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RAPID CLONAL PROPAGATION THROUGH IN VITRO SHOOT TIP AND NODAL EXPLANTS OF *PISONIA GRANDIS* R. BR.

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Abstract

Pisonia grandis R. Br. is a medium size evergreen tree belonging to the family Nyctaginaceae and locally known as Lechaikattai keerai in Tamil. The leaf extracts were reported with numerous bioactive compounds. The cells under culture accumulate large amounts of secondary compounds under specific culture condition. Therefore, in vitro propagation of medicinal plant can be used as fast and reliable method to synthesis the bioactive compounds. Moreover, *P. grandis* is reproduced by vegetative means and success rate of production of viable sapling is also very low. Besides, young saplings were also affected with leaf rust diseases. Therefore, effective strategies to be developed for the production of viable saplings of this multipurpose plant species by using plant tissue culture technique. In the present study, maximum mean numbers (13.12 ± 0.42) of shoots per explants were obtained on MS medium supplemented with 1.0 mg/l BAP. IBA at 4.0 mg/l showed the best results, where 8.26 ± 0.17 mean number of roots and 10.82 ± 0.21 mean number of root length were initiated after 15 days of culture.

Key words: Mass multiplication, Leaf rust, MS Medium, BAP, IBA.

Introduction

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants using modern plant tissue culture methods. Micropropagation is used to multiply novel plants such as those have been genetically modified or bread through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a sufficient number of plantlets for planting from a stock plant which does not produce seeds or does not respond well to vegetative reproduction (Ajithkumar and Seeni, 1998).

The encourage overharvesting of medicinal plants, leading to an extractive exploitation that exposes the species to the threat of extinction. The interest an *in vitro* propagation of medicinal plants is clearly on the rise, because they represent the most important sources of medicines and other pharmaceutical products. Methods of *in vitro* propagation offer highly effective tools for germplasm conservation and mass multiplication of many plant species threatened with extinction (Murch *et al.*, 2000; Pan *et al.*, 2003).

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Pisonia grandis R. Br. is a medium size evergreen tree belonging to the family Nyctaginaceae and locally known as Lechaikattai keerai in Tamil. It is wide spread evergreen glabrous garden tree with young shoots are minutely puberulous. It is a native of Hawai island also naturalized throughout India. It has been considered as an ornamental tree and leaves resembles lettuce in taste. So, it is used as a vegetable and also as salad (Anonymous, 1969) in most part of the India. The plant *Pisonia grandis* extensively used as leafy vegetable and in Indian traditional medicine as an anti-diabetic and for other medicinal purposes. Leaves, stem and root of this species are extensively used by the tribals in the preparation of several folk medicines (Shubashini *et al.*, 2013). It has been extensively used in Indian traditional medicine as an antidiabetic, anti-inflammatory agent, and used in the treatment of algesia, ulcer, dysentery and snake bite (Prabu *et al.*, 2008; Radha *et al.*, 2008; Sunil *et al.*,2009; Will McClatchey, 1996). The roots are used as purgative. The phytochemical study reveals that the presence of steroids likes octocosanol, betositosterol, alphaspinosterol,

dulcitol and flavonoids in the leaves of the plant (Manogaran, 2002).

Pisonia grandis have a numerous bioactive compounds includes Pinnatol, Allantoin, β- Sitosterol, α-Spinasterol, β-Sitostero glucoside, Octocosanal, Dulcitol, Flavonoids and Quercetin. It has been claimed that the compound possesses insulin like effects and also mediate certain actions of insulin (Sivakumar *et al.,* 2010). Among the various bioactive compounds, pinitol has been extensively used in Indian traditional medicine as an anti-diabetic and by chewing two leaves of this tree per day has been traditionally followed to control diabetes. Pinitol or 3-O-methyl D-Chiro-inositol, has been reported to possess antidiabetic and hypoglycaemic activities (Mijares *et al.,* 2013; Sivakumar and Subramanian, 2009). It has been claimed that the compound possesses insulin like effects and also mediate certain actions of insulin (Sivakumar *et al.,* 2010). Clinical trials on the effect of pinitol on glycaemic control and cardiovascular risk factors in patients with type II diabetes mellitus have been successfully carried out (Kim *et al.,* 2005; Min-Jung Kang *et al.,* 2006). Pinitol is reported as safe and nontoxic as an anti-diabetic agent even at high levels (Sarah *et al.,* 2000).

Plant products have gained prominence during the past few years along with the management of traditional medicinal plant resources and it has become a matter of urgency. An ever increasing demand of medicinal plants and its drugs warrants their mass propagation. The in vitro propagated medicinal plants are genetically pure elite. In organ culture, plants have high concentrations of secondary metabolites tend to accumulate in specific cell types during developmental stages. The tissue cultured cells from these plants typically accumulate large amounts of secondary compounds under specific conditions. Based on that, efficient in vitro regeneration of the whole plant can be used as a fast and reliable method to transform *P. grandis* for the synthesis of bioactive compounds and its active principles. Moreover, *P. grandis* non-viable to reproduce through sexually and produced their offsprings through vegetative means only. Besides, major percentages of saplings developed through vegetative cuttings were affected with leaf rust (Ramasubbu *et al.,* 2015). Due to its specific medicinal compounds and varieties of medicinal practices, the plant has been over exploited. Therefore, necessary steps to be taken to save the species for before eradication. In this connection, rapid clonal propagation protocol has been developed the mass multiplication of this medicinally important tree species of *P. grandis* for sustainable utilization.

Materials and Methods

Collection of Plant materials and Surface Sterilization

The explants (shoot tip and nodal) of *Pisonia grandis* were collected from Sri Kaliswari College, Sivakasi, Virudhunagar dist, Tamil Nadu, India. The explants were washed under running tap water 30 min. and then they were washed in an agitated solution of liquid detergent for 5 min. The disinfected materials were removed by rinsing the material with sterilized cooled distilled water for 3-5 times. The explants of *Pisonia grandis* were surface sterilized with 0.1% Mercuric chloride for 3 min. Then, subsequently washed with sterile distilled water. The explants were again sterilized with 4% Sodium hypochlorite for 3 min. and rinsed with sterile distilled water. The material were again surface sterilized with ethanol (70%) for 3 min. finally washed with sterile distilled water and the explants cut the tip end with help of a sterilized blade.

Culture medium and Condition

The sterilized explants were cut into 2-3cm long pieces and cultured on Murashige and Skoog medium containing 30g/l of sucrose and 8g/l of agar was also added as carbon source and gelling agent respectively with the above chemical composition. Along with boiled medium, growth hormones like BAP and Kn were added at different concentrations (0.1-2.0 mg/l) and dispensed into the culture vessels. The medium was sterilized in an autoclave at 121ºC for 30 min. The sterilized culture vessels with MS medium supplemented with different hormones were transferred to the laminar air flow chamber for the successful sterilization.

Culture Initiation and Shoot Multiplication

The explants were carefully inoculated in the culture vessels with MS medium along with various concentrations of growth hormones without any microbial contamination. The inoculated glass vessels were finally kept in the culture rack in the room with proper temperature (25±2ºC) and light intensity of 2500 – 3000 lux. by cool fluorescent lamp. After 30-45 days, the shoot development from the culture was observed carefully and number of shoots and length of shoots developed from the explants were also observed. Callus initiations and its developments if any were also observed in the cultured tubes. Multiple shoots developed from the explants were transferred from the culture tube to a sterile glass plate using sterilized forceps. The leaves were removed from the primary shoots developed in the culture medium and sectioned into one node piece with the leaves. These nodal segments were transferred to the shoot multiplication media with different concentration (0.5-2.5mg/l) of 6 Benzyl amino purine (BAP) and kinetin

(0.5-2.5 mg/l). All the experiments were carried out with extreme care and safe inside the laminar air flow chamber to avoid microbial contamination.

Rooting of the shoots

Auxiliary shoots developed in culture in the presence of cytokinin generally lack roots. To obtain full plant, the developed shoots were transferred to a rooting medium combined with different concentration of IAA and IBA (1.0-5.0 mg/l). Half strength of MS medium is found better for rooting of shoots in large number of plantlets.

Acclimatization

The transfer of regenerated plantlets from the culture vessel to the soil requires a careful stepwise procedure. The rooted of plants were gently washed with sterile double distilled water to remove adhering medium completely without causing any damage to the root. The regenerates were carefully transferred to the hardening medium in combination with sterile coco-peat, compost and sand in the ratio of 1:1:2. The successfully developed plants were transferred to the field for the acclimatization and survival rate was recorded after two months.

Results and Discussion

In the present study, nodal and shoot tip explants of the Pisonia grandis were micro-propagated and the multiple shoot initiation was obtained within 40 days of culture. Both full strength and half strength MS medium were tried for shoot and root induction. Shoot development was obtained in the full strength medium, whereas the root induction was observed in the half strength MS medium. The pH adjusted to 5.8 was optimum for the shoot multiplication, root induction and subsequent regeneration. Culture room with continuous light from fluorescent tube was maintained at a constant temperature of 25 ± 2ºC and 80 ± 5% relative humidity. Nodal and shoot tip explants were used for the in vitro propagation and mass multiplication of large number of plants and more number of shoots were obtained through the nodal explants. The result was supported by the earlier reports Ramasubbu and Chandraprabha (2012) and Divya and Ramasubbu (2013) in which in vitro propagation techniques using shoot tip and nodal segments are important for mass scale multiplication and conservation of an endangered, threatened and medicinally promising species within short period and limited space. Propagation through tissue culture provides solution for mass propagation of plants in general and endemic and threatened plants in particular.

Shoot proliferation

After surface sterilization, shoot explants were inoculated in culture bottles aseptically. For inoculation, explants were transferred to large sterile glass petriplates or glassplate with forceps under strict aseptic conditions. The explants were further trimmed and extra leaves were removed and made into suitable sizes. After cutting the explants into suitable size, 5-6mm shoot tip explants, 20-25mm long nodal and shoot tip explants were inoculated on MS medium supplemented with different concentration (0.5-2.5 mg/l) of Benzyl Amino Purine (BAP) and Kinetin (Kn). Multiple shoots were initiated from all of the explants after 4 weeks of culture and all explants were free from both fungal as well as bacterial contamination. The successfully formed shoots were excised individually from proliferated explants and further cultured on same medium to increase the number of shoots (Plate 1 – A-F). Utilizing an accurate sterilization procedure in tissue culture techniques can save time and energy. The use of field grown plants as direct sources of explants for the production of clean in vitro plantlets, presents a major challenge (Webster *et al.*, 2003). This is because the surface of plants carries a wide range of microbial contaminants. To avoid this source of infection, the explants must be thoroughly surface-sterilized before inoculating them into nutrient medium. In an attempt to obtain clean in vitro cultures, sources of contamination other than surface contaminants need to be considered. Ahmad *et al.*, 2011 concluded the in vitro culture of *Aloe barbadensis* Mill., in which explants can be sterilized without using Mercuric chloride. The most suitable concentration of sodium hypochlorite for sterilization is 5%. In the present study, better results were obtained by the surface sterilization of Pisonia grandis from 0.1% of $HgCl_2$ 4% of Sodium hypochlorite and 70% Ethanol.

Table 1: Rapid clonal propogation of *P. grandis*. on MS medium with different growth regulators.

Concentration of growth hormonemg/L	Mean no. of shoot/ explants		Mean length of shoots (cm)	
	Shoot tip	Nodal	Shoot tip	Nodal
BAP				
0.5	5.12±0.21	6.07±0.13	6.11 ± 0.18*	7.62± 0.31
1.0	8.38±0.16	13.12 ± 0.42	8.12 ± 0.27*	10.31 ± 0.11
1.5	9.34±0.42	10.16 ± 0.37	6.32 ± 0.17	5.87 ± 0.29
2.0	7.20±0.63	5.81 ± 0.37	6.37± 0.41	4.63 ± 0.15
2.5	6.67±0.47	6.88 ± 0.35	4.73 ± 0.27	4.28 ± 0.11
Kn				
0.5	5.19 ± 0.31*	7.81 ± 0.27	5.78 ± 0.47	5.32 ± 0.10
1.0	4.31 ± 0.41	11.73 ± 0.48	9.27 ± 0.13	8.50 ± 0.31
1.5	8.32 ± 0.41	12.14 ± 0.27	13.02 ± 0.40	12.53 ± 0.27
2.0	7.30± 0.41	7.63 ± 0.27*	9.89 ± 0.11	8.01± 0.21
2.5	6.87 ± 0.34	8.02 ± 0.19*	4.03 ± 0.27	4.21 ± 0.50

± Standard error *callus formation along with shoot development.

Table 2: Effect of growth hormones on average rooting of *in vitro* developed root of *P. grandis.*

Growth regulators mg/L	Mean number of roots	Mean root length (cm)
IBA		
1.0	4.90±0.30	4.07±0.21
2.0	5.78±0.30	4.78±0.21
3.0	5.83±0.13	4.73±0.19
4.0	8.26±0.17	10.82±0.21
5.0	8.19±0.10	4.87±0.21
IAA		
1.0	7.05±0.32	4.85±0.67
2.0	6.43±0.47	5.72±0.35
3.0	4.97±0.18	5.92±0.25
4.0	7.72±0.17	8.62±0.55
5.0	5.63±0.27	4.79±0.33

PLATE - 1

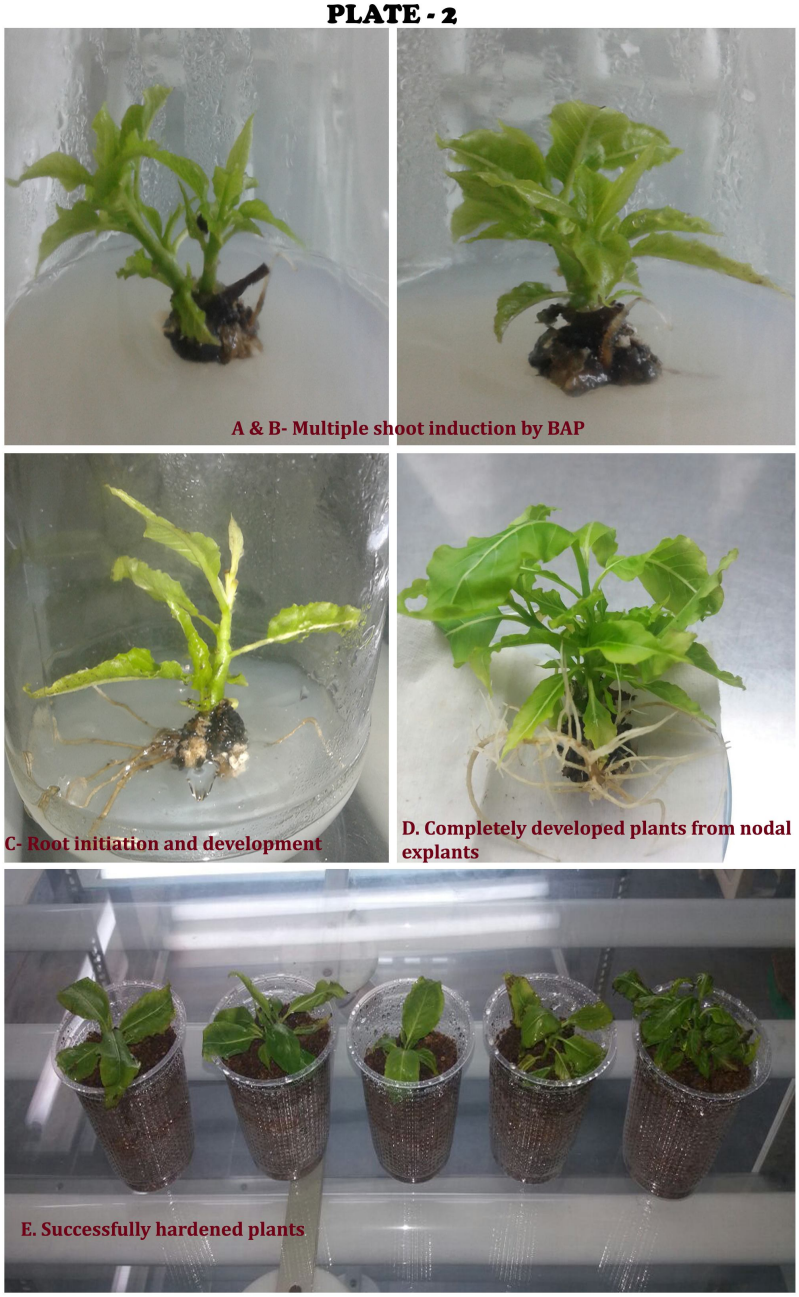
The synergetic effect of BAP (1.5 mg/l) induced 9.34 ± 0.42 mean number of shoots from shoot tip explants of Pisonia grandis. In 1.0 mg/l (BAP) concentration, 8.12 ± 0.27 mean length of shoots was obtained from Shoot tip explants of Pisonia grandis. The number shoot developed or organogenesis was highest (13.12 ± 0.42) in nodal with 10.31 ± 0.11 mean length of shoots in explants treated with 1.0 mg/l of BAP. The mean number of shoot developed was highest (8.32 ± 0.41) with mean length of shoots 13.02 ± 0.40 in 1.5 mg/l of kinetin. In 1.5 mg/l (kn) concentration, the number of shoot organogenesis was highest 12.14 ± 0.27 in nodal with mean length of 12.53 ± 0.27 shoots in nodal explants.

The results of the present study indicated that, the growth regulators in 1.0 mg/l (BAP) concentration induced shoot proliferation and 4.0 mg/l (IAA) concentration induced root proliferation. Selection of appropriate explants material is an important aspect during initial shoot induction. Micropropagation of medicinal plants is quite better than the traditional system of cultivation (Evan *et al.*, 1981). In vitro regeneration of medicinal plants using various explants such as leaf, shoot tip and node is generally practiced by various researchers in varieties of plants (Moura *et al.*, 1993). The best germination and development of seeds was recorded in MS medium supplemented with 4.43µM of BAP and 0.57µM of IAA in combination after 19 days of culture. Although, Dode *et al.*, (2003) used basal MS medium for in vitro seed germination of *O. basilicum* and found addition of growth regulators with MS medium is best for germination. Multiple shoot buds were initiated on the callus cultured in MS medium supplemented with both cytokinins (BAP and Kn) and auxins (IAA and NAA) singly and in combination. The manipulation of plant growth regulators is essential to optimize the induction of callus (Lim *et al.*, 2009).

Effect of plant growth regulators

Number of newly initiated shoot buds depends on the growth regulator concentration and type of cytokinin (BAP and Kinetin) used. The frequency of shoot bud development was increased with the increase in the concentration of cytokinins (BAP). The synergetic effect of BAP (1.5

PLATE - 2



mg/l) induced 9.34 ± 0.42 mean number of shoots from shoot tip explants of Pisonia grandis. In 1.0 mg/l (BAP) concentration, 8.12 ± 0.27 mean length of shoots was obtained from Shoot tip explants of Pisonia grandis. The number of shoot developed or organogenesis was highest (13.12 ± 0.42) in nodal with 10.31 ± 0.11 mean length of shoots in explants treated with 1.0 mg/l of BAP (Table 1). The mean number of shoot developed was highest (8.32 ± 0.41) with mean length of shoots 13.02 ± 0.40 in 1.5 mg/l of kinetin. In 1.5 mg/l (kn) concentration, the number of shoot organogenesis was highest 12.14 ± 0.27 in nodal with mean length of 12.53 ± 0.27 shoots in nodal explants (Plate 2- A&B). Emergence of shoot buds from callus when treated with MS medium supplemented with BAP and IAA singly and in combination showed poor response and eventually turned brown with no further growth. The superiority of Kn over BAP with respect to shoot bud initiation and subsequent proliferation of shoots from auxillary buds has been reported in earlier studies (Bhattacharya and Bhattacharya, 2001; Kumar and Rao, 2007).

By these studies of (Patnaik and Chand, 1996; Begum *et al.*, 2000) multiple roots were observed when the shoots were transferred to MS medium supplemented with 26.85µM of NAA and 02.32µM of Kn. Rooting did not occur or was poor when the shoots were placed in medium supplemented with IAA and BAP in combination or singly. This is in contrast to some of the earlier reports wherein rooting was induced. In the present study, the maximum numbers of shoots (9.34±0.42) were observed in MS media with 1.5mg/l BAP and these results were not supported to the earlier reports.

Rooting

The factor determining the success of micro-propagation is rooting. In vitro multiplied shoots were carefully removed from the culture medium and washed thoroughly with distilled water to remove the excess amount of medium and transfer to the medium with different concentrations (1.0 – 5.0 mg / l) of IAA and IBA for rooting. Among the various growth regulators tested, IBA at 4.0 mg/l showed the best results, where 8.26 ± 0.17 mean number of roots and 10.82 ± 0.21 mean number of root length were initiated after 15 days of culture. It shows the complete plantlets with elongated shoot and root systems ready to transfer to the soil. IBA at lower concentration has produced poor number of roots at culture condition. IBA at 1.0 mg/l produced a mean value of 4.90 ± 0.30 roots (Table 2; Plate 2 – C&D).

Among the growth regulators tested, IAA at 4.0 mg/ l also showed good results, where 8.72 ± 0.17 mean number of roots and 8.62 ± 0.55 mean number of root length were initiated after 15 days of culture. It shows the complete plantlets with elongated shoot and root systems ready to be transferred to the soil. IAA at lower concentration has produced poor number of roots at

culture condition. IAA at 3.0 mg/l produced a mean value of 4.97 ± 0.18 roots. The differences observed with regard to rooting percentage, number of roots/plantlet and number of days taken for rooting due to different auxins and their concentrations were also analyzed.

In the present study, the maximum numbers of roots (8.18±0.79) were observed in MS media with 4.0 mg/l IAA. But the roots developed in the cultured shoots were mostly branched and hairy. It was observed that higher concentrations of NAA inhibited root formation, when combined with Kn. Dode8 also observed that the presence of NAA (auxin) inhibited root formation when combined with different concentrations of cytokinin. Begum4 found that in vitro regenerated shoots rooted best in half-strength MS medium supplemented with NAA only. Gopi *et al.*, (2006) reported root primordial emergence from the shoot base on first week of culture in auxin-supplemented medium of *O. gratissimum*, where rooting in regenerated shoots was observed in half- strength MS medium containing IAA. Patnaik and Chand (1996) reported that shoots of *O. americanum* could be rooted in half- strength MS supplemented with IBA and *O. tenuiflorum* in medium with NAA. This result was corroborated with the earlier above reports.

Hardening process

Regenerated healthy rooted shoots of Pisonia grandis were used for hardening. The plantlets were removed from the culture vessels with the help of a long blunt pair of forceps and were rinsed with tap water for removing the agar medium, care was also taken to avoid damage to the plants. The agar medium sticking to the roots was removed carefully to avoid damage. The plantlets were then transplanted into plastic cups containing different mixtures of Sand + cow dung compost + coco peat (2:1:1) and were carefully sprayed with water and shifted to the glasshouse for hardening of plantlets. The minimum and maximum temperatures of the glasshouse at the time of transplantation were 18°C and 25°C respectively. The relative humidity of the glasshouse was around 70-80%. The plantlets were watered daily (Plate 2- E). Faisal and Amin (2000), who reported 85% survival of chrysanthemum plantlets when transferred to the plastic pots containing soil, sand and cow-dung. Datta *et al.*, (2001) also found similar results by transferring regenerated chrysanthemum plants with well established roots to pots with a mixture of sand: soil: manure (1:1:1) and kept in a chamber for 15 days. Misra and Singh (1999) reported that transfer of Gladiolus plantlets to a sterilized potting mixture containing FYM: sand: loam (2:1:1) resulted in better rooting. For the present investigation, the mixture of sterile soil and vermiculite in the ratio of 1:1 was used to acclimatize the plantlets with newly formed roots in plastic pots. Similar soil composition i.e. Soil and sand was used to acclimatize Dalbergia latifolia for hardening (Mohanraj, and Ramasubbu, 2013).

Manikandan *et al.*, (2017) reported that the regenerated healthy rooted shoots of Dalbergia latifolia were used for hardening. The plantlets were transplanted into plastic cups containing sand + soil + coco peat (1:1:1). The pots were covered with holed polythene bags for about 2-3 weeks and were carefully sprayed with water and shifted to the glasshouse for hardening of plantlets. The minimum and maximum temperatures of the glasshouse at the time of transplantation were 18°C and 25°C respectively. The relative humidity of the glasshouse was around 70-80%. The plantlets were watered daily. The hardened plants of Dalbergia latifolia showed a good survival rate of about 80%.

Conclusion

The present study describes the standardized protocol for rapid clonal propagation of Pisonia grandis using shoot tip and nodal part. It can be concluded that the protocol developed in this investigation is potentially useful in large scale propagation of Pisonia grandis.

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