.*

Materials and Methods

Source of *D. sissoo* explant

D. sissoo is naturally present in Pakistan, India, southern Iran, Afghanistan, Bhutan, Malaysia, Bangladesh. For present research work, plant sample (mature shisham tree) growing in good health was selected and utilized. The length of stems that were separated from juvenile portions ranged from 140-150cm (Van Sambeek and Preece, 1999; Vieitez *et al.,* 1994), were further cut down in to 40cm long stems ranges in diameter from 1.0-4.6cm and were named as logs.

Preparation and disinfestation of explant

Surface disinfection method was used to sterilized the explant material. For 10-15 minutes, in running tap water excised softwood shoots (explant material) were washed. The explant was then surface-disinfected in a flask with laundry detergent. And continuously flask was shaken for 10 minutes. Explant material were being rinsed for many times with distilled water (d.H_2O) after 10 minutes. Explants were then submerged for 10-20 minutes in a solution of 15% NaOCl (Sodium hypochlorite, 3.0% v/v) and Tween 20 (0.1% v/v). Explants were rinsed 7-8 times with autoclaved d.H_2O (distilled water).

Preparation of shoot forcing media

Three types of media (sawdust, cocopeat and sand) were selected to carry out the experimental study. From local market of Lahore, these media were purchased. After that each media is separately put in to a plastic bag and each bag weighs about 5Kg and was tightly tied after being packed twice.

Stock solutions for MS medium

For culturing of softwood shoots that had been grown using softwood shoot forcing, MS basal medium (Murashige and Skoog, 1962) was combined with different growth regulators. Double distilled water (dd.H2O) was used to prepare solutions and kept in refrigerator at 4^{o}C. Stock solutions required for preparing MS basal medium include the solutions of Macro and Micronutrients, Ferric-EDTA, Vitamins, and Plant growth regulators.

a) Macronutrients

Final concentration (20X) for the stock solution of macronutrients of MS medium was prepared. An amber colored bottle was used to hold the stock solution of macronutrients at 4^{o}C in refrigerator.

b) Micronutrients

The stock solution of micronutrients was prepared at final conc. of 100X. And salts for micronutrients are also listed in the same annexure.

c) Iron-EDTA

At a conc. of 200X, the stock solution of Iron-EDTA was prepared. An amber colored bottle was used to store the Iron stock solution at 4^{o}C in refrigerator.

d) Vitamins

At a conc. of 100 X vitamin stock solution was prepared. Vitamins were individually or separately dissolved.

e) Myo-Inositol

At a conc. of 100X, Myoinositol stock solution was prepared.

Growth regulators

Growth regulator stock solution was prepared in millimolar (mM) concentration. By adding a particular amount of d.H_2O, it was further diluted.

For present research work, various types of growth regulator stock solution were prepared. These growth regulator stock solutions consist of auxins, i.e., NAA (Naphthalene acetic acid), IAA (, Indole-3-acetic acid), and IBA (Indole-3-butyric acid), and cytokinins, i.e., BAP (6-benzylaminopurire). For various treatments, PGRs were accurately measured in milligrams according to the experimental requirement and were dissolved in double distilled water.

MS medium from stock solutions

The exact volume of stock solutions i.e., 50mL of macronutrients, 10 mL of micronutrients, 10 mL of Fe-EDTA and 5 mL of vitamins were utilized to prepare MS basal medium (1L). By adding double distilled water, the final volume was set to 1L. The pH of medium was adjusted to 5.75 and drops of sodium hydroxide (1N) and hydrochloric acid (1N) were used for this purpose. Media was solidified by adding agar at a conc. of 6.0g/L. In order to melt the agar, the medium was heated to boil, and then approx. 15 mL of medium was transferred in culture tubes that were sterilized already. Now the tubes were sealed with plastic sheets to make them germ free and zero contact with external environment and were tied with rubber bands.

Sterilization

Sterilization was done for shoot forcing media, tissue culture media, Laminar Air flow cabinet, surgical tools, culture room and transfer room by using standard culturing techniques (Khan et al., 2021).

Explant inoculation

For inoculation, from each culture tube propylene sheet was removed, now the explants were placed on the solidified agar

medium with the help of forceps. Now the opening of culture vessel was slightly heated and again wrapped by polypropylene sheets.

Culture conditions

Furthermore, cool fluorescent tube lights at a temperature of 25±2ºC were used, and cultures were placed under a 16-hour photoperiod (35 µ mol m^{-2} s^{-1}). Temperature and light were kept at their already standardized level. 16-hour photoperiod (35 µ mol m^{-2} s^{-1}) was provided by cool fluorescent tube light and cultures were placed in it.

Forcing of softwood shoots from logs

For current study, 140-150cm long stems of *D. sissoo* were separated from juvenile parts of selected tree of *D. sissoo* by following the protocol of Van Sambeek and Preece(1999), Henry and Preece (1997), and Vieitez *et al*., (1994), and were further cut in to smaller segments of 40cm long stems that ranged in diameter from 1.0-4.6cm are known as logs. A tray of 52cm length, 25cm width and 6.5 cm height was filled with sterilized media (sawdust, cocopeat and sand) and logs are also placed horizontally in it. For each medium and for each condition either laboratory or natural, total 18 trays were used.

For each media 3 trays were used. Randomly 3 logs/tray were selected and embedded in each medium respectively. Without plant growth regulators, the logs were watered daily. To avoid direct contact of water with developing softwood shoot, great care is required. To protect explant from fungal and microbial contamination the logs were sprayed with Hydrogen peroxide (H_2O_2) (Aftab *et al*., 2005).

Experiment lasted from 13th to 30th March, 2014, on which soft shots were harvested. Percentage of sprouting, number and length of forced softwood shoots, number of leaves and nodules after regular interval of time was recorded per experimental unit to collect data for forced softwood shoots.

Rooting of softwood shoots in MS medium

The softwood shoots, after reaching greater than or equal to length of 4 cm (≥ 4cm) were separated from logs to be harvested with caution. Sterilized of softwood shoots was done by washing them with laundry detergent softly. Then these shoots were delicately placed in a solution of 0.7% NaClO (sodium hypochlorite) and 0.1% Polyoxyethylene sorbitanmonolaurate (Tween-20) and was continuously shaken at slow speed for 15 minutes. After that, these shoots were washed with autoclaved d.H_2O thrice to remove the traces of sterilizing solution from them.

In laminar air flow cabinet, culture tubes that contains MS basal medium along with different types of growth regulators

were unfastened in sterilized environment near the flame. In culture vessels, the sterilized explants i.e., softwood shoots were inoculated in a very precise way, not damaging the explant or surface of medium using sterilized forceps. After shifting the explant into the culture tubes, mouth of these vessels were again sterilized in flame and tied with plastic sheets. For further growth, the culture was kept at 25± 2ºC room temperature for 16-hour photoperiod in culture room. On daily basis, the observation on rooting of softwood shoots was recorded.

In vitro rooting off softwood shoots in different medium

To avoid dehydration, greater than or equal to 4 cm (≥ 4cm) long forced softwood shoot was separated with help of sterilized scalpels and placed in a beaker containing d.H_2O. for at least 10 seconds, these softwood shoots were subjected to different growth regulators like Indole-3butyric acid (IBA 1000ppm), Naphthalene acetic acid (NAA 1000 ppm) or combination of both. After that in 3 trays containing sterilized sawdust, cocopeat and sand, softwood shoots were planted. These experimental trays were kept in culture room at 25 ± 2ºC with a photoperiod of 16-hours for further growth. On daily basis or per requirement of experiment these softwood shoots were watered.

Statistical data analysis

For calculation of values, COSTAT was applied. Mean values and standard deviations were calculated. By applying Ducan's Multiple comparison test, the significance of the dependent variables was determined.

RESULTS

The forcing of epicormic latent buds from the large stem segments of older trees was used for clonal propagation of trees as an alternative method. During the spring season, the large stem segments were excised from juvenile portions. The clonal propagation of shisham has been proposed in present study as an alternative method for multiplication and establishment due to numerous limitations in traditional breeding processes.

Forcing of epicormics sprouts from large stem segments

From mature shisham tree a 40cm long stem of varying diameter ranges from 1-5 cm were excised. Flats or trays of 52cm length, 25cm width and 6.5cm height were filled with sterilized sand, cocopeat, sand and logs for forcing epicormic buds were placed horizontally in it. These flats were placed in both laboratory and natural conditions. In terms of forcing epicormic buds, mature stem segments present good response. After 8, 9 and 10 days, sprouting of epicormic buds on sand, cocopeat and sawdust was started.

Under laboratory conditions, the maximum and significant number of sprouting (5.45) was observed on sand. Shisham logs in sand has produced (0.64) number of shoots having (1.06) cm length along with (0.61) number of nodes and (4.91) number of leaves.

However maximum number of growth of shoots (2.91) were seen in cocopeat. Least number of sprouting (2.81) with least number of shoots (0.39), and least number of leaves. (0.77) were observed in sawdust medium under laboratory conditions. Shoots developed from stem logs were thin and light green in color. Leaves were pale yellow to light green in color. Surprisingly, a very significant feature i.e., rooting was also showed by stem logs of shisham placed in cocopeat medium under laboratory conditions. Roots were developed from two logs placed in different trays filled with cocopeat.

Figure 1: A mature Shisham (*Dalbergia sissoo*) tree used as explant source

While under natural conditions sprouting was initiated after 8, 11 and 13 days in each medium (sand, cocopeat and sawdust) respectively. Under natural conditions large number of sprouts (5.40) were developed by *sissoo* logs placed in sand with maximum number of shoots (1.33), shoot length (1.39), nodes (1.83) and leaves (2.89). Least number sprouts (3.28) were observed in sawdust medium with least number of shoots (0.43) and 1.22cm shoot length, (0.72) number of nodes, (1.14) number of leaves under natural environment. Under natural conditions, softwood shoots grew more rapidly in sand then cocopeat and sawdust and these were of bright green in color. Leaves were broad and dark green in color. Similarly, highest number of shoots with greater shoot length were obtained in sand.

A remarkable feature, i.e., the development of inflorescence was also observed in logs placed on all three media (sand, cocopeat and sawdust) in both (natural and laboratory) conditions. More number of inflorescences were observed on logs placed on sand under natural conditions. Flowers were pale white to dull yellow in color. Development of inflorescences in all three media cocopeat, sand and sawdust under both natural as well as laboratory conditions. Although forcing of epicormic buds was possible in all flats of sand, cocopeat and sawdust under natural environment but

further development was restricted due increase in temperature.

Establishment of softwood shoots for rooting

Softwood shoots (≥ 4 cm long) were cut with sharp disinfected blades (sterilized), and then put in a water beaker to avoid desiccation. These shoots were subjected to Indole-3 butyric acid (1000 ppm IBA), Naphthalene acetic acid (1000 ppm NAA) or combination of both (IBA + NAA, 1000 + 1000 ppm). In a tray filled with sterilized sand, cocopeat, and sawdust these softwood shoots were embedded in growth regulators for 10 seconds for rooting.

For further growth the trays were placed in a culture room at 25 ± 2ºC temperature for 16-hours photoperiod. When required d.H_2O was given to shoots. However, the softwood shoots show no sign of germination or rooting and turned necrotic.

***In vitro* establishment of softwood shoots for rooting**

Forced softwood shoots, established for *in vitro* rooting did not show successful results due to the contamination of cultures.

DISCUSSION

Present study included the forced sprouting of soft shoots from logs of *D. sissoo* with their successful rooting *in vitro.* It was reported earlier that rooting of woody species is very complicated and difficult (Kyte and Kleyn 1999). Previously it was reported by Fett-Netto et al., (2001) that due to the age of seedling, the woody species loose the capability of rooting, and hence showed no rooting. In present research work, softwood shoots were excised and placed in sterilized media (sand, cocopeat and sawdust) after embedding them in solutions of growth regulators (1000ppm IBA, 1000 ppm NAA, or both) for 10s (Haroon *et al*, 2018; Henry and Preece, 1997). In all mediums of shoots, no rooting was observed after some days. It was suggested by Balleste *et al*. (1999) that complicated or delayed root induction was linked with maturity or juvenility of shoots.

Earlier Nascimento *et al*. (2018) has also shown the three successful methods of epicromic shoot propagation of *Ilex paraguariensis*. Shukla *et al*., (2020) showed that solid media usually cause the damage to newly growing roots of mericlones or if shifted to other media, it is stuck at some points of roots and causes contamination and causing root decay. Therefore, it is suggested to opt liquid medium for growth of roots in future and then transferring them to cocopeat or other media etc.

Conclusion

In conclusion, softwood shoot forcing from huge stem segments is reliable and cost-effective method for clonal micropropagation. Greater and efficient

growth of *D. sissoo* can be achieved by using various novel methods of micropropagation. In order to improve this method of micropropagation as it is an efficient one, more research work is required. In *in vitro* culturing of softwood shoots, to avoid microbial and fungal contamination more steps or safety measure should be adopted.

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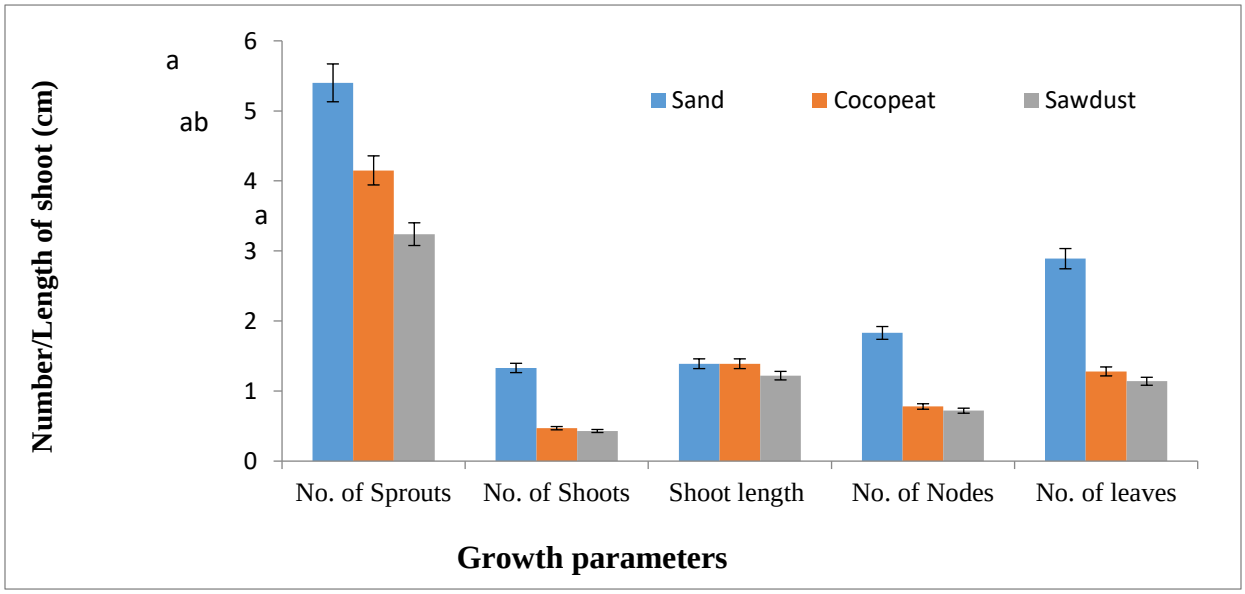

Above the columns, vertical bars are the SE (±) of means. Above the vertical bars, different letters are demonstrating the substantial differences according to Duncan's Multiple Range test at P less than 0.05 (P<0.05).

Figure 2: Effect of different media on epicormic bud induction potential with reference to bud-derived shoot parameters in stem logs of shisham that were present under natural conditions.

Table 1: Studied parameters for micropropagated shoots

Condition	Medium used	Log Diameter (cm)	No. of Sprouts	No. of Shoots	Shoot length (cm)	No. of Nodes	No. of leaves
Lab	Sand	3.05^{a}±0.6	5.45^{a}±0.9	0.64^{c}±0.02	1.06^{b} ±0.65	0.61^{bc}±0.01	4.91^{a}± 0.49
	Cocopeat	2.94^{a}±0.5	4.69^{a} ±0.6	2.91^{a} ±0.06	2.56^{a} ±0.65	1.94^{a}±0.04	4.92^{a} ±0.39
	Sawdust	3.11^{a}±0.4	2.81^{d}±0.3	0.39^{c} ±0.08	0.83^{c} ±0.06	0.31^{c}±0.06	0.77^{d} ± 0.04
Natural	Sand	3.06^{a}±0.5	5.41^{a}± 0.2	1.33^{b}± 0.01	1.39^{b} ±0.09	1.83^{a}±0.00	2.89^{b}± 0.11
	Cocopeat	3.28^{a}±0.4	4.15^{ab}±0.8	0.47^{c} ±0.04	1.39^{b} ±0.09	0.78^{b}±0.01	1.28^{c}± 0.96
	Sawdust	3.28^{a}±0.5	3.24^{c}±0.6	0.43^{c} ±0.01	1.22^{b}±0.07	0.72^{b}±0.01	1.14^{c}± 0.76

Data signified the means of 9 logs per medium/ location. Three logs were placed in each medium. Diverse letters inside a detailed column represent noteworthy modification at $P < 0.05$ according to Duncan's Multiple Range Test.