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Research Article

# Silver nitrate and silver-thiosulphate mitigates callus and leaf abscission during Shisham clonal micro-propagation

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**Abstract** Basal callus formation and leaf abscission is a problem in clonal micropropagation. We have described an *in vitro* clonal propagation protocol of *Dalbergia sissoo* Roxb (shisham) 'FRI-14' in which $AgNO_3$ played important role not only in mitigating problem of leaf abscission and basal callus, but also improved shoot induction and multiplication. Best induction and shoot multiplication was obtained on MS media with 1.5 mg/l 6-BAP and 10 mg/l $AgNO_3$ and half-strength MS media with 0.5 mg/l 6-BAP, 2 mg/l $AgNO_3$ and 50 mg/l Adenine sulphate whereas best *ex vitro* rooting was obtained with 200 mg/l IBA in pulse treatment.

**Keywords** Clonal propagation, Ethylene inhibitors, Tissue-culture, Tahli, Shisham, Callus

## Introduction

*Dalbergia sissoo* is a fast growing, nitrogen fixing, multi-purpose timber yielding tree species which belongs to family Fabaceae. This perennial tree is indigenous to India, Bangladesh, Pakistan, Nepal and Afghanistan can thrive in diverse range of soil types, elevations, climate and pHlevels (Majeed et al. 2019; Orwa et al. 2009). This is pioneer tree of primary serale so excellent for land restoration and afforestation programs (Tiwari 1994). Though it is largely being grown in agroforestry, lately the genus *Dalbergia* declared endangered by IUCN (Chauhan et al. 2021). Moreover, it is a known indigenous therapeutic plant as it contains many bioactive compounds such as isoflavones, coumarins, flavones, and quinines, dalbergin etc. (Majeed et al. 2019). A clonal highly productive and disease resistant variety of this species FRI 14 has been released for cultivation under farm and agroforestry system in northern India (Chauhan et al. 2021). Clonal varieties are propagated through either macro or micro propagation. Micro propagation has some distinct advantages over other conventional method of vegetative propagation as it has the potential to grow thousands of infection free plants of desirable genetic material, also maintain vigour by producing virus free plants through apical meristem (Shahzad et al. 2017). There are many reports of micro-propagation of *D. sissoo* by using explants derived from seedlings (Chand and Singh 2004, 2005; Das et al. 1997; Pattnaik et al. 2000; Singh and Chand 2003) and mature explants (Datta and Datta 1983; Joshi et al. 2003; Thirunavoukkarasu et al. 2010; Vibha et al. 2014).

The most important challenges reported in the micro-propagation of *D. sissoo* are basal callus formation, leaf fall, shoot tip necrosis (Datta and Datta 1983; Joshi et al. 2003; Thirunavoukkarasu et al. 2010; Vibha et al. 2014). Often, premature leaf fall, reduced growth and develop-menthave been associated with excess amount of ethylene produced in *in vitro* culture vessels. Callus can be a spoilsport, as regeneration through this pathway may produce genetically dissimilar plantlets instead of clones due to somaclonal variation (Bairu et al. 2011; Currais et al. 2013). Chemical compounds like $AgNO_3$ are known to block the action of ethylene, consequently promote the growth of plant cultures and reduce the leaffall in shoots (Lemos and Jennet 2015; Ravi et al. 2019). Moreover, $AgNO_3$ hastens *in vitro* callus formation.

This study was an attempt to develop a complete micropropagation protocol for an elite variety of *D. sissoo*

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'FRI 14' alongwith mitigating problem of premature leaf fall, shoot tip necrosis and basal callus formation using ethylene inhibitors like $AgNO_3$ and silver thiosulphate during clonal micropropagation.

## Material and Methods

The clonal variety of 'FRI 14' is being grown in a vegetative multiplication garden of *D. sissoo*and branches from rejuvenated shoots were collected as explant in the month of March to Junefor *in vitro* propagation. Nodal segments were wiped with 70% alcohol followed by soaking of nodal cutting in aqueous solution of Citramide® (3 ~ 4 ml in 100 ml distilled water) for 15 minutes. Nodal segments were washed thoroughly with sterile distilled water followed by treatment with a fungicide 0.2% Nativo® for another 15 minutes. Finally, explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 8 ~ 10 minutes in aseptic condition and washed thoroughly with sterile water to remove traces of mercuric chloride.

### Axillary bud induction

Sterilized nodal segments were cultured on MS (Murashige and Skoog 1962) media supplemented with different concentration and combination of 6-Benzylaminopurine (BAP), Kin (Kinetin), $AgNO_3$ and silver thiosulphate (STS). Prior to adding 7 % (w/v) agar, the pH was adjusted to 5.6 with the help of a pH meter. Equal amount of media prepared was aliquoted into glass tubes (25×150 mm) and autoclaved for 15 minutes. Cultures were maintained at a uniform condition throughout experiments with 26°C temperature and 50 $\mu$ mol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) and photoperiod of 16/8 (light/dark).

### Shoot multiplication

*In vitro* raised shoots were sub-cultured on MS media augmented with different concentrations and combinations of 6-Benzylaminopurine (BAP), Ads (adinine sulphate) and $AgNO_3$ (silver nitrate). MS Media without any plant growth regulators was considered as control. Cultures were repeatedly transferred on fresh media with same combination of plant growth regulators after every two weeks. After six weeks of subculturing, data in terms of shoot length (in cm), shoot number (≥ 0.5 cm) and leaf fall percentage was recorded.

### *Ex vitro* rooting

Healthy *in vitro* grown microshoots (3 ~ 4 cm) were trimmed at their basal end and washed with sterile water in septic condition. Microshoots were pulse treated with different concentrations of NAA (100 ~ 300 mg/l) and IBA (100 ~ 300 mg/l). The shoots treated with auxins for root induction were transferred into jam bottles containing autoclaved vermiculite. Thereafter, properly caped jam bottles were placed inmost humid section of green house. Once rooting initiated, the caps of the bottles were loosened to reduce the humidity and were moved near fan section. Finally, the plantlets were planted on a mixture containing equal amount of soil and sand. Root length and root number were calculated after four weeks.

### Statistical analysis

Experiment conducted were set up in a completely randomized design (CRD). Each experiment had nine replicates. Shoot number, shoot length, leaf fall, root number and root length calculated were analysed statistically. The analysis of variance (ANOVA) was determined by using SPSS. Tukey's HSD test was used for post hoc and mean were compared at $p \leq 0.05$.

## Result and Discussion

Effect of different combination of hormones on *in vitro* bud induction is shown (Table 1). Shoot bud initiation was observed in all media combinations. However, of both the cytokinins (6-BAP and Kin) tested, 6-BAP was found more effective than Kin. Our results are in conformity with earlier report on the micropropagation of *D. sissoo* (Vibha et al. 2014). BAP is a synthetic cytokinin which helps in inducing bud break, help in axillary shoot development and shoot multiplication (Sadeghi et al. 2015). The best response was noted in MS media fortified with 1.5 mg/l BAP and 10 mg/l $AgNO_3$ (Fig. 1b) with maximum shoot number (2.6) and shoot length (4.4 cm) followed by MS media containing 1.5 mg/l BAP and 15 mg/l $AgNO_3$. However, the further increase in the concentration of BAP resulted in basal callus formation and reduction in the overall development of shoots (Fig 1c). Similar observations were reported in *Moringa oleifera* (Ravi et al. 2019; Riyathong et al. 2010).

Both $AgNO_3$ and STS showed a synergetic effect with BAP on the recorded parameters. Growth of *in vitro*

**Table 1** Effect of different cytokinin (BAP & Kin) and different additives (Silver nitrate and Silver thiosulphate on axillary bud induction and shoot growth of *D. sissoo*

| BAP (mg/l) | Kin (mg/l) | $AgNO_3$ (mg/l) | STS (mg/l) | Shoot No. (± SD) | Shoot Length (± SD) (cm) |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | | | | 1.5 $\pm0.74^{de}$ | 2.5 $\pm0.37^{e}$ |
| 1.5 | | | | 2.0 $\pm0.38^{bcd}$ | 3.3 $\pm0.27^{d}$ |
| 2.5 | | | | 1.5 $\pm0.52^{de}$ | 1.4 $\pm0.52^{g}$ |
| 1.5 | | 5 | | 2.4 $\pm0.74^{ab}$ | 3.9 $\pm0.38^{bc}$ |
| 1.5 | | 10 | | 2.6 $\pm0.83^{a}$ | 4.4 $\pm0.41^{a}$ |
| 1.5 | | 15 | | 2.4 $\pm0.63^{ab}$ | 4.1 $\pm0.55^{ab}$ |
| 1.5 | | | 5 | 1.7 $\pm0.70^{cde}$ | 3.9 $\pm0.36^{bc}$ |
| 1.5 | | | 10 | 2.2 $\pm0.56^{abc}$ | 3.8 $\pm0.62^{c}$ |
| 1.5 | | | 15 | 1.9 $\pm0.35^{cde}$ | 2.5 $\pm0.35^{e}$ |
| | 0.5 | | | 1.4 $\pm0.51^{e}$ | 1.7 $\pm0.33^{fg}$ |
| | 1.5 | | | 1.9 $\pm0.70^{bcd}$ | 2.0 $\pm0.38^{f}$ |
| | 2.5 | | | 1.9 $\pm0.70^{bcd}$ | 1.9 $\pm0.61^{f}$ |

Where Kin = Kinetin, STS = Silver thiosulphate
Means followed by the same letter within columns are not significantly different ($p < 5\%$) using Tukey's HSD test

cultures is often inhibited by excess amount of accumulated ethylene which leads to leaf fall (Burg 1968; Kao and Yang 1983), cultures deterioration and browning of medium and explants. $AgNO_3$ is known to restrict the effect of ethylene hormone on plant. In this study, addition of 10 mg/l $AgNO_3$ had a significant effect on shoot number and shoot length. It was also observed that the leaf fall, basal callus formation and necrosis of shoot tips reduced drastically in shoots treated with $AgNO_3$. Hassanein et al. (2018) reported that the shoot multiplication of *M. oleifera* was improved by addition of $AgNO_3$ in the culture media. Similar results have been observed in other species such as pomegranate (Naik and Chand 2003), black gram (Mookkan and Andy 2014), banana (Tamimi 2015), sunflower (Mirzai et al. 2015) and cherry (Sarropoulou et al. 2016).

Shoot multiplication

*In vitro* grown shoots were transferred repeatedly on fresh half strength MS media with different concentration of BAP, $AgNO_3$ and adenine sulphate (Ads). The maximum number of shoots was observed after fourth passage of subculturing. Of all the media combinations used, the best result was noted on half strength MS Media supplemented with 0.5 mg/l BAP, 2 mg/l $AgNO_3$ and 50 mg/l Ads. Maximum number of shoots (10.7) was recorded on this media combination. Ads was added to multiplication media as adinine in Ads is known to induce cell growth and shoot bud formation (Ahmad et al. 2018). For shoot multiplication, half strength MS media was found superior to full strength MS media. There was an increase in both the number of shoots and shoot length with the addition of $AgNO_3$. Moreover, it was observed that $AgNO_3$ reduced the premature leaf fall of *in vitro* grown shoot cultures (Table 2, Fig. 1d). In cultures of *Annona squamosa*, silver nitrate was found to be an effective inhibitor of abscission of leaves (Lemos and Jennet 2015). Similarly, silver nitrate is reported to block the action of ethylene which stimulates the leaf abscission in cotton species (Beyer 1975; 1976a; 1976b; 1979). Ravi et al. (2019) noticed that application of silver nitrate (2.5 μM) reduced the leaf fall (20.6%) significantly in cultures of *M. oleifera.*

*Ex vitro* rooting

Effect of different concentrations of different auxins on *ex vitro* root initiation in shoots of Dalbergia sissoo is shown in Table 3. *Ex vitro* rooting helps in reducing cost, rooting time and acclimatization during micropropagation of plants and therefore it is a very effective method of inducing rooting *in vitro* cultures (Phulwaria et al. 2012b; Yan et al. 2010).

Furthermore, *ex vitro* differentiated roots are more adapted to the anchoring medium. The shoots multiplied and established *in vitro* conditions were treated with auxins (IBA and NAA) of different concentration for root

**Table 2** Effect of different concentrations of plant growth regulators and additives (BAP, Ads and $AgNO_3$) on multiplication of shoots of *D. sissoo*

| Media combinations | Shoot length (cm) (± SD) | | Shoot number (± SD) | | Leaf fall (%) (± SD) | |
|---|---|---|---|---|---|---|
| 0 | 0 | | 0 | | 0 | |
| 0.5 BAP mg/l (MS) + 50 mg/l Ads | 4.5 | ±0.23$^{d}$ | 5.7 | ±0.46$^{c}$ | 37.64 | ±1.57$^{bc}$ |
| 0.25 mg/l BAP (1/2 MS) + 50 mg/l Ads | 5.0 | ±0.30$^{c}$ | 7.6 | ±0.64$^{b}$ | 35.65 | ±0.7$^{6c}$ |
| 0.5 mg/l BAP (1/2 MS) + 50 mg/l Ads | 4.1 | ±0.45$^{e}$ | 4.8 | ±0.70$^{cd}$ | 39.10 | ±1.33$^{b}$ |
| 1 mg/l BAP (1/2 MS) + 50 mg/l Ads | 3.8 | ±0.40$^{e}$ | 3.9 | ±1.03$^{d}$ | 41.26 | ±1.46$^{a}$ |
| 0.25 mg/l BAP + 2 mg/l $AgNO_3$ (1/2 MS) + 50 mg/l Ads | 5.5 | ±0.50$^{b}$ | 7.8 | ±0.46$^{b}$ | 28.75 | ±0.98$^{e}$ |
| 0.5 mg/l BAP + 2 mg/l $AgNO_3$ (1/2 MS) + 50 mg/l Ads | 6.0 | ±0.54$^{a}$ | 10.7 | ±0.64$^{a}$ | 30.57 | ±2.05$^{de}$ |
| 1 mg/l BAP + 2 mg/l $AgNO_3$ (1/2 MS) + 50 mg/l Ads | 6.1 | ±0.34$^{a}$ | 7.8 | ±0.91$^{b}$ | 32.07 | ±1.39$^{d}$ |

Where, Ads = Adenine sulphate
Means followed by the same letter within columns are not significantly different ($p < 5\%$) using Tukey's HSD test

**Table 3** Effect of different concentrations of auxins (IBA, NAA) on *Ex vitro* rooting initiation for *in vitro* raised shoots

| IBA (mg/l) | NAA (mg/l) | Root Number (± SD) | | Root Length (cm) (± SD) | |
|---|---|---|---|---|---|
| 0 | 0 | 0 | | 0 | |
| 100 | | 4.56 | ±0.527$^{a}$ | 4.1 | ±0.10$^{b}$ |
| 200 | | 4.89 | ±0.782$^{a}$ | 5.1 | ±0.25$^{a}$ |
| 300 | | 4.67 | ±0.866$^{a}$ | 3.8 | ±0.16$^{c}$ |
| | 100 | 1.67 | ±0.500$^{c}$ | 2.1 | ±0.31$^{e}$ |
| | 200 | 3.00 | ±0.500$^{b}$ | 2.4 | ±0.27$^{d}$ |
| | 300 | 2.22 | ±0.833$^{c}$ | 2.3 | ±0.20$^{d}$ |

Means followed by the same letter within columns are not significantly different ($p < 5\%$) using Tukey's HSD test

induction. The effect of different rooting hormones in terms of root length and root number were analysed after four weeks of treatment. IBA was found better than NAA for rooting of cultured shoots. Earlier studies on *in vitro* propagation of *D sissoo* also suggest that IBA is superior rooting hormone than other hormones tested (Thirunavoukkarasu et al. 2010; Vibha et al. 2014). IBA in woody species, has been reported to stimulate strong rooting response (Eeswara et al. 1998; Husain et al. 2008). The best result was observed with 200 mg/l IBA which produced an average of 4.89 roots per shoot which was significantly higher than NAA treated shoots (Fig. 1e and 1f). However, there was no significant difference in the number of roots between different concentration of IBA. The lowest number of roots (2.2) was found in 1 mg/l NAA treated shoots. It was observed that the lateral roots developed were without callus formation at the basal ends of *in vitro* raised shoots, which is another advantage of *ex vitro* rooting over *in vitro* rooting.

## Conclusion

In summary, an efficient protocol was developed for direct organogenesis and multiplication of an elite germplasm of *D. sissoo*. Best result for axillary bud induction and shoot development was observed on MS media containing 1.5 mg/L BAP and 10 mg/l $AgNO_3$. Different media combinations were used for multiplication of *in vitro* raised shoots and the maximum number of shoots (10.7) were noted in MS media supplemented with 0.5 mg/l BAP, 2 mg/l $AgNO_3$ and 50 mg/l Ads. Further, the shoots obtained after four weeks of subculturing, shoots obtained were pulse treated with different auxins (NAA, IBA) for *ex vitro* root induction. Finally, the rooted shoots were transferred in pots for the hardening of the plantlets. Results of present study also suggest that addition of $AgNO_3$ not only played a positive role in growth and maintenance of shoots but also helped in reduction of premature leaf fall. The protocol is suitable for commercial production of *D. sissoo* variety 'FRI-14'.

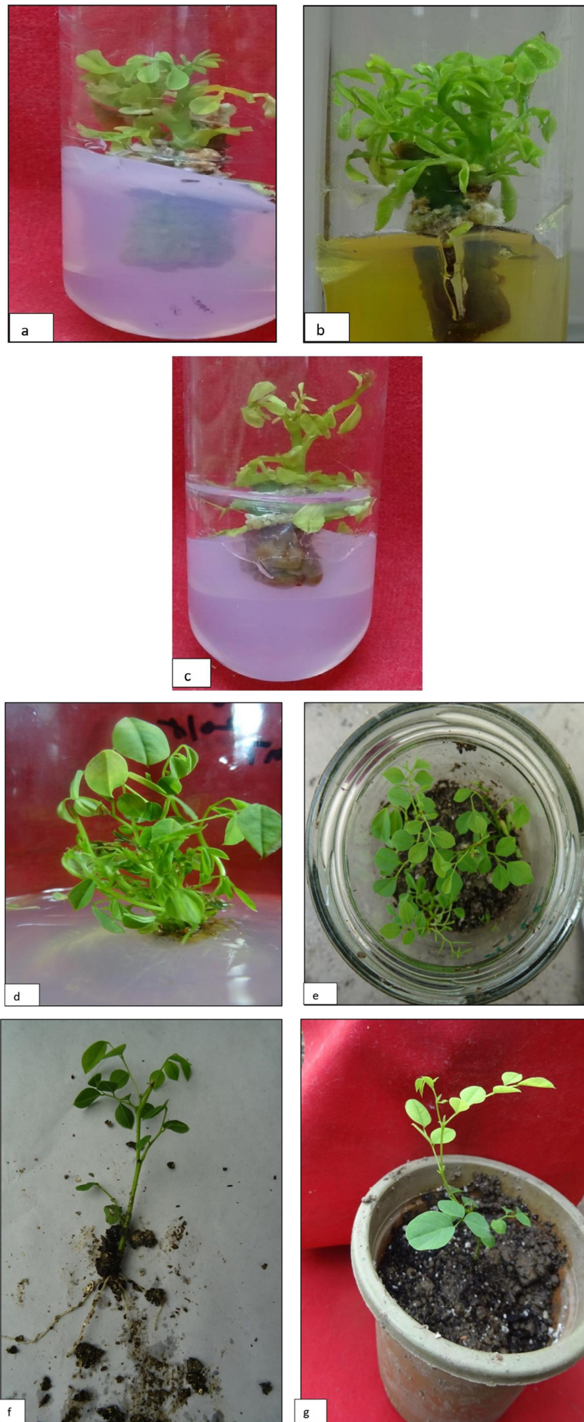


**Fig. 1** Micropropagation of D. sissoo. (a) Shoot bud induction on media supplemented with 1.5 mg/L BAP (bShoot bud induction on media supplemented with 1.5 mg/L BAP and 10 mg/L $AgNO_3$. (c) leaf fall in 2.5 mg/L BAP. (d) Shoot multiplication on ½ MS media supplemented with 0.5 mg/L BAP + 2 mg/L $AgNO_3$ + 50 mg/L Ads (e) Shoot cultured in Vermiculite for root induction (f) *ex vitro* rooting on ½ strength MS supplemented with 0.5 mg/L IBA (g) Plantlets transferred in pots

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## Contribution

MR and AT has jointly conceptualized the study, AT guided and facilitated the study and MR executed research work, MR and AT has jointly analysed data and written manuscripts.

## Conflict of interest

Authors have no conflict of interest

## Announcement for submission

Authors declare that this manuscript has not be submitted fully or partially anywhere.

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