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In-vitro establishment, germination and growth performance of Red Sandalwood (*Pterocarpus santalinus* L.)

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ABSTRACT

For the selection of a suitable surface sterilization procedure for *in-vitro* establishment of seeds, 0.1 % Mercuric Chloride was used with five different exposure times (5, 10, 15, 20, and 25 min). Pods with their highest external diameter of < 3, 3-4, 4-5, and > 5 cm were used to examine the effect of pod size on *in-vitro* germination and pods harvested at light brown stage and, stored at ambient temperature (28 ±2 °C) for 1, 2, 3, and 4 weeks, were used to examine the effect of storage time on *in-vitro* germination. To study the effect of different culture conditions on *in-vitro* germination, seeds were cultured on hormone free Murashige & Skoog (1962), Anderson (1980), Vitis (1987), and Woody Plant Medium (1980) with (1 g/l) and without activated charcoal. To study the *in-vitro* growth performance of red sandalwood seedlings, germinated seeds were cultured on hormone free MS, Anderson, and Woody Plant Medium at their full strength incorporated with 1 g/l of activated charcoal. Dipping the pods in 0.1 % $HgCl_2$ with 2-3 drops of Tween 20 for fifteen minutes was suitable for surface sterilization. Pods with <3 cm of diameter did not contain viable seeds. Seeds obtained from pods with >4 cm in diameter showed a significantly higher germination (90 %) ability, while time taken for germination was not affected by pod diameter. Success of seed germination was reduced with increasing storage time. Seeds cultured within one week of harvest showed the highest germination (96 %) and short germination time (6 days), while seeds cultured after 4 weeks showed relatively less germination (61 %) and longer germination time (10 days). Germination percentage was significantly higher (92%) in Anderson medium without charcoal, and low germination percentages of 62, 62 and 61 % were recorded on seeds cultured on woody plant medium (with charcoal), MS and Vitis medium (without charcoal), respectively. Seeds cultured on Anderson, Vitis and woody plant medium took almost similar time (6-8 days) for germination. Seeds cultured on Vitis medium (with charcoal) showed the longest hypocotyls length, 13.8 mm. The highest plant height of 9.9 cm and highest number of nodes per seedling (7 nodes/shoot)

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were recorded for seedlings in woody plant medium while MS medium showed the lowest number of nodes (6 nodes/shoot) and lowest plant height of 7.3 cm. Leaf formation was best in woody plant medium, and the average leaf diameter is 10.8 mm. The root system developed well in all media. The highest mean score for the appearance of the plant was recorded in woody plant medium (3.8). Therefore, Anderson medium was suited for *in-vitro* establishment of red sandalwood seeds. However, after seed germination, for further growth of the seedlings, germinated seeds should be transferred to Anderson medium incorporated with 1 g/l of activated charcoal.

Key words: Browning, Germination, I*n-vitro*, *Pterocarpus santalinus*, Red Sandalwood,

INTRODUCTION

Red Sandalwood (*Pterocarpus santalinus* L.) belonging to the Family *Fabaceae*, is one of the most valuable medicinal plant species. It is used as an external application for curing inflammations of skin diseases (Bhattacharjee, 2004), treating bone fracture, leprosy, spider poisoning, and scorpion sting (Aslam *et.al*., 1998). Sri Lanka is highly dependent on India for importing Red Sandalwood for Ayurvedic purposes. Since this species has been included in red list of endangered plants under the IUCN guidelines (Joseph and Abeysekara, 2004), India has recently banned the export of Red Sandalwood. Hence the cultivation of the plant in Sri Lanka to satisfy the local demand is very important and urgently needed. Conventional clonal propagation methods like grafting and rooted cuttings have not been very successful (Sita *et al.*, 1992) and natural germination by seeds is also highly restricted due to its hard seed coat. According to Kumarasinghe *et al*., (2004) only about 49 % of germination was recorded when a seed treatment (alternate soaking and drying of scarified pods) was applied. On the other hand, available fruit bearing plants are very limited in Sri Lanka and thus collection of seeds is also very difficult since matured plants are extremely tall. This necessitates a non-conventional *in-vitro* rapid propagation system for the production of planting material locally. Therefore, this study was carried out to study the *in-vitro* germination and growth performance of Red Sandalwood in different culture conditions.

MATERIALS AND METHODS

This research was carried out at the Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, University of Ruhuna. For the selection of suitable surface sterilization procedure, extracted seeds were thoroughly washed with a detergent (5% v/v Teepol^{TM}) and kept under running tap water for 2 hrs. Then they were dipped in 0.1% $HgCl_2$ with two drops of Tween 20 for five different time durations of 5, 10, 15, 20, and 25 min. Thereafter, seeds were thoroughly washed with autoclaved distilled water for three times each with 10 min time period and cultured on full strength hormone free Murashige and Skoog (1962) basal medium. Number of seeds contaminated and germinated were recorded after three days of culture initiation and thereafter every 3rd day for 2 weeks.

To study the effect of pod size on *in-vitro* germination, pods were categorized into four groups according to their highest external diameter as < 3 cm, 3-4 cm, 4-5 cm, and > 5 cm including the wing. Seeds were then extracted and surface sterilized using 0.1 % $HgCl_2$ for 15 minutes and cultured on full strength hormone free Murashige and Skoog (1962) basal medium. Time taken for germination and number of germinated seeds were taken as observations, after three days of culture initiation and thereafter every 3rd day for 2 weeks.

To study the effect of storage time of pods on *in-vitro* germination, pods were stored at room temperature (28 ±5 ºC) for 1 week, 2 weeks, 3 weeks, and 4 weeks time period. At the end of storage period, seeds were extracted, surface sterilized using $HgCl_2$ 0.1 % for 15 minutes and cultured on full strength hormone free Murashige & Skoog (1962) basal medium. Time taken for the germination and number of germinated seeds were taken as observations, after three days of culture initiation and thereafter every 3rd day for 2 weeks.

To select a suitable establishment media for *in-vitro* propagation, seeds were surface sterilized using 0.1 % $HgCl_2$ for 15 minutes and cultured on hormone free Murashige & Skoog (1962), Anderson (1980), Vitis (1987), and Woody Plant Medium (1980) prepared at their full strength with and without charcoal (Norit PN.5TM). Time taken for germination, number of seeds germinated and hypocotyls length was recorded, after three days of culture initiation and thereafter every 3rd day for 2 weeks.

To study the effect of different culture medium on *in-vitro* growth performance of red sandalwood seedlings, *in-vitro* germinated seeds (just after the appearance of the hypocotyls) were cultured on hormone free, Murashige & Skoog (1962), Anderson (1980), and Woody Plant Medium (1980) prepared at their full strength incorporated with 1 g/l of activated charcoal (Norit PN.5TM). Plant height, number of leaves, leaf diameter, number of shoots, and overall appearance of the plant were taken as observations after one month of culture initiation.

Statistical analysis was performed using the SAS systems for Widows Release 6.12. Data were subjected to Two-way Analysis of Variance, and treatment means were compared using Least Significant Differences ($P<0.05$). All treatments were replicated ten times and cultures were kept at 23±2ºC and given 12 hrs photoperiod light regime.

RESULTS AND DISCUSSION

Dipping the seeds in 0.1% $HgCl_2$ for 15 min was found to be effective in surface sterilization. More than 90% of seeds were not contaminated and established well in the medium. When the exposure time in $HgCl_2$ was lower than 15 min., seeds were contaminated with either bacteria or fungi. When the exposure time increased up to 20 or 25 min, very low percentage of contamination was observed (Figure 01).

According to Narayanaswami (1999), for woody species, underground tubers and heavily infected plants from the field, treatment with $HgCl_2$ dissolved in water (0.1 % w/v) for 5 min for several hours was resorted to, but may cause erratic growth or affect cell proliferation unless thoroughly washed in order to completely

remove all traces of mercury still adhering to the material.

According to Rajkumar (1999), micropropagation studies conducted in India have shown that mercuric chloride at the concentration of 0.1 percent for 1 min was effective as a surface sterilant in aseptic cultures of *P. santalinus*. Conger (1981), reported that during the experiments carried out on Alfalfa *(Medicago sativa L.),* scarified seeds were surface sterilized in 95 % ethanol on shaker for 15 min followed by 2.5 min in 1.3 % sodium hypochlorite, 5 min under vacuum in 1.3 % sodium hypochlorite, and 2.5 min in fresh 1.3 % sodium hypochlorite. After three consecutive shakes (each for 5 min) in sterile distilled water, seeds have been successfully established in culture medium.

Seed germination was also reduced significantly ($p \leq 0.05$) due to fungal and bacterial contaminations with less exposure time (Figure 02). With increasing exposure time, embryo was damaged because $HgCl_2$ may have been toxic to the embryo due to longer exposure time. Therefore, though the contamination was lower with higher exposure times (> 15 min), % germination was lower due to the damage of embryo. However, since plant parts are contaminated with a wide range of fungi and bacteria it is necessary to disinfect tissues with a strong sterilant causing minimum amount of cellular damage to the host tissue.

Fresh plant tissue is usually less contaminated, especially with spores, than dormant tissues such as seeds. The latter, however, will withstand more rigorous disinfection treatments without damage (Narayanaswami, 1999). Though some herbaceous plant materials may pose no problem in sterilization, woody materials need specific sterilization treatments. Nek rosova (1988) has reported high contamination rates for several fruit crops until optimum sterilization times are determined using 0.1% $HgCl_2$.

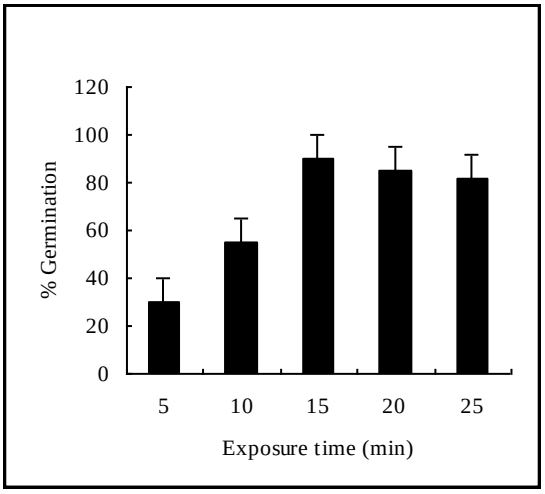

Figure 01: Effect of exposure time in 0.1% $HgCl_2$ on contamination percentage

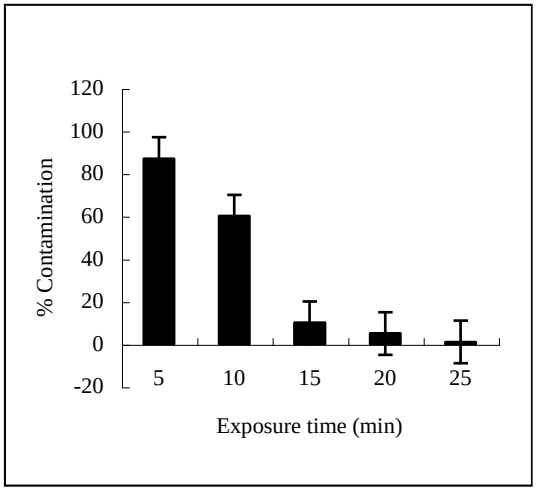

Figure 02: Effect of exposure time in 0.1% $HgCl_2$ on *in-vitro* germination

Pods should be thoroughly washed with a detergent material, and kept under running tap water for about 2 hours prior to surface sterilization. Continuous shaking throughout the time was necessary and washing the seeds with autoclaved distilled water for three or four times was effective to remove the sterilizing agent. According to Kumar and Kumar (1998), pretreatment of explants in running water overnight was very helpful. Surfactants (like Tween 20) reduced the surface tension of material being cleaned, thereby making the disinfecting solution more effective. It is important to remember that bacterial and fungal populations could build up on crevices, dense hairs, can all harbor microorganisms. Therefore, careful attention should be paid in explant selection to avoid explant sources that are more likely to harbor microorganisms and that by virtue of the explant surface or character may escape the effects of surface sterilants (Debergh and Zimmerman, 1991).

All damaged pods and those covered with mould on the surface should be discarded as they cause contaminations. Only light brown seeds should be taken for germination. Most of the dark brown seeds were found to be either not viable or embryo and cotyledons contaminated with fungi or bacteria. The rotten seeds could be identified when soaked in water after they were kept under running water. Rotton embryos come out between the seed testa and fingers when the seed was pressed between thumb and forefinger, whereas viable seeds feel hard. Anis *et al* (2005) have conducted experiments on *in-vitro* plantlet regeneration of *Pterocarpus marsupium*, a species very similar to red sandalwood, belonging to the same genus. The stony fruit coat of *P. marsupium* was removed manually with the help of a cutter and the seeds exposed to running tap water for 30 min, and then soaked for 24 h in distilled water. Seeds were then treated with liquid detergent for 5 min, followed by thorough washing. They were then surface sterilized with 0.1 % (w/v) aqueous $HgCl_2$ for 5 min, washed 4-5 times in sterile double distilled water and then implanted aseptically on half strength MS basal medium.

Most pods (> 99 %) with diameter less than 3 cm were seedless. If seeds are present, they were very fragile and had shriveled appearance. Therefore, no germination was recorded in pods with diameter less than 3 cm. These pods could be discarded directly after collection so that no labor and time is wasted in removing the hard seed coat. Pods with diameter above 3 cm contained seeds. *In-vitro* germination of red sandalwood seeds increased significantly ($p \leq 0.05$) with increasing pod diameter. Pods having a diameter above 4 cm showed a significantly higher (90 %) germination ability, while pods with 3-4 cm diameter showed less germination (42 %) ability (Figure 03).

However, there was no significant effect of pod diameter with time taken for seed germination. Pods in each three category took similar time (7 –8 d) to germinate (Figure 04). Therefore, pods having a diameter of more than 3 cm could be used for germination. However, as the pods with diameter of more than 4 cm showed a significantly ($p \leq 0.05$) higher germination, it is advisable to use those pods for germination to reduce time and labor. There were few pods (0.1 %) with two seeds inside the pod. This could be observed in pods with a diameter of more

than 5 cm. According to Sita *et al* (1992), in *P.santalinus* of the two ovules per flower, only one ovule produced seed in all modes of pollination but rarely both ovules produced seeds in xenogamous fruits. These pods also should be discarded as seeds show poor germination, due to compaction of two seeds inside a pod. For better results, seeds having shriveled appearance or very dark brown testa should also be discarded, as they do not produce plantlets, and increase contaminations.

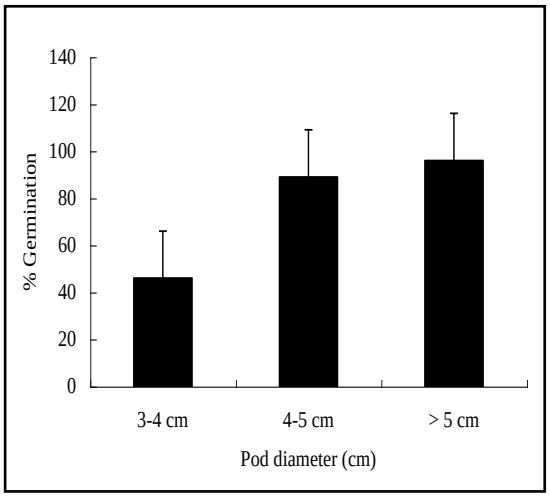

Figure 03: Effect of pod diameter on *in-vitro* germination

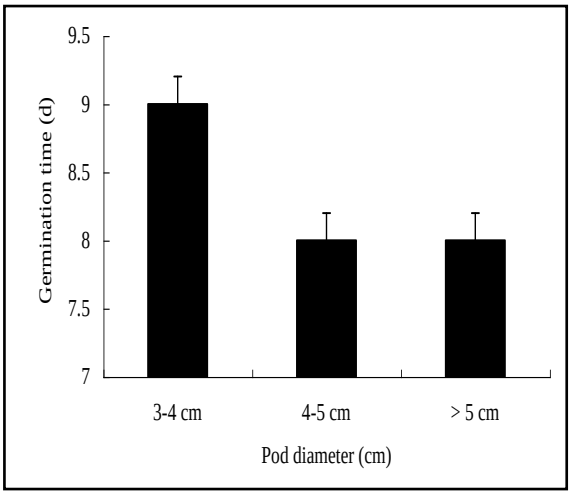

Figure 04: Effect of pod diameter on *in-vitro* germination time

Storage time of pods also showed a significant effect on *in-vitro* germination of seeds. Seed germination decreased with increasing period of storage. The highest germination (96 %) was recorded for seeds extracted from pods, stored only for one week. When pods were stored beyond one week, germination ability gradually declined and pods stored for three weeks, seed germination was about 60 % (Figure 05). According to Subasinghe *et al* (2003), the viability of seeds decreased rapidly after harvest and, for this reason pods could be stored only for a short period of time. Therefore, it is advisable to use the seeds for germination as early as possible when seed propagation is practiced *in-vivo*. The same time period applies for the *in-vitro* germination. To ensure higher germination, pods should be cultured *in-vitro* within one week of harvest. According to Kumar and Kumar (1998), generally, more juvenile the plant material, greater the chances of success in culture initiation.

Table 01: Effect of storage time on *in-vitro* germination of red sandalwood seeds

Treatment number	Storage time	Germination percentage	Time taken to germinate (d)	Browning
1	One week	96^{a}	8^{b}	Low
2	Two weeks	76.5^{b}	8^{b}	Medium
3	Three weeks	63.2^{c}	9^{ab}	Medium
4	Four weeks	61.8^{c}	10^{a}	High

(Means with same letter are not significantly different at $p \leq 0.05$)

Time taken for seed germination also increased significantly ($p \leq 0.05$) with increasing storage time. In pods cultured after one week of storage, seed germination was observed within 8 days, while for those pods stored for four weeks, seeds took more than 10 days to germinate (Figure 06).

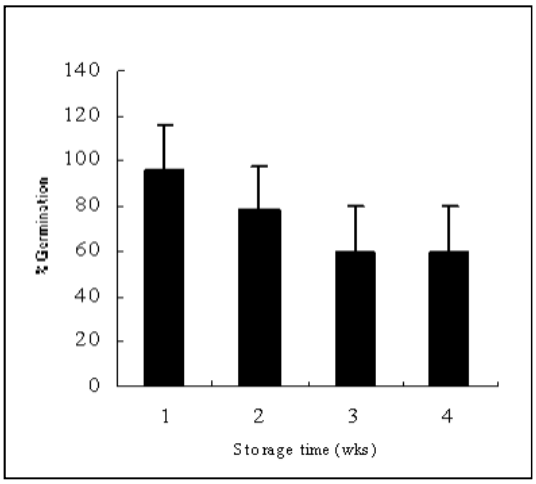

Figure 05: Effect of storage period on *in-vitro* germination

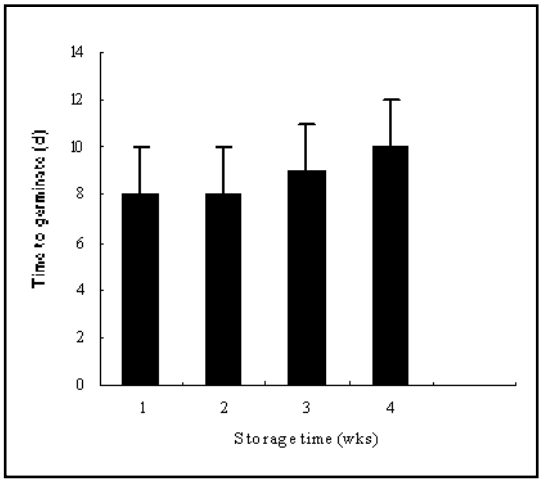

Figure 06: Effect of storage period on *in-vitro* germination time

The different media used for seed establishment had a significant ($p \leq 0.05$) effect on *in-vitro* germination of red sandalwood seeds. Germination percentage was significantly higher (92%) for seeds cultured in Anderson medium without charcoal. In contrast low germination percentages of 62, 62 and 61 % were recorded for seeds cultured in Woody Plant medium (with charcoal), MS medium (without charcoal) and Vitis medium (without charcoal), respectively (Figure 07). However, According to Rajkumar (1999), for direct embryogenesis, Murashige and Skoog medium was found to be better than Gamborg's medium for *Pterocarpus santalinus*. Suitability of MS medium for seed establishment was also reported by Anis *et al* (2005) for *P. marsupium*. They have implanted *P. marsupium* seeds on half strength MS basal medium containing 3 % sucrose and gelled with 0.8 % agar. Seeds cultured in Anderson medium, Vitis medium, or Woody plant medium with or without charcoal took almost similar time (6-8 days) for germination and showed no significant difference in germination time. However, seeds cultured in Anderson medium (without charcoal) took the shortest time (6 days) for germination when compared to those cultured in other media. Seeds cultured in MS medium (without charcoal) took much longer time (10 days) for germination and was significantly ($p \leq 0.05$) different from other media used (Figure 08).

Seeds cultured in Vitis medium (with charcoal) showed the longest hypocotyl length, 13.8 mm. Average growth of the hypocotyl of seeds cultured in Anderson medium (without charcoal) was 10 mm. The shortest hypocotyl length (2.1 mm)

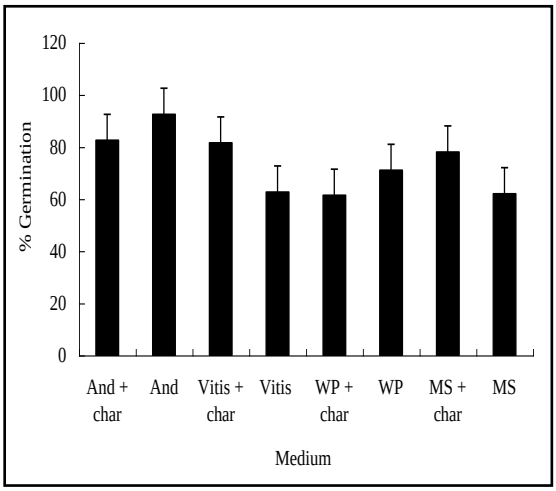

Figure 07: Effect of culture media on *in-vitro* germination percentage

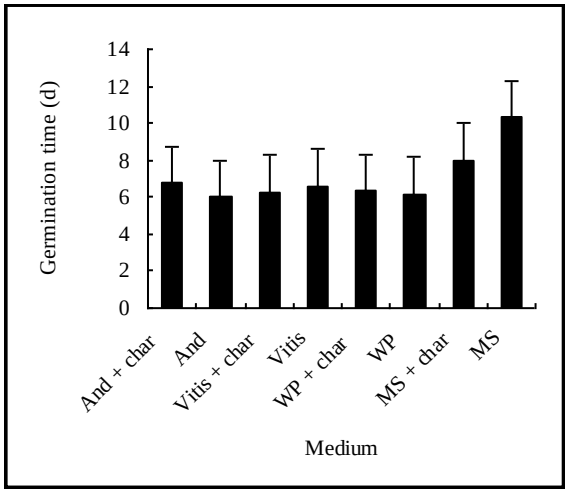

Figure 08: Effect of culture media on *in-vitro* germination time

was recorded for seeds cultured in MS me dium (without charcoal) and showed a significant ($p \leq 0.05$) difference.

When charcoal was added, medium turned dark and it enhanced hypocotyl growth and development. Thus, hypocotyl length increased when charcoal was added to the medium. The appearance of the seedling and root system improved when charcoal was incorporated into the medium.

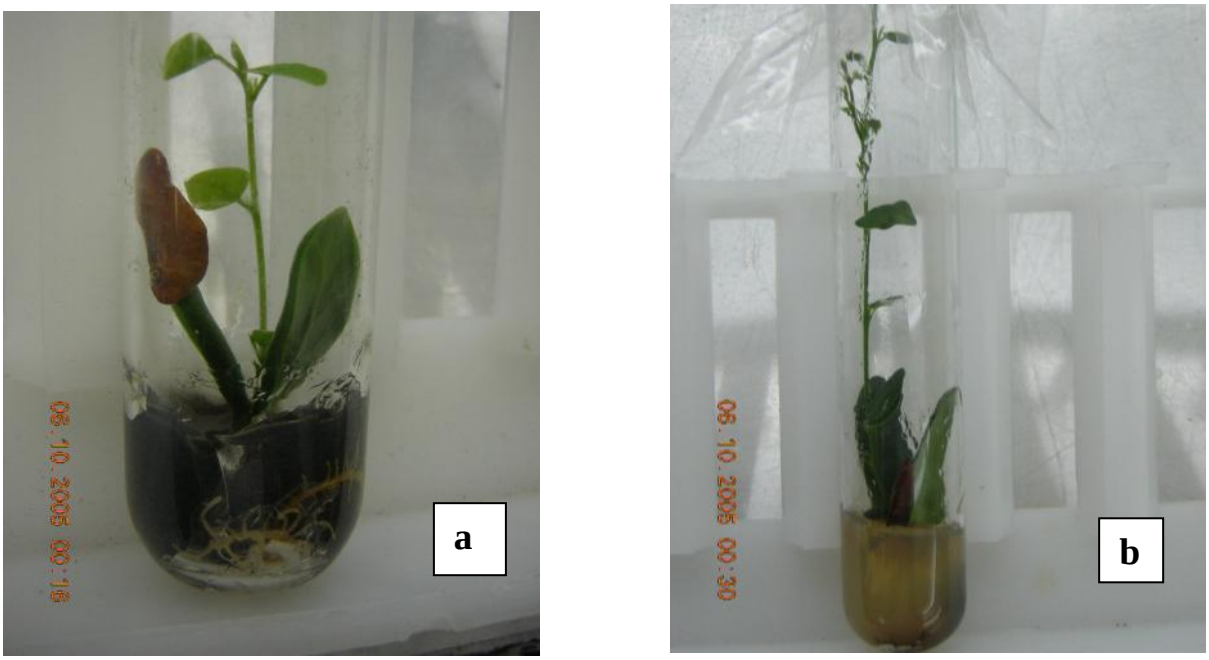

Plate 01: (a) Seedling in charcoal (1 g/l) medium, (b) without charcoal medium

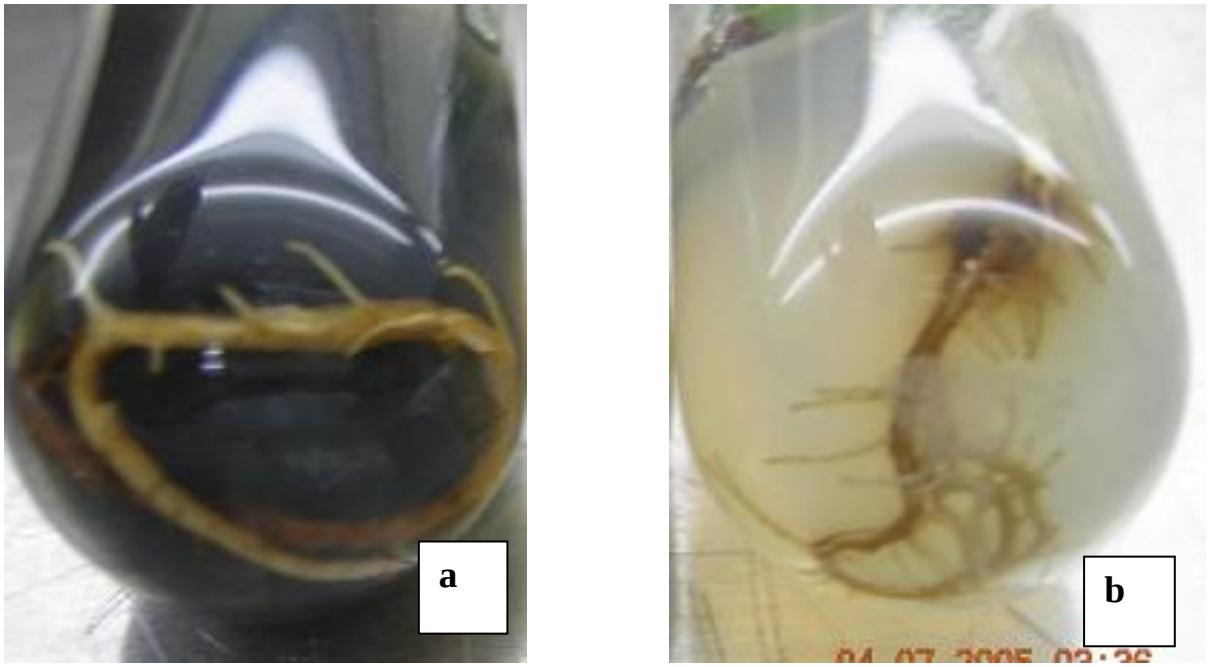

Plate 02: (a) root development in charcoal (1 g/l) medium (b) Root development without charcoal medium

Plate 03: Stages of seed germination in red sandalwood in the medium incorporated with 1 g/l activated charcoal

The cultures which lack activated charcoal turned browned very soon (3-4 days) after culture establishment. However, Woody Plant Medium showed the highest amount of browning than other media used. The lowest extent of browning was shown in seeds cultured on Vitis medium. Through visual observations it was evident that the culture media incorporated with charcoal produce a healthy seedlings (Plate 02 & 03).

The use of activated charcoal at concentrations ranging between 0.2-3 % (w/v) has been found to induce beneficial as well as deleterious effects on cultures. Activated charcoal has a very fine network of pores with enough inner surface area to facilitate the adsorption of solid compounds as well as gases. Addition of activated charcoal to culture media has been found to stimulate growth and differentiation in Orchids (Wang and Haung, 1976) and Carrots (Fridborg and Krikson, 1975). The promotion of growth in cultures with the help of activated charcoal is due to its ability to adsorb toxic brown phenolic compounds exuded by explants during culture initiation. Promotive effects on somatic embryogenesis in anther cultures of *Anemors* and *Nicotiana* as well as stimulation of growth and organogenesis in woody species have also been observed by addition of activated charcoal to culture media. As activated charcoal adsorbs various organic compounds like auxins, cytokinins, vitamins, iron chelates: inhibition of growth in presence of activated charcoal, if any, may be attributed to the phenomena (Kumar and Kumar, 1998).

Table 02: Effect of different culture media and activated charcoal on *in-vitro* establishment and germination of red sandalwood seeds

Medium	**Germination time (d)**	**Germination%**	**Hypocotyl length (mm)**	**App. of the seedling**	**App. of the roots**
Anderson + AC	6.75^{ab}	82.50^{ab}	12.7^{a}	Good	VG
Anderson	6^{c}	92.50^{a}	10.00^{ab}	Good	Good
Vitis + AC	6.25^{bc}	81.50^{ab}	13.80^{a}	Good	VG
Vitis	6.57^{bc}	62.67^{c}	12.90^{a}	Average	Average
WPM+ AC	6.33^{bc}	61.40^{c}	10.90^{ab}	Good	Good
WPM	6.14^{c}	71.00^{bc}	11.60^{ab}	Average	Average
MS+ AC	8.0^{b}	78.00^{b}	8.20^{ab}	Average	Good
MS	10.33^{a}	62.00^{c}	2.10^{b}	Poor	Poor

AC-Activated charcoal,WPM-Woody plant medium, MS- Murashige and Skoog medium, VG-Very good.
(Means with same letter are not significantly different at $p \leq 0.05$)

The highest plant height of 9.9 cm and highest number of nodes per seedling (7 nodes/shoot) were recorded for seedlings in woody plant medium incorporated with 1 g/l of activated charcoal. Seedlings cultured in MS medium showed the lowest number of nodes (6 nodes /shoot) and lowest plant height of 7.3 cm. In contrast seedlings grown in Anderson medium had the medium number of nodes (7) and plant height (8.4 cm) (Fig 09 & 10). Seedlings cultured in Woody Plant medium showed a higher number of shoots (3 shoots/ seedling) at emergence, followed by 2.37 shoots /seedling in MS medium. The lowest number of shoots (2.25 shoots /seedling) was recorded in Anderson medium (Fig 11). Leaf formation was shown to be in woody plant medium as it produced eight leaves per plant, and the average leaf diameter was 10.75 mm. Seedlings in MS medium showed comparatively lower number of leaves (5) and smaller leaf size. In contrast seedlings cultured in Anderson medium showed an average growth (Fig 11 & 12).

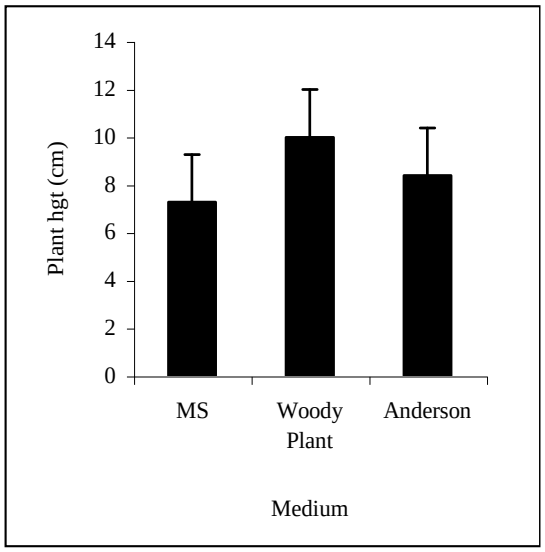

Figure 09: Effect of culture media on plant height

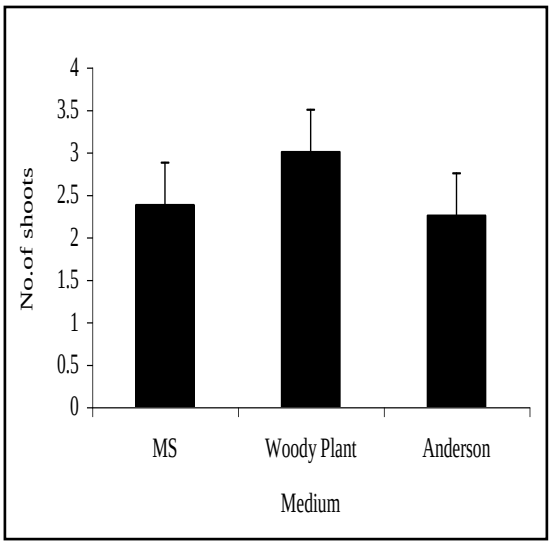

Figure 10: Effect of different culture media on shoot number

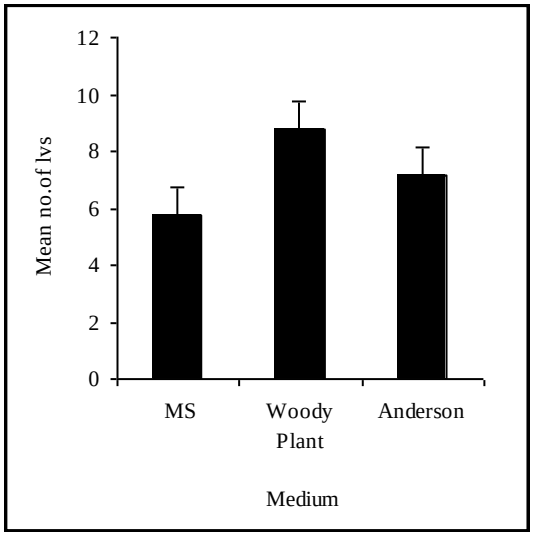

Fig 11: Effect of different culture media on average leaf diameter

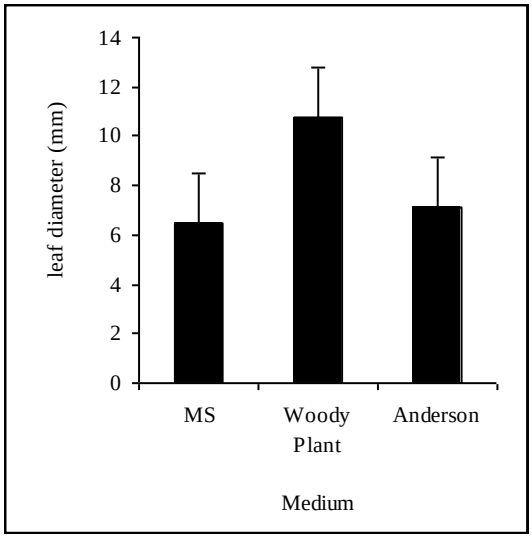

Figure 12: Effect of different culture media on leaf formation

Table 3: Effect of different culture media on *in-vitro* growth performance of red sandalwood seedlings

	MS + AC	**WPM + AC**	**Anderson + AC**
Number of leaves	5.75[a]	8.75a	7.12a
Number of shoots	2.37[a]	3.00a	2.25a
Plant height (cm)	7.26[a]	9.97[a]	8.37a
No. of nodes in the highest shoot	6.25[a]	7.12[a]	7.37a
Leaf diameter (mm)	6.50[a]	10.75[a]	7.12a
Overall appearance of the seed-ling	2.12	3.75	2.75
Overall appearance of the root system	4	4	3.88

AC- Activated charcoal, WPM-Woody plant medium, MS- Murashige and Skoog medium (Means with same letter are not significantly different at $p \leq 0.05$)

The root system developed well in all media. The mean score was similar in MS medium and Woody Plant medium (4), while it was marginally low (3.875) in Anderson medium. The highest mean score for the appearance of plant was recorded in woody plant medium (3.75) while in MS and Anderson media, it was 2.13 and 2.75, respectively (Table 3). Seedlings cultured in woody plant medium showed the highest value for most of the parameters. However, no significant difference was observed among these parameters. Hence there was no significant effect of the selected culture conditions on *in-vitro* growth performances of red sandalwood seedlings at $p \leq 0.05$.According to the results Woody Plant Medium incorporated with 1 g/l activated charcoal showed marginally higher performance in contrast to other culture media tested, woody plant medium can be successfully applied as the growth medium for *in-vitro* culturing of germinated red sandalwood seeds (Plate 05).

Plate 05: Growth of red sandalwood seedling in WP medium + 1 g/l activated charcoal

However, according to Narayanaswamy (1999), selection of a culture medium for *in-vitro* studies would seem baffling in view of several well-established media and their numerous modifications. Optimum factors influencing growth and morphogenesis vary with the genotype, substrate and the immediate milieu in which the tissue grows. These are important parameters in selecting the medium. Even closely related species could differ in their cultural requirements. It would be a good idea to first consult literature concerned with *in-vitro* studies of a few related species before choosing a medium. A second approach would be to tryout a few well-known media and select the one that is best suited.

For micro-propagation of large number of plant species, the salt mixture of MS medium has proven quite satisfactory. However, for some plants, the levels of salts in MS medium may be unnecessarily high or even toxic. For micro-propagation of such species, the salt concentration of MS medium may be suitably lowered or any other suitable culture medium may be attempted (Kumar and Kumar, 1998). The physical and chemical state of various types of culture media has a major effect on the plant's developmental pathway. In addition to the physiological state of the medium, various dissolved constituents also affect plant morphogenesis. There appears to be differences between species in their response to levels of carbohydrates in the medium. In *Philodendron*, addition of Manitol has increased chlorophyll levels in the leaves, but had not improved the growth. Media rich in mineral nutrients, such as MS were shown to promote vitrification and slow growth in some species,

while using media with lower levels of minerals or only half of the MS salts improved plant development (Debergh and Maene, 1985).

CONCLUSION

For surface sterilization of red sandalwood seeds, $HgCl_2$ could be used successfully. Dipping the seeds in 0.1 % $HgCl_2$ with two drops of Tween 20 for 15 minutes was found to be effective in reducing fungal and bacterial contaminations and to ensure higher germination percentage.

Pods with external diameter of more than 4 cm could be used for effective *in-vitro* germination. Pods should be sorted after collection and, those with external diameter less than 3 cm should be discarded to reduce labor, as they do not contain viable seeds. Results revealed that germination ability reduced rapidly with time after harvest. Therefore it is advisable to culture the pods *in-vitro* within one week after harvest to obtain higher germination percentage.

Among the media tested, Anderson medium was suited for *in-vitro* establishment of red sandalwood seeds as it showed higher germination rate and took less time for germination than the other media tested. However, after seed germination, for further growth of the seedlings, germinated seeds should be transferred to Anderson medium incorporated with 1 g/l of activated charcoal.

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