ISSN 2673-1320

Floriculture Research in Sri Lanka

Proceedings of the National Symposium on Floriculture Research

2023

Department of National Botanic Gardens
P.O. Box 14, Peradeniya, Sri Lanka

Sri Lanka Council for
Agricultural Research Policy
114/9, Wijerama Mawatha, Colombo 07, Sri Lanka.

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National Symposium on Floriculture Research (NaSFloR)

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114/9,Wijerama Mawatha,Colombo–07

Department of National Botanic Gardens

Peradeniya

ISBN: 978-955-9224-73-0

Designed by

National Agriculture Information & Communication Centre,
Department of Agriculture

Published by

Sri Lanka Council for Agricultural Research Policy
114/9,Wijerama Mawatha, Colombo–07 and
Department of National Botanic Gardens, Peradeniya

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NaSFloR **2023**

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Department of National Botanic Gardens survey on the Suwahas mal programme Department of National Botanic Gardens

Yapa Y.M.U.A.*, Senarathne M.M.D.J.

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ABSTRACT

Floriculture sector is a profitable agribusiness in Sri Lanka. Department of National Botanic Gardens implemented Suwahas mal programme in 2005 to enhance economic capacity of medium and small scale floriculture growers in Sri Lanka. The programme has already registered 216 Suwahas mal societies in 24 districts with nearly 5732 grower members. The survey presented here was conducted to assess the situation of the Suwahas mal growers' island wide, with the aim of preparation of an updated computer database of growers for formulation of future directives to develop floriculture sector in Sri Lanka. Data was collected using a detailed questionnaire and interviews, compiled and analyzed. In this study randomly selected 1030 (18%), out of 5732 Suwahas mal growers in 24 districts had been subjected to the survey. Results of the survey revealed that, the majority of the growers involved in this sector were females (87%). Among the respondents 50% were in the age category between 46-60 years. Highest average monthly production of the cut flowers is for the Chrysanthemums with ca. 20,850 bunches, although represented only by NuwaraEliya and Badulla districts. Badulla district shows the highest land consumption in which average land area per person was 1867.02 m^2, while Kandy district shows the lowest land consumption representing of an average land area per person of 302.76 m^2. Highest average income per month of Rs. 104,265.00 was also earned by Badulla district growers from sales of their produce to the local market. Major constraints faced by 'Suwahas mal' growers were the lack of a target floriculture market and the lack of quality seeds and planting materials.

Keywords : Floriculture, Suwahas mal programme, Suwahas mal growers

INTRODUCTION

Floriculture in Sri Lanka started as an industry in 1970's and has grown during the last few years substantially to become one of the major foreign exchange generating industries (Srikrishnah *et al.*, 2011). In general it is an intensive type of agriculture and income per unit area is much higher than any other branches of

Agriculture sector. Sri Lanka produces floriculture products ranging from tropical to temperate crops due to varying climatic conditions from cooler hill country climate to lowland tropical climate.

Department of National Botanic Gardens implemented Suwahas mal programme in 2005 to enhance economic level of medium and small scale floriculture growers in Sri Lanka resulting an enhanced island wide spread of floriculture sector involved growers (Department of National Botanic Garden, 2011). The main objectives of this programme was congregation of flower growers to act in unity to achieve uplifted livelihood of flower growers by dissemination of relevant technical knowledge through training programs and workshops for growers, provision of subsidies for improvement of floriculture crop production, provision of facilities to conduct

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Suwahas mal exhibitions and create links with local and export markets to enhance income generation (Department of National Botanic Garden, 2018).

The growers in the inception of the programme presented their products for the local market but are now entering the foreign floriculture fairs with the intervention of the role played by the Department of National Botanic Gardens (Floriculture Research and Development Division, 2020; 2021). Europe is the main market for **floriculture products**, 60% of **Sri Lankan exports** are destined to the Western continent while Japan, Middle East, the USA and Korea make up the other key markets (Export Development Board, 2023).

Hence, this survey was conducted to assess the present situation of the Suwahas mal growers island wide, preparation and updating of a comprehensive computer database of growers, forecasting and preparation of future plans and projections and identification of growers having an export potential.

MATERIALS AND METHODS

This survey was carried out with 1030 Suwahas mal growers in 24 districts, including producers of cut flowers, cut foliage, potted ornamental plants and other floriculture crops. The total sample was randomly selected from each district by using Suwahas mal reports published by Floriculture Research and Development Unit of the Department of National Botanic gardens in Royal Botanic Gardens, Peradeniya.

Following main data focuses were collected through a structured questionnaire and interviews.

1. Demographic information
2. Land extent and geographical location
3. Production and sales
4. Monthly income
5. Constraints

The questionnaire was prepared after discussion with officers who supervise the Suwahas Mal societies attached to the Department. Direct field observations, published and unpublished reports and interviews were used to collect primary data and secondary data. Descriptive analysis were done using Microsoft Excel.

RESULTS AND DISCUSSION

In this study randomly selected 1030 (18%), out of 5732 Suwahas mal growers in 24 districts were surveyed. The survey results reveal that majority of the registered Suwahas mal societies are represented by Gampaha district (31 societies). This can be attributed to Gampaha district having a high population density of 1761 persons km^{-2} (Department of Census and Statistics, Sri Lanka 2021). This is followed by Kurunegala (22) and Kegalle (21) districts, while lowest number of societies are recorded in Jaffna, Vauniya, Mullaitivu, Kilinochchi, Batticaloa, Ampara, Trincomalee and Poḷonnaruwa districts.

Furthermore, most of the members registered under Gampaha, Kurunegala, Badulla districts are as 1334 (23.27%), 707 (12.33%) and 473 (8.25%) sequentially while lowest number of members are registered in Ampara and Polonnaruwa districts. From the total number of active Suwahas mal growers 4966 (87%) are females while only 766 (13%) are males (Table 1). This explains that the majority of the growers are women engaged in floriculture sector as housewives who had chosen this sector as a self-employment while engaged in day-to-day household chores to earn an extra income for the family.

According to the survey data Suwahas mal growers are mostly engaged in cultivation of Chrysanthemums, Roses, Gerberas and Gypsophila. Among these cut flower crops Chrysanthemums show the highest average

monthly production of 20,850 bunches (Table 2 & Figure 2), although Chrysanthemums are cultivated as cut flowers restricted to Nuwara Eliya and Badulla districts only. The main reason for this is the low production and maintenance cost incurred, especially low cost for planting materials due to the majority of growers keep their own mother plant collections and Chrysanthemums have three production seasons per year compared to Roses, Gerberas and Gypsophila. Gypsophila is also only cultivated in Nuwara Eliya and Badulla districts. Gerberas are cultivated in the Colombo, Kandy, NuwaraEliya, Badulla, Rathnapura, Puttalam and Batticaloa districts. The average monthly production of Gerbera as a cut flower is 5,120 flowers and 5948 flowers for Roses.

Table 1 : Percentage of Suwahas mal growers according to their Gender

Gender	Number of Members	Percentage (%)
Female	4966	87
Male	766	13

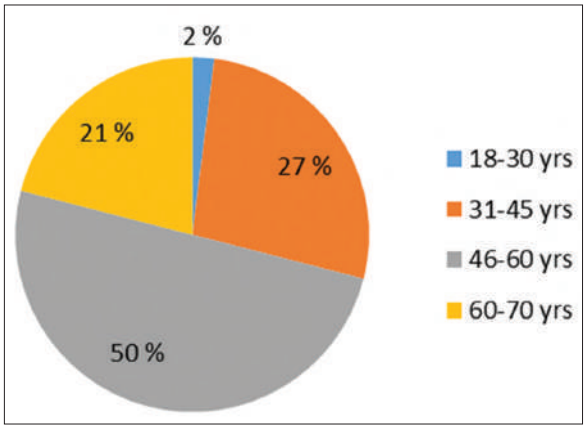

Figure 1: Percentage of Suwahas mal growers according to their Age categories

Table 2: Average Monthly Cut Flower Production by Suwahas mal growers (2020)

Variety	Average Monthly Production (2020)
Chrysanthemum	20,850 Bunches
Rose	5,948 Flowers
Gerbera	5,120 Flowers
Gypsophila	639 Bunches

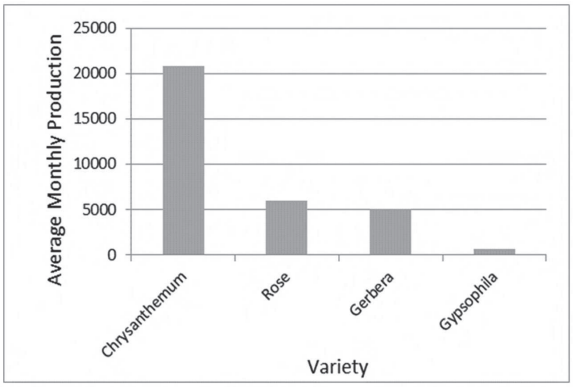

Figure 2: Average Monthly Cut Flower Crop Production by Suwahas mal growers

According to survey data shown in Figure 3, Badulla district shows the highest land consumption represented by average land area per person of 1867.02 m^2 in terms of the amount of land allocated for flower cultivation per member associated with the Suwahas mal programme. As Badulla district shows less population density of 312.8 persons per km^2 (Department of Census and Statistics, Sri Lanka 2021), more lands are available for floriculture per person compared to other districts. Other than that, Badulla district has favourable climatic condition for temperate cut flowers like Chrysanthemums, Roses and Gerberas. Due to this situation Badulla district has more potential for enhancing floriculture production, especially for cut flowers in future.

Moreover, this is followed by districts of Gampaha, Jaffna, Vavuniya, Kilinochchi, Kurunegala and Kegalle in flower cultivation, while Kandy district shows the lowest land consumption with an average land area per person of 302.76 m^2 (Figure 3). However, the higher population density of 773.7 persons km^{-2} (Department of Census and Statistics, 2021) represented by Kandy district can be the reason for lowest average land consumption per person in floriculture production.

The survey results also revealed that the highest average income per month of Rs. 104,265.00 was earned by Badulla district

growers by selling their products to the local market, but not in export oriented markets. This is followed by Puttalam with Rs. 86,696.00 of an average income per month (Figure 4).

In addition, the results of the survey reflected that the highest land consumption in floriculture sector and highest average monthly production of 20,850 bunches of Chrysanthemums are the main reasons for the highest average income per month in Badulla district (Figure 4).

Gampaha District shows an average monthly export income of Rs. 10,728.00 among 24 districts, and also this indicates that majority of Suwahas mal growers tend to cater to the local market than the export market (Figure 4). The main reasons behind this is the strict export regulations with more documentation processes and lack of awareness on floriculture export market potential. This reflects that most of the Suwahas growers are comfortably earning their income by selling their floriculture products locally to enhance their livelihood by day-to-day income, but does not engage in long term plans to cater to the export market with improved cultivation practices and quality standards associated.

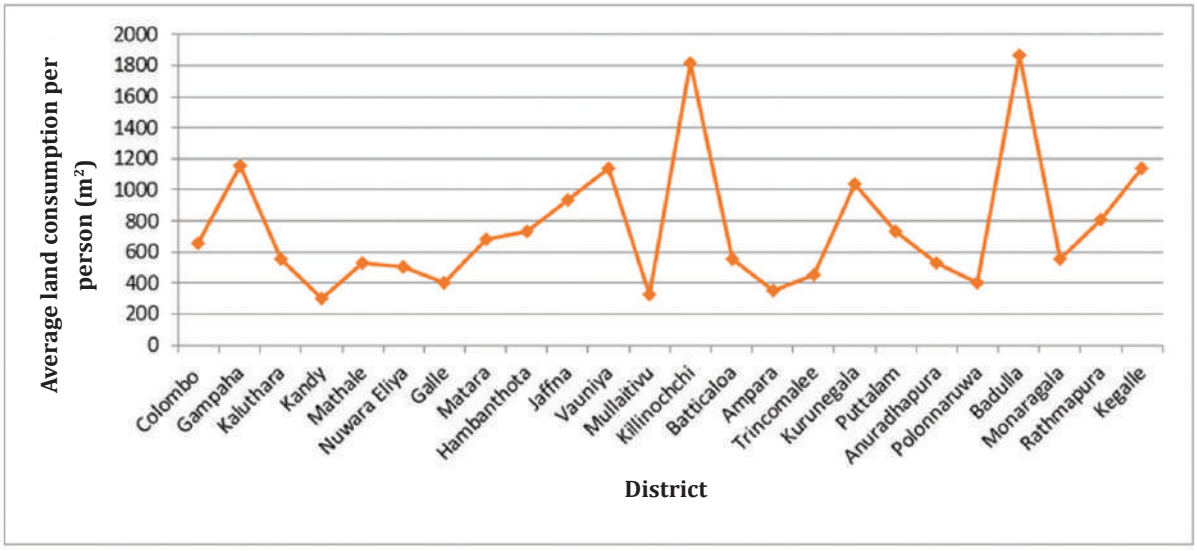

Figure 3: Average Land Consumption per person by Suwahas mal growers

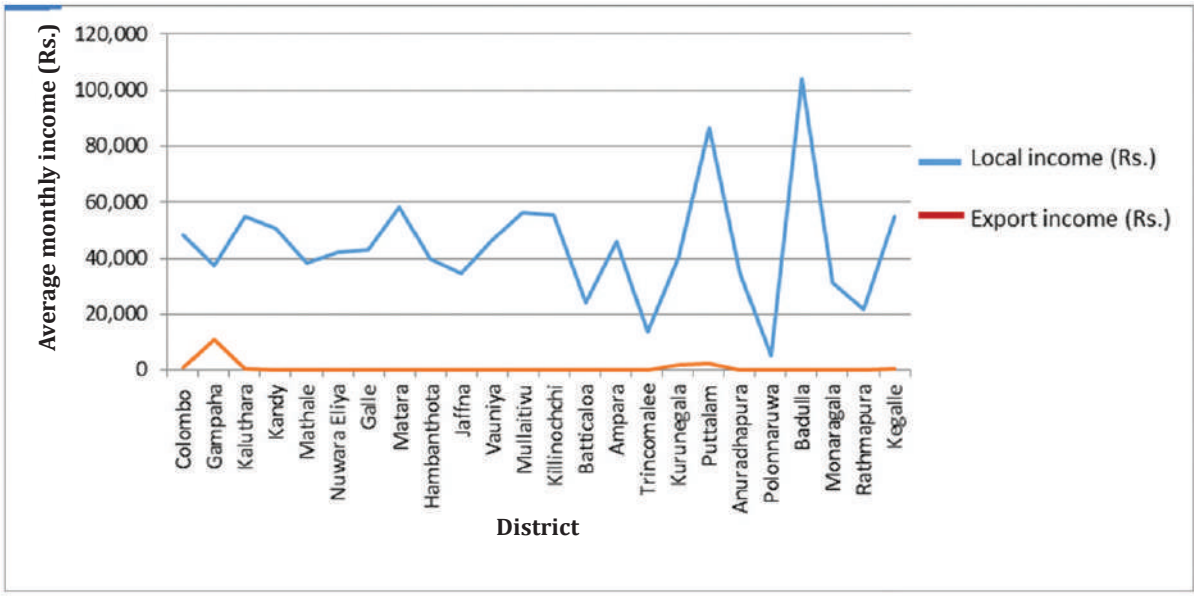

Figure 4: Average income per month of Floriculture Market for Suwahas mal growers

Further more, the study identified the major constraints faced by growers are difficulties in procuring land and capital expenditure, lack of specific locations for sales, lack of floriculture target markets and difficulties in acquiring quality planting materials, insufficient technical knowledge and storage facilities, insufficient staff for supervision to guide the trade and lack of fixed prices for cut flowers (Table 3).

Table 3 : Constraints faced by Suwahas mal growers

Constraints	Percentage (%)
Lack of floriculture target market	76
Lack of quality seeds and planting materials	75
Lack of specific locations for sales	72
Insufficient technical Knowledge	66
Insufficient staff for supervision 65	
Difficulties in procuring land and capital Expenditure	59
Lack of fixed prices for cut Flowers	55
Insufficient storage facilities	14

CONCLUSION

Gampaha district represents the highest number of Suwahas mal growers. The majority of Suwahas mal growers involved in floriculture were females, the age category falls between 46-60 years. Chrysanthemums shows the highest average monthly production, although restricted only to Nuwara Eliya and Badulla districts, while Badulla district shows the highest land consumption and highest average income per month through floriculture. Major constraints faced by 'Suwahas mal' growers were attributed to lack of floriculture target markets as well as lack of quality seeds and planting materials.

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Optimization of Fast and Cost-effective Protocols for DNA Extraction from Fresh and Dry Rhizome of *Alpinia calcarata* (Heen Aratta)

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ABSTRACT

The rhizome of *Alpinia calcarata* is highly used as herbal medicine and is very potential for adulteration in Ayurvedic medicine. Authentication of the rhizome is difficult by morphological characteristics. DNA technology can be used as an accurate and fast method for authentication. In DNA technology high-quality DNA is required for downstream applications such as PCR. Hence the DNA extraction method should be a cost-effective and suitability for commercial requirements. This research was done to develop a low-cost effective method for DNA extraction from the dry rhizomes of *Alpinia calcarata*. Three modified methods; SDS, 4x CTAB, and CTAB series were followed to extract the genomic DNA from dry rhizomes, After the extraction, the quality and the quantity of the extraction DNA were checked and PCR amplification was done using the universal barcoding regions. The purity ratio of extracted DNA ranged from 1.42 - 1.72, 1.53 -1.89 and 1.58 – 1.76 and quantity roughly around 753.65, 437.49, and 447.22 ng/µl from Modified SDS, Modified 4X CTAB and CTAB series methods respectively. Spectrophotometric and electrophoretic analysis showed that the isolated DNA according to three methods was highly pure and could be amplified using universal barcoding regions.

Keywords : Alpinia calcarata, DNA isolation, modified SDS, modified CTAB, CTAB series, universal barcoding regions.

INTRODUCTION

Alpinia calcarata belongs to the family Zingiberaceae and the plants are perennial aromatic herbs and distributed through India, China, Thailand, Malaysia, Indonesia, and Sri Lanka. Many Alpinia species are very valuable because of their medicinal properties such as properties of hypertensive, anti-emetic,anti-oxidantandanti-inflammatory effects (Rahman and Islam, 2015). But in the herbal medicine industry substitution and adulteration occurs with closely related species. Due to these adulterations, efficacy of the drug decreases, and in some cases, can be lethal if it is substituted with toxic adulterants (Li *et al*., 2011). Rhizome of *Alpinia calcarata* and other related species of family Zingiberacea is the most common part used in herbal medicine and adulteration may occur due to similar macroscopic and phytochemical characteristics. Various traditional methods are available for the identification of herbal medicines such as organoleptic methods (senses: taste, sight, smell, touch), macroscopic and microscopic methods (identification by shape, color, texture) and chemical profiling (e.g. TLC, HPLC-UV, HPLC-MS). These methods cannot be used for the identification of related plant species in raw or processed products. Since, when related species are in the processed form it requires trained persons for the correct identification and is difficult to identify organoleptic properties, microscopic properties etc. (Techen *et al*., 2014).

DNA technology can be used as an accurate and fast authentication method for medicinal

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plants and processed products. The isolation of pure and high molecular weight genomic DNA is a pre-requisite for many molecular biology applications including the Polymerase Chain Reaction (Dey *et al*., 2010).

An array of DNA isolation protocols are available for the extraction of DNA from various plant parts of different species. However, DNA isolation from the rhizome of *Alpinia calcarta* posed problems because of naturally occurring secondary metabolites such as polyphenols and polysaccharides (Kress *et al*., 2002). Complications encountered in the isolation and purification of DNA especially from medicinal and aromatic plants include degradation of DNA by endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Moreover, contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Dey *et al*., 2010).

In this present study, we attempt several protocols for DNA extraction from the dried rhizome of *Alpinia calcarata* with modifications that used several compounds for countering these secondary metabolites. We also compared yield and quality of isolated DNA using three modified methods and their acceptability based on their efficiency. The reproducibility of extracted DNA was tested using PCR techniques.

MATERIALS AND METHODS

MATERIALS

Dried rhizomes of *Alpinia calcarata* samples were collected from Diyathalawa Ayurvedic Hospital and Ayrvedha Medicine shops in the Badulla area. Collected rhizomes were washed three times with sterilized distilled water and 70 % ethanol and dried. Samples were separately cut into small pieces, 100 mg was weighed out, covered with aluminum foils and kept in a -80 oC freezer overnight before extraction.

Extraction buffer

Method 1: The protocol described by Nagaraj *et al*., 2018 was modified. It consisted of 1.5% SDS, 250mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0) and 300mM NaCl. 20ml of Extraction buffer was used for extraction from 1g of tissue.

Method 2: Extraction buffer prepared broadly based on Doyle and Doyle's (1990) CTAB buffer. It consisted of 4% CTAB, 100 mM Tri-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.8 M NaCl, 2% PVP and 0.5% β-mercaptoehanol. 20ml of Extraction buffer were used for extraction from 1g of tissue.

Method 3: Three extraction buffers were prepared according to the protocol described by Cheng *et al*., 1997.

2X CTAB extraction buffer - 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol

10X CTAB buffer - 10% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.7 M NaCl, 0.2% β-mercaptoethanol

1X CTAB buffer - 1% CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1.4 M NaCl, 0.2% β-mercaptoethanol

DNA isolation

Method 1: Modified SDS method

Overnight frozen dried rhizome samples were taken and quickly ground with frozen (-80^{o}C) mortar and pestle. The crushed homogenate was immediately transferred to extraction buffer warmed at 65^{o}C and incubated for 15 minutes with gently mixing

the homogenate twice by inverting the centrifuge tubes. The incubated samples were centrifuged at 13000 rpm for 10 minutes and the clear supernatant was collected to a fresh sterile centrifuge tube. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for about 20 minutes and centrifuged at 13000 rpm for 10 minutes. The aqueous phase was pipetted out, an equal volume of ice-cold isopropanol was added and centrifuged at 13000rpm for 10 minutes. After centrifugation, the supernatant was discarded and 70% ethanol (200 µl) was added to the pellet and washed twice by centrifugation at 5000 rpm for 5 minutes. The pellet was air dried at room temperature for complete removal of ethanol and then the pellet was re-suspended in 50 µl of TE buffer.

Method 2: Modified 4X CTAB method

Overnight frozen dried rhizome samples were taken and quickly ground with frozen (-80^{o}C) mortar and pestle. The crushed homogenate was immediately transferred to extraction buffer warmed at 65^{o}C and incubated for 1 hour and 30 minutes with occasional gently shaking. After incubation, samples were centrifuged at 1000 rpm for 30 minutes. The pellet was discarded and the supernatant was transferred into a fresh sterile centrifuge tube. This was followed by the addition of equal volumes of Phenol: Chloroform: Isoamyl alcohol (25:24:1) (v/v) mixture. The samples were mixed well by constant shaking for 5 minutes and centrifuged at 10000 rpm for 30 minutes. The upper aqueous layer was collected into a fresh sterile centrifuge tube and 10 M ammonium acetate (10 µl for 1000 µl of supernatant) and an equal volume of ice-cold isopropanol were added to retrieve the aqueous fraction. The mixture was mixed well, kept in an ice bath for 15 minutes and centrifuged at 10000 rpm for 30 minutes at 4^{o}C. The supernatant was discarded and the pellet was washed in 200 µl of 70 % ice-cold ethanol by centrifugation at 6000 rpm for 10 minutes. The pellet was dried at room temperature and resuspended in 50 µl of TE buffer.

Method 3: CTAB series method

The frozen sample was crushed into powder with pre-chilled mortar and pestle with 1.5 ml of 2X CTAB extraction buffer. The suspension of samples were incubated at 90°C for 30 minutes with occasional shaking, and then centrifuged at 12000 rpm for 10 minutes. The supernatant was separated, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged at 12000 rpm for 10 minutes. This step was repeated twice and supernatant was separated. The 0.1 volume of 10X CTAB buffer (preheated at 65^{o}C) was added and at the same time equal volume of the supernatant Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added. The samples were centrifuged at 12000 rpm for 10 minutes and separated from the supernatant. Subsequently again an equal volume of 10X CTAB buffer was added to the separate supernatant and centrifuged at 12000 rpm for 10 minutes. The supernatant was separated, 0.6 volume of ice-cold isopropanol was added and kept in an ice bath for 15 minutes. Then the samples were centrifuged at 10000 rpm for 30 minutes to obtain the pellet. The pellet was washed with 70% ethanol by centrifugation at 6000 rpm for 10 minutes and allowed to complete drying of ethanol. Then the pellet was re-suspended in 50 µl TE buffer.

Estimation of DNA quality and quantity

The yield and the purity of DNA were measured using Nano-drop Spectrophotometer at 260 nm and by the ratio of absorbance at 260 nm and 280 nm respectively. Quality of the extracted DNA was tested by running the DNA on 0.8% agarose gel. The gel was examined using Gel

documentation unit (Model - Quantum ST – 4, France).

PCR amplification

The two barcode loci for the coding genes ITS1 and rbcL were amplified using primers recorded in table 01. Amplification of the PCR protocols for the two primer regions were done according to Hearth *et al*., 2019. The PCR amplicons were separated on 1.5% agarose gel, and visualized with the gel documentation unit (Model - Quantum ST – 4, France).

Table 01: Primer sequences with number of bases

Primer	Primer sequence	No of bases
ITS1 F	5'CGT AAC AAG GTT TCC GTA GGT GAA C 3'	25
ITS1 R	5' TTA TTG ATA TGC TTA AAC TCA GCG GG 3'	26
rbcL F	5'ATG TCA CCA CAA ACA GAA AC 3'	20
rbcL R	5' TCG CAT GTA CCT GCA GTA GC 3'	20

RESULTS AND DISCUSSION

Plants in the family Zingiberaceae contains a high amount of secondary metabolites. *Alpinia calcarata* rhizomes contain various secondary metabolites such as phenols and polysaccharides that can interfere with the DNA extraction protocols. In the DNA extraction procedure these phenols oxidized and covalently bind to proteins and DNA, giving DNA a brown color and making it useless for downstream applications. In addition to phenols, there may be high viscous pectin like polysaccharides in extracted DNA samples. They tend to co-isolate with DNA and interfere with biological enzymes such as polymerase, restriction endonuclease and ligases etc. (Mathew *et al*., 2013).

Protocols of DNA extraction that have been proposed so far exclusively for the Zingiberaceae family, are specialized protocols concentrating on extracting DNA from leaves or any other tender tissue. Bhadra and Bandyopadhyay (2015) proposed a protocol to extract DNA from different plant parts including rhizome of eight selected species of the family Zingiberacea and Syamkumar *et al*., (2003) proposed a protocol to extract DNA from the rhizome of turmeric (*Curcuma longa*) and ginger (*Zigiber officinale*). Kress *et al*., (2002) also used a modified protocol of Doyle and Doyle (1990) to extract DNA from the Zingiberacea family for the molecular level characterization using gene specific markers. However, they used leaf samples for the DNA extraction procedure. Thus, a convenient, fast and cost-effective protocol to extract DNA from the rhizome of *Alpinia calcarata* is not available.

In this study, our focus was to extracted high quality DNA from the dried rhizomes of *Alpinia calcarata* for downstream application xlike PCR. Here three different modified protocols were used and a comparison was done.

Both qualitative and quantitative estimations of the extracted genomic DNA from three different protocols showed the presence of high quality DNA. Gel image (Figure 1) of agarose gel electrophoresis of extracted DNA sampled showed a single clear sharp band in each sample that proved the structural integrity and purity of genomic DNA. The ratio of absorbance at 260 nm and 280 nm ranged from 1.42 - 1.72, 1.53 -1.89 and 1.58 – 1.76 from the modified SDS, modified 4X CTAB and CTAB series methods respectively. The amount of DNA obtained from the modified SDS, Modified 4X CTAB and CTAB series methods were roughly around 753.65, 437.49, and 447.22 ng/µl respectively. High quantity DNA was obtained from the modified SDS method while the other two methods approximately give the same quantity.

Table 2: Nano-Drop spectrophotometric results of extracted DNA

Sample number	Extraction method	A260/ A280	A260/ A230	Concentration (ng/µl)
1	Modified SDS method	1.42	0.56	605.49
2		1.68	0.55	794.48
3		1.71	0.68	776.85
4		1.72	0.58	789.25
5	Modified 4X CTAB method	1.82	0.89	321.90
6		1.53	0.78	451.61
7		1.89	0.75	498.29
8		1.75	0.69	478.14
9	CTAB Series method	1.64	0.82	456.89
10		1.58	0.83	446.89
11		1.72	0.75	421.23
12		1.76	0.74	463.85

The quantity of extracted genomic DNA is shown in table 2 and proved that quantity of extracted DNA is suitable for downstream applications like PCR. PCR amplifications were done using two universal Barcoding primers. All the primers were able to amplify the extracted DNA obtained from three different modified protocols (Figure 2) and it ensures the reproducibility of the extracted DNA. PCR amplicons of ITS1 regions showed sharp clear band without any primer dimers for all the samples

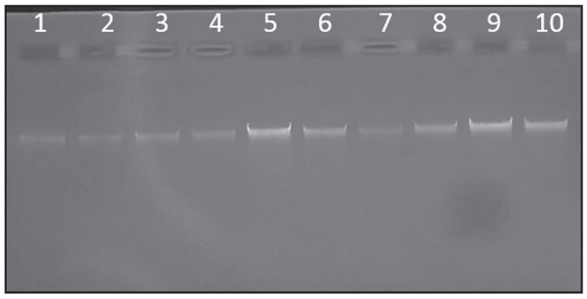

Figure 1: Genomic DNA visualized in 0.8% Agarose gel. Lane 1: Sample 1, Lane 2: Sample 2, Lane 3: Sample 3, Lane 4: Sample 5, Lane 5: Sample 6, Lane 6: Sample 7, Lane 7: Sample 9, Lane 8: Sample 10, Lane 9: Sample 11, Lane 10: Sample 12

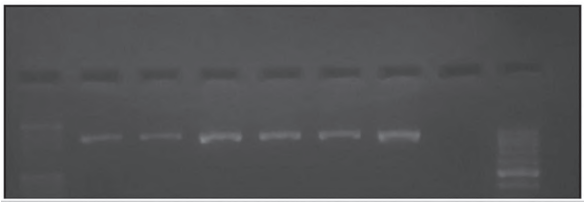

Figure 2: PCR products of amplification (a) ITS1 (b) rbcL nuclear regions visualized in 1.5% Agarose gel. Lane 1: 100 bp DNA ladder, Lane 2: Sample 3, Lane 3: Sample 4, Lane 4: Sample 5, Lane 5: Sample 7, Lane 6: Sample 11, Lane 7: Sample 12, Lane 8: Negative control

Plant leaves contain low water content compared with the rhizome and quality DNA can be easily obtained. Here in our protocols, pre-chilled rhizomes samples were used for the DNA extraction, hence a powder for the DNA isolation step was easily obtained, with a lower cost. According to Bhadra and Bandyopadhyay (2015), use of liquid nitrogen during crushing remains the most effective method for recalcitrant plant parts (like rhizome) reporting that pre-chilling cannot be used

During homogenization of crushed samples, polyphenols are released from vacuoles and interact irreversibly with DNA. PVP has been used mostly in CTAB extraction procedures to minimize polyphenol oxidation (Singh *et al*., 2014). In this study PVP is not used in all proposed protocols. Mathew *et al*., (2013) showed that CTAB along with NaCl can be used to remove polysaccharides. Thus in the proposed protocols, polyphenol browning did not interfere with the quality of extracted DNA, as buffers were modified accordingly. In the CTAB series method a series of CTAB buffers were used and therefore it may have efficiently minimized the polyphenol oxidation that interferes with the quality of extracted DNA. In addition, β-Mercatoethanol being a reducing agent inhibits the oxidation process.

In conventional protocols generally Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixture or Chloroform: Isoamyl alcohol (24:1) mixture will be used twice to eliminate secondary metabolites and protein impurities from samples. These steps mainly affect the quality and quantity of extracted DNA because of the increased number of precipitation steps that might hinder long-term storage (Moore, 1998). In the modified SDS protocol Chloroform: Isoamyl alcohol (24:1) was used only once and quality DNA was extracted with the modification done to extraction buffer composition. This may have prevented unnecessary DNA loss without compromising the quality of DNA and also reduces health hazards (Russell and Sambrook, 2001).

A High concentration of EDTA at pH 8.0 was used in every extraction buffer as a chelating agent, which facilities the removal of Mg ions, leading to a decrease in the activity of DNase. In the buffer solution, Tris-HCl provides a buffering capacity because low or high pH damages the DNA.

Increasing the number of washing in 70 % ethanol gave better DNA because it helped to complete removal of residual NaCl and CTAB (Dey *et al*., 2010). And in this study, the efficiency of the ethanol washing is increased by using centrifugation at medium level rpm.

With all these modifications for the extraction buffer and procedures, high quality and quantity DNA was obtained from all developed protocols. Generally, CTAB extraction protocol takes roughly around 6 hours to complete the extraction (Nagaraj *et al*., 2018) All the modified protocols for the DNA extraction reduced the time of DNA extraction significantly to only a few hours without reducing the quality and yield of the DNA.

Yield and quality of DNA significantly affect the efficiency of DNA amplification by PCR reaction (Das *et al*., 2012). The optimized protocols described in Herath *et al*., 2019 were used to amplify genomic DNA extracted from dried rhizome and it produces clear scorable amplified products suitable for molecular level characterization.

Though yield of DNA in the modified 4X CTAB and CTAB series methods were less compared to the modified SDS method, they had a high purity ratio with the same reproducible ability in PCR.

CONCLUSION

The proposed three methods can be efficiently used for DNA extraction from dried rhizome without using liquid nitrogen and give high quality and quantity DNA for downstream applications. Moreover, all three methods require a minimal amount of reagents. Time required for DNA extraction from these methods varies from 3 – 4 hours. Thus, it can be concluded that the three methods described here can be used to isolate DNA from rhizome parts of *Alpinia calcarata* depending on the laboratory setup and objectives of isolation. The isolated DNA can be used for a downstream applications like PCR, sequencing etc.

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Review of Morphological Characteristics of *Lepironia articulata* (Retz.) Domin and Identification of its importance

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ABSTRACT

Lepironia articulata belongs to the family Cyperaceae and is a typical plant of wetlands.*L. articulata* can also toleratebrackish areas. Objectives of this review was to compare the quantitative and qualitative parameters with literature review and identify characters of *L. articulata* to be selected as an ornamental plant and to identify uses according to its specific characters to botanic gardens Sri Lanka. The *L. articulata* rhizomes were cultivated in marshy lands, Pond, and Top soil under dry condition. The. They were not supplied with any special treatments. Thereafter, following parameters were compared with literatures. Average shoot length of *L. articulata* was recorded as (180-200cm), Average shoot Diameter (7mm), Days required to first flower initiation after planting (45-50 Days), diameter of a pod (8mm), length of a pod (1-1.5cm), length of shoot tips to the base of the pod (2-3.5cm), shoot color (Grey green) and intervals to initiate a new shoot from the rhizome (50-55 Days) were identified.

Keywords : Lepronia, Wet land, Morpology, Muthurajawela

INTRODUCTION

Lepironia articulata sedges are mostly found in Madagascar, India, Sri Lanka, Southern China and Southeast Asia.(Domyos & Te-chato, 2013). Prychid & Bruhl, 2013 reported that plants of *Lepironia articulata* are tall perennials from coastal freshwater wetlands and rivers from China south to warm-temperate south-eastern Australia, Sri Lanka and Madagascar. It is an evergreen perennial sedge. This plant is a tall tightly subaquatic, rush-like, leafless, rhizomatous, perennial that forms large dense clump. They growup to 2 m in height and rhizomes creep horizontally to a few centimeters depth below the mud surface Shoots colors are grey-green and horizontal rhizomes are covered with ovate, ferrugineous scales(Ooi & Ang, 2015)

a. Morphological Characters

Stems (culms) close together, arranged in one row along the rhizome.They are smooth and hollow but transversely septatewith around 0.5-1cm distance between two septa. Stems are erect, slenderLeaves are reduced to bladeless sheaths.They grow in an upright direction. Inflorescence consisting of a single spike-like cluster.Its flowers are arranged in an inflorescence known as a spikelet. The spikelets occur singly on each stem. each ovoid to oblong ellipsoid, and 1–4 × 5–15 mm. (Ooi & Ang, 2015).

This cluster is ellipsoid. (Brotonegoro, *et al*., 2003)Inflorescence sarepersistent, bearing spirally arranged tightly and densely imbricate flowers. Inflorescence is apseudo-lateral, many-flowered spike,2 cm long, ovoid-obovoid or ellipsoid, yellowish brown todark brown at maturity.(Saji, *et al*., 2009)

Glumes are red-brown and ovate to obovate-orbicular. Flowers consist of a cyme with a terminal pistillate flower and up to

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about 15 cm small, hyaline, hypogynous scales, each subtending a single staminate flower.

Stamen 2.5 – 3 mm long withdark extended connective. It takes 3 months for *Lepironia articulat*a culms to start flowering and 7 months to reach their maximum dry weight of about 3.5 g. Reed swamps dominated by *L.articulata* contain an average of 30 clumps and 140 culms per m2 with a standing biomass (including rhizomes) of up to 850 g dry weight per m2. Furthermore, the stem of *L. articulata* are cylindrical (Brotonegoro, *et al*., 2003)

Ikusima,1978 reported that leafless culms grow up to 2 m in height andreaches 850 g dry weight m−2. The spatial distribution pattern of the apex of rhizomes tends to approach a random pattern. *L.articulatata* about 7 months for culms to mature and the maximum mean dry weight of a culm reaches 3.40g in the unburnt area and 2.25g in the burnt area. The mean lifetime of culms is estimated at 227 days (unburnt area) and 309 days (burnt area). The mortality of young culms is not as high as in seedlings of many other plants.

b. Botanical Classification

This sedge belongs to,

Kingdom - Plantae,

Order -Poales,

Family - Cyperaceae,

Genus -*Lepironia*,

Species -L.*articulata*

Botanical Name -*Lephronia articulata*

Inflorescences are narrow-ovid to ellipsoid. The flower and seed head is conical and is carried near the top of the reed stem and below the stem top. It contains a small cigar-like (conical) flower pod which is brown in colouras well as very tough and versatile. (Brotonegoro, *et al*., 2003)

c. Growth habit

Lepironia articulata is found in shallow water (usually less than 0.8 m deep) in open swampy locations. It grows in oligotrophic, slightly acid (pH 5.0-6.5) water. It grows in full sun to 50% shade. In Sri Lanka it can be found from Muthurajawela Marshy land and along Hamilton Canal in the Negambo area and Kalutara. It can also be seen in Baddagama Biodiversity Park and Bird Sanctuary Park. (Dassanayake,1980). It grows in ephemeral swamps and the margins of freshwater swamps.Up to 7m rhizomes were found per m^2, at 5-7 cm below the mud surface and new shoots arise from the rhizome every 51-55 days (Brotonegoro, *et al*., 2003).

d. Physiochemical characters

According to phytochemical screening, *L. articulata* contains a variety of active compounds, including tannins, saponins, flavonoids, alkaloids, and steroids. (Fern *et al*.,2014). Finding was reported that 6 different phenolic compounds were extracted from *L. articulata*. They are, 4-Hydroxybenzoic acid, Caffeic acid ,Vanillic acid and trans-p-Coumaric acid . It was reported that *L. articulata* can be exploited as a new source of natural colorants that have the potential uses in the dye and food colorant industries. (Othman, *et al*., 2020)

Katayon, *et al*., 2008 reported that *Lepironia articulata* has been shown to have antimalarial and antioxidant activity and reddish-brown in color, chelated taste, and had a specific odor.

e. Importance to Phytoremediation

Phytoremediation is a plant-based approach, which involves the use of plants to absorb and remove elemental pollutants or reduce their bioavailability in soil (Berti and Cunningham,

2000). Sand, gravel medium, and aquatic plants are frequently utilized by wetlands. Primarily to reduce pollutants from greywater (Ismail, *et al*., 2015) It is reported that *L. articulata reduces* the concentration of dissolved manganese (Mn) and ferrous metals (Fe) and raises the pH of acid mine water (Hendri *et al*.,2023). Furthermore, wetlands possess an undeniable capacity to absorb and hold on to nutrients, particle matter, and other contaminants that are released from greywater alongside the roots of plants (Wurochekke *et al*., 2014). *L. articulata* can be used as phytoremediation also *L. articulata were* effective in the removal of phosphorus and nitrogen from domestic wastewater. (Tenzin, *et al*., 2021) as well as Cr like heavy metals (Syukora, *et al*., 2015) agents of acidic land or drainage canals contaminated with acidic pollutants, this is because this sedge has a high tolerance to metals and some other acidic substances as well as a root system that is resistant to pollutants Fira *et al*., 2023). Asadullah, *et al*., 2019 was reported that Hydrochar and modified biochar prepared from a tropical biomass (Lepironiaarticulata) were successfully used for the removal of Cr(VI) from aqueous solution. The finding was reported that *L. articulata* can be used as a phytoremediation agent of CPME with an initial COD concentration of 7290 mg/L and AN concentration of 376 mg/L. (Said , *et al*., 2021)

(ALSbani, *et al*., 2021) reported that *L. articulata* can tolerate to diesel better and also indicate that the phytoremediation was effective in PAHs remediation from wastewater. *L. articulata* was efficient in PAH removal (up to 96%) and it demonstrated the tolerance to diesel toxicity until 3% plants can tolerate and survive.

1. METHODOLOGY

The *L. articulata* rhizomes were cultivated in marshy lands, Pond, and Top soil under dry condition. They were transplanted in Marshy land, top soil, lake and Pots. containing 20-25 shoots The. They were not supplied with any special treatments. After the full maturity stage, quantitative and qualitative traits were recorded and compared with literatures. They were shoot length, shoot diameter, number of shoots per bush, Number of flowers per shoot, Distance to the shoot tip and to the flower base, color of the flower, length of flower, diameter of a flower, the color of shoots, intervals between new shoots initiate from the rhizome and days to initiate 1st flower from the shoot. Thereafter average plant morphological characters were identified. Days neededfor first flower to emerge wasrecorded from after planting to 1st flowering stage.

RESULTS AND DISCUSSION

Phenotypic Parameter	Unit	Average readings
Shoot Color	-	Grey green
Seed Color	-	Brown
Average shoot length	cm	180-200
Average shoot Diameter	mm	6-8
Average inflorescence Diameter	mm	7-9
length of shoot tips to the base of the inflorescence	cm	3-4
Length of a seed	cm	1-1.5
Leaf shape	-	Cylindrical
inflorescence shape	-	Ovid-ellipsoide
Days require a flower initiate	Day	45-50
intervals to initiate a new shoots from the rhizome	Days	50-55
No of inflorescence per shoot	-	1

According to the results, all parameters were same to the literature review. **Importance for Gardens and Environment.**

According to the characters in *L. articulata* it has a high potential to be used as anornamental plants by value addition due to its grey green color,ability to remain long period without drying or withering and its durable. Besides stems are very rigid and easy to propagate through rhizomes. It has robust form, with both submerged and emergent portions of culms that are more rigid and of a greater diameter. it forms important vertical structural habitat for birds, fish and aquatic invertebrates(Marshall & McGregor, 2011).This plant can be established in gardens and can be maintained wet landsas well as dry areas. These areas contain a diversity of plants, animals and Fresh water and marine life that provide beautiful places for bird watching, sightseeing, hiking, fishing, hunting, boating and photography (Mishra *et al*.,2015). Hence it can be used for landscaping in gardens. It was reported thatthe plant has been used extensively for planting in wetlands constructed for urban run-off management and for decorative ponds and lakes. It is also useful as a filter plant in a natural swimming pool, providing stable habitat and food for water birds(Domyos & Te-chato, 2013) also Lepironiaarticulata has the potential to be used as a sustainable acoustic absorber(Shafii & Zainulabidin, 2021)

This plant can maintain water quality by absorbing many pollutants in surface water as well as can be grown along rivers and streams. The management of *L. articulata* by forest able to reduce illegal logging and forest fires. (Kissinger, *et al*., 2024) It absorbs energy and stores water during heavy rainy season, which reduces downstream flood damage and reduces the risk of floods. Additionally, the slow release of this stored water over some period of time can help keep streams flowing during periods of drought. Moreoverits fibers root system can bind soil particles preventing excessive erosion and sedimentation downstream. (Chucheep *et al*., 2022).

This sedge is a fiber-producing plant and can be used to make daily necessities such as woven bags, mats and ropes etc. Brink & Escobin, 2003 reported that *L. articulata* could be used for weaving purpose.Therefore, *L. articulata* plays a vital role in income generation for self-employees(Wunbua, *et al*., 2012) and (Sinaga *et al*., 2021). *L. articulata* can be harvested year-round and is done by pulling up 10-20 flowering stems together and cutting them to the required size. Brotonegoro, *et al*., 2003 reported that *Lepironia articulata* can be harvested twice a year.

CONCLUSION

In conclusion, the compared parameters were same to the literature review and it is recommended that *L. articulata can* be cultivated as an environment-friendly option to improve aesthetic value in Botanic garden as well as improve economic value of the land. *L. articulata* can be selected as an ornamental plant for indoors as well as outdoorsand to identify the uses according to its specific characters to botanic gardens in Sri Lanka.

SUGGESTIONS

Experiments should be developed to find out genetic characters to develop improved varieties by mutagenesis.

Experiment should also be carried outto improve it as an ornamental plant in outdoor gardens as well as indoor gardens.

To develop a “sedge garden”as part of landscaping for value addition and scenic beauty.

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Antifungal activity of selected plant extracts to eliminate fungal endophytes present in *Dracaena sanderiana*.Mast.

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ABSTRACT

In this study antifungal activity of three plant species was evaluated against fungal endophytes present in *in vitro Dracaena sanderiana* Mast. using the poisoned food technique which was used to determine mycelial growth inhibition percentage. Plant extractions namely guava (*Psidium guajava L.*) leaf extract, turmeric (*Curcuma longa L*) rhizome extract, and neem (*Azadirachta indica A. Juss.*) leaf extract with different concentrations were tested for their antifungal activity against two endophytic fungi, *Fusarium oxysporum* and *Epicoccum ilaticum* isolated from the *in vitro* grown *D. sanderiana* by calculating inhibition percentage. *In vitro* antifungal activity was tested along with a commercially available standard fungicide tebuconazole as the positive control. There no growth of fungi resulted on the positive control. Highest growth inhibition percentages against *Fusarium oxysporum* were obtained at 0.5% concentration of guava leaf extract (84.64%), 20% concentration of turmeric rhizome extract (62.79%) and 3% concentrations neem leaf extract (68.50%). *Epicoccum italicum* showed 100% mycelia growth inhibition percentage for all the tested concentrations of all three plant extracts. Thus, methanolic extract of *Psidium guajava* leaves showed stronger antifungal activity for *Fusarium oxysporum* and *Epicoccum ilaticum* than turmeric rhizome extract and neem leaves extract.

Keywords : *Dracaena sanderiana*; Antifungal activity; *Psidium guajava*; *Curcuma longa* L; *Azadirachta indica; Fusarium oxysporum; Epicoccum italicum.*

INTRODUCTION

Dracaena sanderiana is one of the most popular indoor ornamental plants that has high commercial value in the national and international floriculture trade. It ranks seventh among the top ten potted plants in the world. (Jazib *et al*., 2020). *D. sanderiana* is commercially propagated by stem cuttings. However, production of the required amount of high quality planting materials can be achieved only by applying micropropagation (Beura *et al*., 2007). However, microbial contaminations are Major problem in *in vitro* plant propagation. Recently, the elimination of pathogens from *D. sanderiana* was made possible through nanoparticles (NPs). It was found that Ag/Fe-TiO2 sterilization controls *Escherichia coli*, *Staphylococcus aureus*, and *Fusarium spp*. (Seneviratne *et al*., 2023) However, the efficacy of surface sterilization is not sufficient to avoid contamination due to micro-organisms that are present in internal tissues of plants. They are referred to as endophytes and usually do not cause any harm to the host plant. Among the microbial contaminations, endophytes are a major problem in *in vitro* propagation. Endophytes have symbiotic relationship with the host plant. The first study of fungal endophytes was carried out in temperate regions, but now it has also extended to tropical plants. All plants are associated with fungal endophytes. The most common fungal endophytes belong to Ascomycetes

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and *Basidiomycetes*. They mostly inhabit tissues of bark, leaves, flowers, seeds, roots, stems and etc. (Castillo *et al*.,2017) However, application of fungicides and other chemicals for control fungal endophytic contaminations can negatively impact plant health, human health and acquire greater resistance to active compounds. Plant extracts are therefore believed to be more acceptable and less hazardous than synthetic chemicals. (Praveen *et al*., 2013) Hence, the objective of this study was to investigate suitable natural antifungal compounds from plant extracts to effectively eliminate fungal endophytes present in *D. sanderiana.*

In this study, methanolic plant extractions namely guava (*Psidium guajava)* leaf extract, turmeric (*Curcuma longa* L) rhizome extract, and neem (*Azadirachta indica*) leaf extract with different concentrations were tested for their antifungal activity against isolated fungi from the tissue cultured *D. sanderiana*. Guava leaves are rich in phenols and flavonoids. (Oluwajobi *et al*., 2019) The active compounds in guava help to treat infectious diseases and particularly diseases caused by fungi. These treatments are common practices registered in countries such as Cuba, Brazil and South Africa. (Maria F.B *et al*., 2017) Turmeric is a well-known spice from ancient times not only as a flavoring agent but also as a food preservative and traditional medicine in China, India and Southeast Asia. (Radwan *et al*., 2014) Curcuminoids are phenolic compounds that cause the color of turmeric. (Tanzeela *et al*., 2015) Researchers have identified three curcuminoids from turmeric rhizomes which show in vivo antifungal activity for the control of plant diseases (Cho *et al*., 2005). Neem leaves were the third plant material used in this study. It is called as a village pharmacy in India because almost all the parts of *Azadirachta indica*, have medicinal properties (Arul *et al*., 2015). These plant materials were included in this study due to the fact that they are readily available, and several studies have found that methanolic extract of the same exhibited stronger antifungal activity against different fungal strains (Oluwajobi *et al*., 2019, Abdelgaleif *et al*.,2019 Mondall *et al*.,2009). The research was conducted with the objective of identifying, evaluating, and comparing antifungal activity of guava, turmeric and neem plant extracts to determine their effectiveness in eliminating fungal endophytes in *D. sanderiana* while promoting the quality of *in vitro* propagated dracaena plants.

METHODOLOGY

Sample collection Strategy of the study

Healthy *Dracaena sanderiana* plants of var. 'White' and 'Gold' were collected from Omega Green (Pvt) Ltd, Athuruguriaya.

Preparation of Medium

The culture medium used for *D. sanderiana* explants was half strength Murashige and Skoog's (MS) (1962) basal medium, supplemented with 3% sugar and 100 mg/L myoinositol . The pH of the medium was adjusted to 5.8. Then the medium was boiled in the microwave oven until agar was completely dissolved. When the agar medium cooled down to below 50 ^{0}C (when it can be held comfortably by hands), Augmentin (0.05g/L) was added to the medium and mixed thoroughly to obtain an even concentration throughout the medium. Culture tubes were sterilized with 5% Clorox™ solution called CSUP method (Peiris *et al*., 2012) prior to pouring.

Surface sterilization and establishment of explants

Dracaena sanderiana plant nodes were detached from mother plants and collected

into a plastic bag with water to avoid drying during transport. All samples were handled carefully and processed as quickly as possible. They were washed by rubbing gently but thoroughly with liquid soap under running tap water in the laboratory and separated into single nodes by cutting the stems. Nodes were agitated vigorously in distilled water with a few drops of liquid soap using an orbital shaker at 200rpm for 30 minutes. Thereafter, explants were washed with 10% sodium hypochlorite (Clorox™) and liquid soap by agitating vigorously again by keep on an orbital shaker at 200rpm for 10 minutes. This was repeated with a fresh solution of 10% Clorox. In the laminar flow cabinet, the nodes were rinsed with 70% IPA for 30 seconds. Soon after nodes were rinsed with autoclaved distilled water 3 times. Unwanted parts which were damaged by Clorox were removed and nodes were dipped in Augmentin (0.1g/L) for 10 minutes and then cultured in the freshly prepared medium aseptically

Identification of fungal endophytes

Potato dextrose agar (PDA) supplemented with Augmentin (0.1g/L) was used as the medium to obtain endophytic fungal growth isolated from dracaena nodal explants (Figure 1). Morphological properties of fungal colonies growing on culture plates were determined by examining their color, colony texture, and other aspects of their growth. Two endophytic fungi isolated from *Dracaena sanderiana* culture tubes were identified by comparing their color and the colony texture with reference studies.

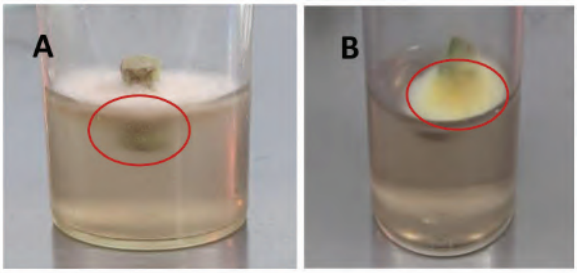

Figure 1: A- *Fusarium oxysporum*, B- *Epicoccum italicum* fuzzy growth on the medium

Preparation of plant crude extracts

Psidium guajava leaves, *Azadirachta indica* leaves and Curcuma longa rhizomes were cut into pieces and dried in an oven at 45°C for 48 hours. Dried leaves and turmeric rhizomes were ground to obtain a fine powder and stored in air-tight polythene bags. The methanol extract of three plant materials was prepared by mixing 20g of powder with 100ml of methanol. (1:5). After, the mixture was sonicated at 40°C for 6 hours, it was centrifuged at 3000rpm for 5 minutes and the supernatant was collected. Then the collected extract was poured into the evaporating discs and kept in a water bath at 45°C to evaporate methanol completely and the crude extracts of plant materials were collected.

Determination of Antifungal Activity

Crude methanolic extracts of plant materials were used to prepare different test concentrations by dissolving the plant crude extract in Dimethyl Sulfoxide (DMSO). Six different concentrations (0.1%, 0.3%, 0.5%, 1%, 5%, 10%) of the guava extract (Dhiman *et al*., 2011), four different concentrations (5%, 10%, 20%, 30%) turmeric rhizome extract (Shukla, 2012) and four different concentrations (0.5%, 1%, 2%, 3%) of neem leaves extract (Mondall *et al*, 2009) were prepared to test antifungal activity against the isolated fungi.

5 Antifungal activity of all three plant extracts were evaluated against two fungal strains by poisoned food technique which was used to determine mycelial growth inhibition percentage (Mondall *et al*, 2009). About 1ml of each plant extract was mixed with 9ml of PDA medium and poured into separate petri plates. Once the media was solidified, a 7mm diameter plug from the 7 days old pure fungus culture was placed on the center of each petri

plate. Then plates were incubated at 28°C for seven days. The colony diameter of each plate was measured on the 8th day of the incubation period. Each treatment was replicated three times.

The fungal growth inhibition percentage was calculated through the following formula,

$$\% FG = \frac{Dc - Dr \times 100}{Dc}$$

% FG = Percentage inhibition fungal growth

Dc = Diameter of control, Dr = Diameter of test

RESULTS AND DISCUSSION

Methanolic extracts of Guava (*Psidium guajava*) leaves, Turmeric (*Curcuma longa L*) rhizomes, and Neem (*Azadirachta indica*) leaves showed significant antifungal activity against both *Fusarium oxysporum* and *Epicoccum italicum*, fungal endophytes that were isolated from *D. sanderiana*.

Identification of the fungal endophytes

Among all the isolated fungi cultures, only two of them were subcultured. One fungus isolated was similar to the published information of Chen *et al.*, (2017) which can be identified as *Epicoccum italicum*. The colony on PDA appeared yellow with white margin and a black center. The reverse side of the fungus appears salmon to saffron color with a yellow margin. They grew 35-39mm in diameter after seven days. The agar medium became yellow discoloration when it was exposed to NaOH.

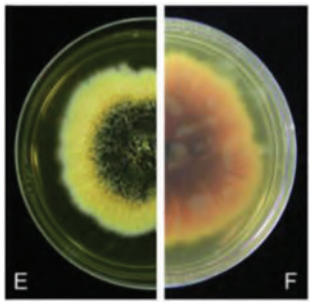

Figure 2: Colony on PDA (front and reverse)

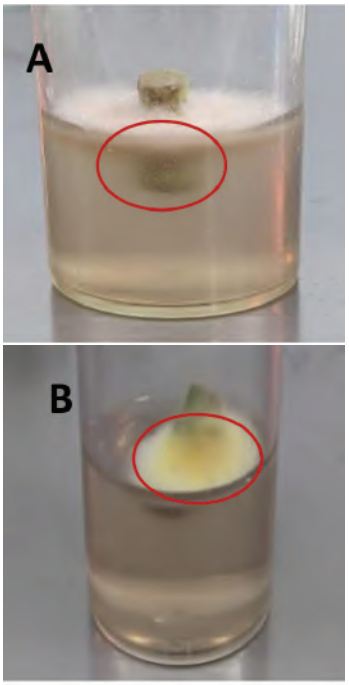

Figure 3: A- Front view of colony on PDA, B- Reverse view of colony on PDA

Antifungal Activity of Guava Leaves Extract Against Isolated Fungal Endophytes

Results of this study showed that *Fusarium oxysporum* was effectively inhibited at concentrations 0.1%, 0.3% and 0.5%, as 81.42%, 82.85% and 84.64%, respectively. (Table 1) Highest inhibition was showed at 0.5% concentration of guava leaf extract. However, the growth inhibition percentage of *Fusarium oxysporum* decreased after a certain (1%) concentration of guava leaf extract. In the previous study of Djeuani *et al.*, (2014) mentioned that it is possible that high concentrations of *Psidium guajava* in the medium does not given a high percentage of growth inhibition. However, the interaction between *Fusarium oxysporum* and guava leaf extract may be complex and various bioactive compounds can be involved with different modes of action in high concentrations. Therefore, those compounds may have interacted in ways that decrease the overall growth inhibition of *Fusarium oxysporum* at the higher concentrations.

Table 1: Mycelial growth inhibition percentages against guava extract

Concentration Treatment	Mycelial Growth Inhibition (%)					
	0.1%	**0.3%**	**0.5%**	**1%**	**5%**	**10%**
Fusarium oxyporum	81.42	82.85	84.64	83.92	81.07	76.42
Epicoccum italicum	100	100	100	100	100	100

Antifungal Activity of turmeric rhizome Extract Against Isolated Fungal Endophytes

Table 2: Mycelial growth inhibition percentages against turmeric extract

Concentration Treatment	Mycelial inhibition (%)					
	5%	**10%**	**20%**	**30%**	**5%**	**10%**
Fusarium oxysporum	60.46	61.62	62.79	83.92	81.07	76.42
Epicoccum italicum	100	100	100	100	100	100

Comparing the guava leaves extract, turmeric rhizome extract showed less antifungal activity against *Fusarium oxysporum*. Turmeric rhizome extract showed growth inhibition against *Fusarium oxysporum* at 5%, 10% and 20%. (Table 2) The largest growth inhibition percentage was shown at 20% as 62.79%. Similar to the guava extract, mycelia growth inhibition percentage of *Fusarium oxysporum* decreased at higher concentrations of turmeric rhizome extract. Shukla *et al*., (2012) has conducted an investigation by using a similar concentration series of turmeric extract for *fusarium oxysporum* and they have mentioned 87.96% mycelial growth inhibition at 15% concentration (Shukla *et al*., 2012) Other than the complex interaction between turmeric rhizome extract and *Fusarium oxysporum*, the reduction of mycelia growth inhibition percentage can be a result of stimulation of fungal growth at higher concentrations. Some compounds of turmeric rhizome extract might act as growth promoters or nutrients for *Fusarium oxysporum* at higher concentrations. This can lead to an increase in the fungal growth of the medium while decreasing mycelial growth inhibition percentage. However, methanolic extract of turmeric rhizomes were highly active against *Epicoccum italicum*. According to results show in table 2, growth of *Epicoccum italicum* was 100% inhibited by turmeric rhizome extract in concentrations 5%, 10%, 20% and 30%.

Antifungal Activity of Neem Leaves Extract Against Isolated Fungal Endophytes

Results also indicated that various concentrations of neem leaf extract led to the significant inhibition of *Fusarium oxysporum* (Table 3). *Fusarium oxysporum* shows growth inhibition percentages 62.20%, 64.56%, 66.92% and 68.50% at concentration (0.5%), (1%), (2%) and (3%) respectively. Among them the highest mycelial growth inhibition was 68.50% shown by 3% concentration. *Epicoccum italicum* showed 100% mycelia growth inhibition percentage for all the tested concentrations of neem leaves extracts.

Table 3: Mycelial growth inhibition percentages against neem extract

	Mycelial Growth inhibition (%)			
Concentration Treatment	0.5%	1%	2%	3%
Fusarium oxysporum	62.20	64.56	66.92	68.50
Epicocum Italium	100	100	100	100

According to the overall results of this study, 0.5% concentration of guava leaf extract, showed highest growth inhibition percentage against *Fusarium oxysporum* and 100% mycelial growth inhibition percentage against *Epicoccum italicum*. Besides at 20% concentration turmeric rhizome extract showed highest growth inhibition percentage against *Fusarium oxysporum* and 100% mycelial growth inhibition percentage against *Epicoccum italicum*. Moreover, 3% concentration of neem leaf extract also showed highest inhibition percentage against *Fusarium oxysporum* and 100% mycelial growth inhibition percentage against *Epicoccum italicum*.

Thus, it is clear from the above results that extracts from all three plants have effective antifungal activity against *Fusarium oxysporum* and *Epicoccum italicum* fungal endophytes that were isolated from culture tubes of *Dracaena sanderiana* explants. *Psidium guajava* leaf extract showed stronger antifungal activity for *Fusarium oxysporum* than *Curcuma longa* L rhizome and *Azadirachta indica* leaf extracts. Furthermore results exhibit that all three plant extracts have similar stronger antifungal activity against *Epicoccum italicum*. The mycelial growth inhibition percentage of the fungi strain depends on not only the concentration of plant extract but also the characteristics of fungi strain.

Study by Das and Goswami (2021) reported that the growth of Aspergillus niger and Saccharomyces cerevisiae fungal strains were inhibited strongly by guava leaf extract. Also their phytochemical analysis have proven that guava leaf extract was rich in a wide range of polyphenols and they are the probable cause of anti-microbial properties of guava leaves. (Das *et al*., 2019)

In addition, a study by Arul *et al*., (2015) on antifungal activity of Malaysian neem leaf extract against selected antifungal species causing otomycosis in *in vitro* culture medium had resulted in significant antifungal effect towards the Aspergillus niger and Candida albicans. Similarly the study by Mondall et al. (2009) on antifungal activities and chemical characterization of neem leaf extracts on growth of some selected fungal species in *in vitro* culture medium, resulted in the most effective antifungal activity against Aspergillus spp and Rhizopus with the alcoholic neem leaf extract.

CONCLUSION

The value of tissue cultured plants depends on their quality and health. The objective of this investigation was to find a natural plant extract which has antifungal potential to eliminate fungal endophytes in *Dracaena sanderiana*. Finding of this research will be an important step towards the tissue culture of dracaena. Evaluating guava leaf, turmeric rhizome, and neem leaf extracts, the study revealed notable inhibition percentages against *Fusarium oxysporum* and *Epicoccum italicum*. Guava leaf extract remarkably surpassed turmeric and neem, displaying high efficacy even at low concentrations. Specifically, at 0.5% concentration, guava exhibited an 84.64% inhibition against *Fusarium oxysporum*, marking its superiority. Yet, further research is essential to determine the optimal concentrations for inhibiting these fungal endophytes effectively. Overall, the methanolic

extract of guava leaves emerged as the most effective against the targeted fungi, offering a promising path for

advancing *Dracaena sanderiana*'s tissue culture industry.

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Efficacies Of Sodium Hypochlorite And Ethanol Against The Mite Attacks In Plant Tissue Culture

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ABSTRACT

Mites are tiny arthropods that can negatively impact plant tissue culture. This study aimed to determine the efficacy of sodium hypochlorite (NaOCl) and ethanol (C_2H_5OH) in controlling mites that may naturally occur during the sub-culturing process of in-vitro-produced orchids. A preliminary assessment of the phytotoxic effects of the treatments on tissue-cultured orchids was conducted. Three concentrations of NaOCl (3.75%, 7.5%, 15%) and four concentrations of C_2H_5OH (60%, 70%, 80%, 90%) were tested at exposure time periods of 5, 10, 15 minutes and 30, 60, 90 seconds, respectively. A control treatment was performed using distilled water. Mite counts were obtained by immersing shoots in the respective solutions, shaking them, and recording live mite counts if any were collected. The survival of mites and plant growth parameters were evaluated for each test chemical at all concentration levels. Data were analyzed using a Generalized Linear Model followed by paired comparisons. Results indicated that all NaOCl concentrations and exposure times did not induce phytotoxic effects, whereas the 90% C_2H_5OH treatment at all exposure time periods exhibited phytotoxicity. NaOCl at all concentration levels and exposure times, except for 5 minutes, demonstrated statistically higher mite mortality compared to ethanol treatments. However, neither NaOCl nor C_2H_5OH affected the growth parameters of orchid plants. The study suggests that sodium hypochlorite is more effective than ethanol in managing mite infestation during the sub-culturing process, recommending the lowest concentration of NaOCl for a 10-minute exposure time for commercial applications.

Keywords : Sodium hypochlorite, ethanol, mite infestation, phytotoxicity, mite mortality

INTRODUCTION

Plant tissue culture involves the cultivation and multiplication of plant cells, tissues, and organs in a controlled, sterile environment using specialized solid or liquid mediums (Stasolla and Thorpe, 2011). The primary benefit of plant tissue culture lies in its ability to swiftly generate diseasefree, superior-quality, and consistently uniform planting material. Moreover, this method enables the multiplication of valuable and significant plant species in a controlled environment, independent of season or weather conditions, throughout the entire year (Ahloowalia *et al*., 2004).Adherence of aseptic conditions is an important aspect of tissue culture, if not contaminations may occur due to chemical contaminants or biological contaminants. Different types of biological contaminants are bacteria, yeast, molds, viruses, mites, and thrips. Among different types of biological contaminants, mites stand out as a significant threat, capable of inducing adverse effects on tissue culture media, culture vessels, and plants.

Mites are tiny arthropods that belong to the order Acari and class Arachnida. The smallest mites are about 0.1 mm long, while the largest are about 6 mm long (Britannica, 2023). The Tarsonemidae family swiftly concludes its life cycle in under a week, often with only two active phases: nymphs and adults (Kaundil,

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2022). The four stages of mite development are egg, larva, nymph, and adult. The reproductive stage is the adult (Government of Newfoundland and Labrador, 2018). Mite populations increase rapidly due to their short development time and high ability to produce offspring. They can reproduce without mating when males are not present (Chong, 2022). In a wet condition, mold mites were able to survive without food for a duration of 31 days at temperatures between 20°C and 25°C (Sass, 2006). Mites thrive in high temperatures and low relative humidity conditions. Diverse categories of mites exist based on their distinct habits and habitats, including plant mites, gall mites, animal mites, poultry mites, and more (Damle, 2016). Animals' stomachs, lungs, or deeper body regions may be habitat to parasitic forms. Some mites are disease carriers for both humans and animals. Plant-feeding mites harm plants by feeding on leaf tissues or by spreading viruses that cause illnesses (Britannica, 2023). The most common cause of mite or thrip contamination in tissue culture rooms is the habit of bringing plant material into a lab (Koolaard *et al*., 2004).

Mites and thrips can cause severe problems because they introduce bacteria, yeasts, and fungal contaminants into plant tissue cultures. Mites are very small organisms therefore they can easily enter most tissue culture vessels. Once infestation takes hold within growth rooms, the migrating mite stages serve as carriers, facilitating the transfer of contaminants from contaminated cultures to uncontaminated ones (Leifert and Waites, 1994). 1 harmful mite population in 30- 40 days can be increased 25000 in theory, then can reach 5000000 after 60 days (Pype *et al*., 1997).

At the Royal Botanic Gardens in Peradeniya Sri Lanka, an infestation of mites affecting tissue culture bottles containing plants prompted an investigation. To ascertain the specific mite species responsible, samples were sent to the Horticultural Crops Research and Development Institute in Gannoruwa. The identified mites in the tissue culture bottles were classified as "Tread Footed Mites" belonging to the family Tarsonemidae.

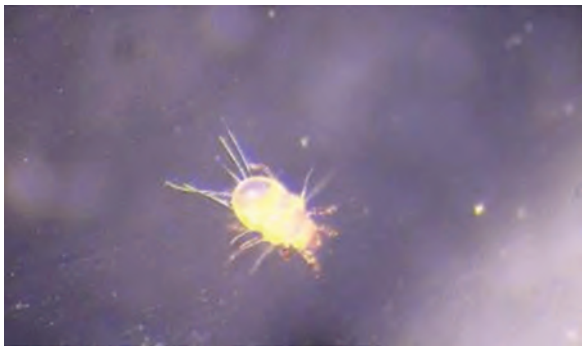

Plate 1.1: Thread Footed Mite (×10 by dissecting microscope

The occurrence of biotic contamination in tissue culture arises from the introduction of pathogens and other microbial impurities carried by the initial explants used to initiate the cultures. Additionally, biotic contaminants can infiltrate during the in vitro culture phases from the surrounding environment, along with microorganisms and microarthropods. Typically, the biotic contaminants linked to the initial explants used for culture establishment comprise crop pathogens or organisms that opportunistically colonize the plants. (Cassells and Tahmatsidou, 1996). Mobile mites can exit their habitat, infiltrating containers through the smallest openings, targeting plant material. Juvenile mites employ modified chelicerae to attach to plants and extract cell sap (Johan *et al*., 1997). Moreover, mite infestations can introduce bacteria, fungi, and yeast contaminants into tissue culture vessels. This poses a significant threat to tissue culture plants due to their heightened sensitivity to various biotic and abiotic conditions. In previous research (Johan *et al*., 1997; Epenhuijsen and Koolaard, 2004), miticide/ acaricide and mechanical methods have been

explored to prevent mite attacks in tissue culture. NaOCl, a disinfectant effective against diverse biological contaminants, is utilized to sterilize explant surfaces during tissue culture procedures (Khanam and Chandra, 2017). Ethanol, another potent sterilizing agent in tissue culture, also has the potential for controlling arthropod pests, however, limited attempts have been made for testing their efficacies against the mite infestations. Therefore, a study was conducted to assess the comparative efficacy of NaOCl and C_2H_5OH in controlling mites occurring during the sub culturing phase in *in-vitro* propagation of orchids

1.1 Objectives

Main objective

To identify the most effective concentration and treating time of Sodium Hypochlorite and Ethanol to control mites during the subculturing process.

Specific objective

To identify the effects of Sodium Hypochlorite and Ethanol on the shoot growth

MATERIALS AND METHODS

1.1 Treatments

Table 2.1.1: Treatments of NaOCl and C2H5OH experiment

Treatment factor	NaOCl	C2H5OH
Concentration	0%	0 %
	3.75 %	60 %
	7.5 %	70 %
	15 %	80 %
		90 %
Treating time	5 minutes	30 seconds

Table 2.1.2: Treatment combinations of NaOCl experiment

Treatments	Concentration (%) ×Time (minutes)
T1	0 × 5
T2	0 × 10
T3	0 × 15
T4	3.75 × 5
T5	3.75 × 10
T6	3.75 × 15
T7	7.5 × 5
T8	7.5 × 10
T9	7.5 × 15
T10	15 × 5
T11	15 × 10
T12	15 × 15

Table 2.1.3: Treatment combinations of C_2H_5OH experiment

Treatments	Concentration (%) × Time (minutes)
T13	0 × 30
T14	0 × 60
T15	0 × 90
T16	3.75 × 30
T17	3.75 × 60
T18	3.75 × 90
T19	7.5 × 30
T20	7.5 × 60
T21	7.5 × 90
T22	15 × 30
T23	15 × 60
T24	15 × 90

1.1 Experiment 01 - Investigation of Phytotoxicity

Healthy orchid shoots of uniform size were selected randomly, and their growth parameters (shoot height, number of leaves and number of roots) were recorded separately.

Shoots were immersed in 50 ml solutions of NaOCl at varying concentrations (3.75%, 7.5%, 15%) for specific durations (5 minutes, 10 minutes, 15 minutes), as two shoots per

replicate. Shoots were also subjected to immersion in 50 ml solutions of C_2H_5OH at different concentrations (60%, 70%, 80%, 90%) for specified time intervals (30 seconds, 60 seconds, 90 seconds), as two shoots per replicate.

Plate 2.2.1: Dipping shoots in respective concentrations of NaOCl and C_2H_5OH

After being treated with NaOCl or C_2H_5OH, the shoots were transferred to fresh media, with two shoots per replicate and kept for three weeks under aseptic conditions in the laboratory. Each treatment was replicated thrice.

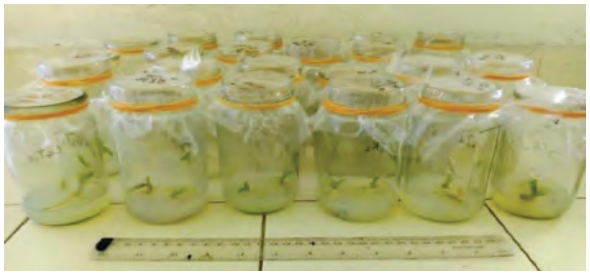

Plate 2.2.2: Transferring the shoots into new media

After three weeks, the shoots were taken off the culture bottles and growth parameters were recorded.

Plate 2.2.3: Recording growth parameters

2.3 Experiment 02: Efficacies of NaOCl and C2H5OH against Mite Infestations

Mite infested orchid shoots of uniform size were selected randomly and growth parameters (shoot height, number of leaves and number of roots) were recorded. Shoots were immersed in 50 ml solutions of NaOCl at varying concentrations (3.75%, 7.5%,

15%) for specific durations (5 minutes, 10 minutes, 15 minutes), as two shoots per replicate. Shoots were also subjected to immersion in 50 ml solutions of C_2H_5OH at different concentrations (60%, 70%, 80%,

90%) for specified time intervals (30 seconds, 60 seconds, 90 seconds), as two shoots per replicate. Water samples were collected and live mite numbers were counted (X). Shoots were transferred into new media, with two shoots per replicate and the bottles were sealed with Parafilm. Shoots were allowed to grow for three weeks.

Plate 2.3.1: Three weeks old shoots after subculturing

Shoots were removed from the media after three-week and growth parameters were recorded again.

Plate 2.3.2: Removing the shoots from culture media after three weeks

Subsequently shoots were dipped into 50 ml of distilled water solutions for desired time periods

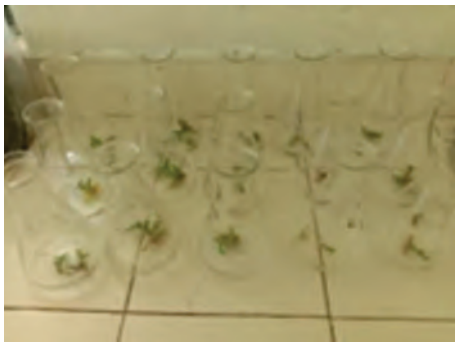

Plate 2.3.3: Dipping shoots in distilled water

Plate 2.3.4: Collecting free moving mites in culture bottle

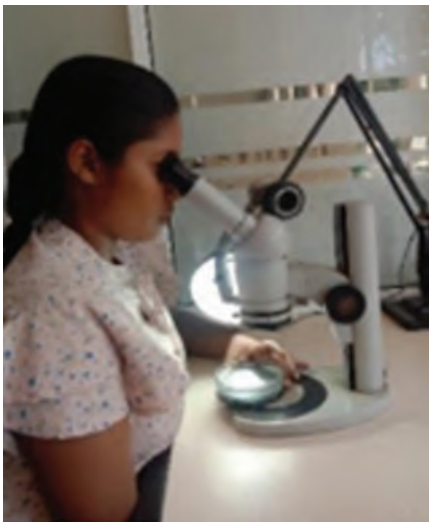

Plate 2.3.5: Counting the live mite number

The water samples were collected, and live mite number was recorded with use of a dissecting microscope (X). It was assumed that this mite count was inclusive of free moving mites too in the culture bottle. Final average live mite count per replicate was calculated as X + Y.

2.4 Data analysis

Data were analyzed using Minitab 17 software. Total survival mite count of each replicate was used for the analysis. Generalized Linear Model followed by paired comparisons was run for mite counts corresponding to treatment combinations of each test chemical. Interactive effect of test chemicals on mite survival and shoot growth was employed.

RESULTS AND DISCUSSION

3.1 Experiment 01 – Investigation of Phytotoxicity

Findings of this experiment indicated that a phytotoxic effect was only observed in plants exposed to 90 % ethanol for all treatment times.

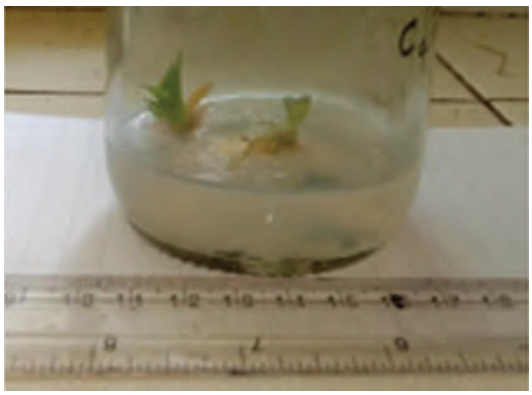

Plate 3.1.1: Leaves showing necrosis (90% ethanol, 30 seconds)

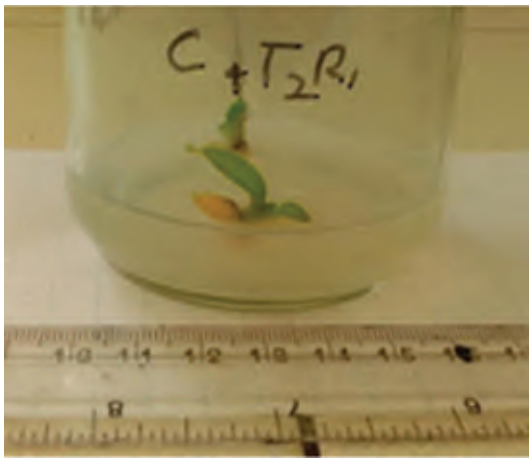

Plate 3.1.3: Leaves showing necrosis (90 % ethanol, 90 seconds)

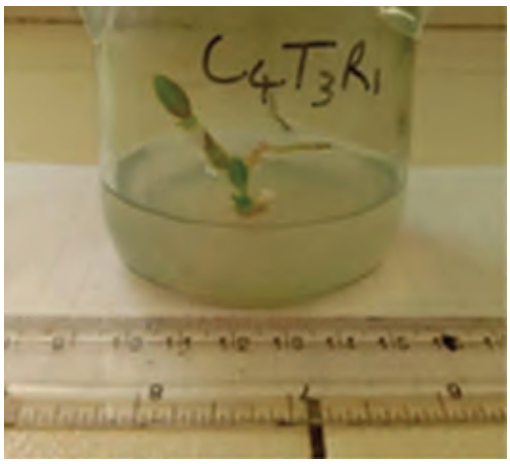

Plate 3.1.4: Leaves have bleached (90 % ethanol, 90 seconds)

The concentration of NaOCl and C_2H_5OH used for mite control in tissue culture should be carefully determined to avoid phytotoxicity while ensuring effectiveness against mites. NaOCl at such high concentrations can cause severe damage to plant tissues and negatively affect their growth and development. After treating plant material or surfaces with NaOCl, it is essential to rinse them thoroughly with sterile water to remove any residual bleach. Residual NaOCl may have phytotoxic effects on tissue cultures, so thorough rinsing is necessary to minimize any potential harm. According to results of this experiment, three concentrations of NaOCl that were used for the experiment did not show any phytotoxic effects for treated plants.

3.2 Experiment 02 - Selecting the Best Treatment among NaOCl and C_2H_5OH

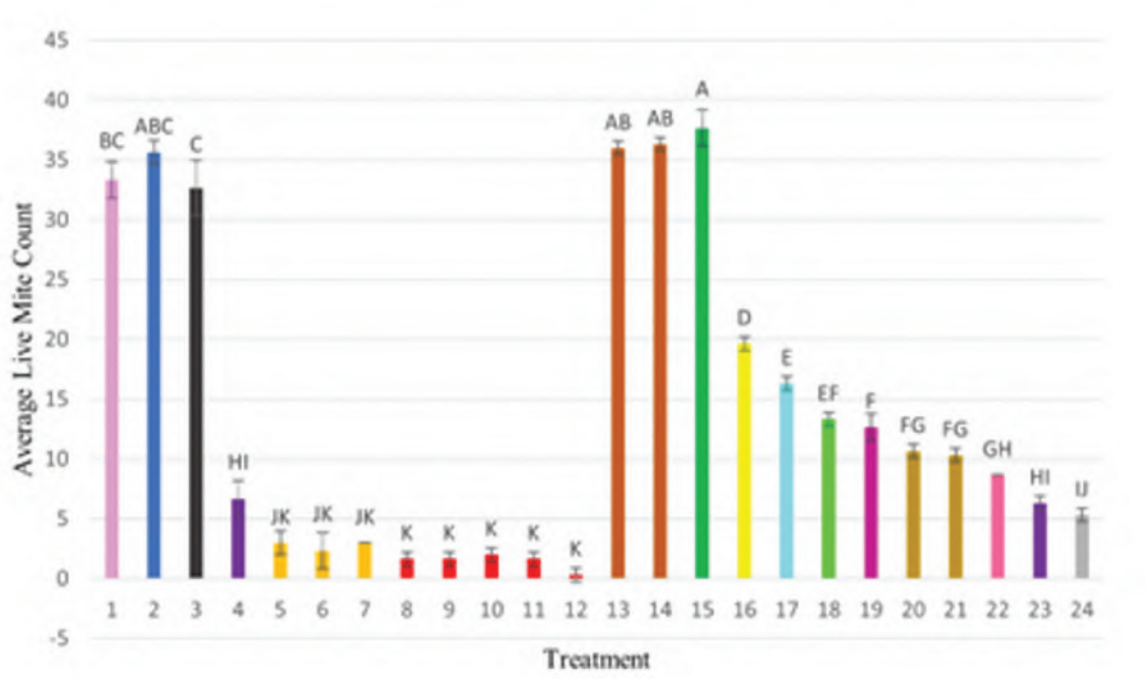

Table 3.2.1: Mean live mite number after treatment with NaOCl and C_2H_5OH

Treatments	Average live mite number
T1	33.3333BC
T2	35.6667ABC
T3	32.6667C
T4	2.3333HI
T5	3JK
T6	6.6667JK
T7	3JK
T8	1.6667K
T9	1.6667K
T10	2K
T11	1.6667K
T12	0.3333K
T13	36AB
T14	36.3333AB
T15	37.6667A
T16	19.6667D
T17	16.3333E
T18	13.3333EF
T19	12.6667F
T20	10.6667FG
T21	0.3333FG
T22	8.6667GH
T23	6.3333HI
T24	5.3333IJ

According to mean live mite number recorded three weeks after application of NaOCl, it was apparent that mean dead mite number of all treatments had drastically increased. T12 (0.3333) registered the lowest live mite number, which was statistically on par with T11 (1.6667), T10 (2.00), T9 (1.6667), T8 (1.6667), T7 (3.00), T6 (6.6667), T5 (3.00) except T4 (2.3333). T1 (33.3333), T2 (35.6667), T3 (32.6667), T13 (36), T14 (36.3333) and T15 (37.6667)

recorded high average live mite number (low mortality) because all were control treatments, and they were statistically on par with each other.

Referring to the mean live mite count three weeks after treatment with ethanol, it is evident that the mean dead mite numbers increased across all treatments, excluding control treatments. Specifically, T17 (16.3333), T18 (13.3333), T19 (12.6667), T20 (10.6667), T21 (10.3333), T22 (8.6667), T23 (6.3333), and T24 (5.3333)

demonstrated statistical parity with each other. Notably, all ethanol treatments, except for T23, exhibited a higher average live mite count compared to the NaOCl treatments.

Figure 3.2.1: Average live mite number after treated with NaOCl and C_2H_5OH

Masood *et al*., (1998) reported that the relationship between NaOCl concentration and mite survival exhibited a linear response, where increasing the concentration of NaOCl resulted in a corresponding increase in mite mortality. Further, it was revealed that the increase in mite mortality was observed as the duration of exposure to NaOCl increased from 5 to 15 minutes. Findings of the present investigation also clearly revealed that there was an interaction effect of NaOCl concentrations and exposure times.

Sterilizing with ethanol can be used as a control method to help eliminate mite attacks in tissue culture to some extent. It is a commonly used disinfectant and has shown effectiveness against various pests, including mites. However, the effectiveness of ethanol as a mite control agent may vary depending on factors such as the concentration of ethanol used, exposure time, and specific conditions of the tissue culture environment. In a study conducted on monitoring and controlling mold mites in tissue culture facilities, it was observed that spraying the mites with aerosols and 70 % ethanol did not effectively kill them (Epenhuijsen and Koolaard, 2004). The findings of this experiment revealed that 70 % ethanol can control mite attacks in tissue

culture, however it cannot eradicate mite attacks.

All NaOCl and C_2H_5OH treatments, excluding control treatments, demonstrated effective control over mite infestation in tissue culture. Furthermore, neither NaOCl nor C_2H_5OH exhibited any discernible impact on the growth parameters of tissue-cultured orchid plants.

With the exception of the 5-minute exposure time, all concentration levels of NaOCl, along with their respective exposure durations, exhibit greater efficacy in controlling mite attacks compared to C_2H_5OH.

Among all treatments of NaOCl, the lowest concentration (3.75 %) with its 10 minute-exposure time can be recommended for commercial applications considering their advantages such as cost effectiveness and time saving.

3.3 Effect of NaOCl and C_2H_5OH on Plant Growth

Table 3.3.1: P value for relevant growth parameters on shoot growth

	Shoot height	Number Leaves	Number of roots
NaOCl P value	0.05<	0.05<	0.05<
C_2H_5OH P value	0.05<	0.05<	0.05<
	0.179	0.257	0.672

Data on shoot height, number of leaves, and number of roots were collected both before and three weeks after sub-culturing. Analysis revealed no interaction effect between NaOCl and C_2H_5OH concentrations and exposure times on the growth of tissue culture plants. NaOCl nor C_2H_5OH has affected growth parameters of Orchid plants.

CONCLUCIONS

Results indicated an absence of phytotoxic effects across all NaOCl concentrations and exposure times, whereas the 90% C_2H_5OH treatment displayed phytotoxicity throughout its all exposure times. With the exception of the 5-minute exposure time, all NaOCl concentration levels exhibited significantly higher mite mortality rates compared to ethanol treatments. However, neither NaOCl nor C_2H_5OH influenced growth parameters of orchid plants. These findings suggest that sodium hypochlorite is more effective than ethanol in managing mite infestation during the sub-culturing process. The most suitable recommendation for practical applications would be the lowest concentration of NaOCl (3.75%) with a 10- minute exposure time due to its cost- effectiveness and time efficiency.

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Antibacterial Activity of *Azadirachta indica* A.Juss. (Neem) Leaf Extract to Eliminate Endophytic Bacteria Present in *Dracaena sanderiana* Mast.

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ABSTRACT

Dracaena sanderiana Mast.is the Sri Lankan number one exported ornamental plant. It is among the top 10 ornamental plants in the world. Presently, the utilization of plant tissue culture technology is employed to promote this valuable cultivation and fulfill the demand to restore plant productivity. Endophytic bacterial contamination causes a significant threat, leading to considerable losses in in vitro cultures. This research was initiated with the objective of using neem leaf (*Azadirachta indica* A.Juss.) extraction for the eradication of endophytic bacteria present in *Dracaena sanderiana* explants. Eight morphologically distinct bacterial varieties were observed by plating microbial growth from contaminated dracaena in vitro cultures. Analysis of contaminated cultures revealed the presence of the two most common strains. Concentrations of 200mg/mL, 100mg/mL, 80mg/mL, 60mg/mL, 40mg/mL, 20mg/mL, and 10 mg/mL of methanolic neem leaf extraction were tested using the well method for antibacterial activity for the selected bacteria isolated. Research outcomes unequivocally demonstrate that methanolic extracts obtained from neem leaves illustrates notably potent antibacterial effects hostile to the isolated strains. The concentration of 200mg/mL of the plant extraction showed maximum antibacterial activity with a mean inhibition zone of 22mm. The next phase of research involves incorporating these neem leaf extracts into the tissue culture medium.

Keywords : *Dracaena sanderiana*; Endophytic bacteria; *Azadirachta indica*;

INTRODUCTION

Lucky bamboo plant – *Dracaena sanderiana* is one of the important products of the foliage industry of Sri Lanka. It is a member of the Agavaceae family and native to India and Africa (Galus *et al*., 2019). This foliage plant is cultivated as an indoor plant in areas beyond tropical zones and shows optimal performance when placed in containers with partial outdoor shade or when situated indoors under bright yet indirect light. Several species of dracaena possess medicinal values such as antimalarial, antileishmanial, molluscicide, etc. (Aslam *et al*., 2013). *Dracaena sanderiana* is commercially propagated by vegetative means and micropropagation proves an efficient method for its propagation. In-vitro micropropagation of *D. sanderiana* comprises sterilization, shoot proliferation, rooting, and acclimatization. Mass production caters to the increasing demand, seed dormancy, inferior rate of germination, and variation in progeny that has increased in vitro propagation of this foliage plant (Aslam *et al*., 2013).

Tissue culture or micropropagation is a process for making an entire plant by using in vitro culture techniques of cells, tissues, or organs of plants under an aseptic and controlled environment. Tissue culture comprises five key stages: the selection and maintenance of the mother plant, in vitro establishment, in vitro multiplication, in vitro rooting, and acclimatization (Rout *et al*., 2006).

Microbial contamination is one of the greatest problems in tissue culture. Normal contaminants in tissue culture include bacteria, fungi, molds, and yeasts. Any step

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during the process can lead to contamination of cultures. Basically, two types of bacteria associated with tissue culture are endophytic and epiphytic bacteria. However, in host plant structures where disinfectants cannot penetrate, epiphytic bacteria like any other microorganisms may inhabit the host (Szewczyk Taranek *et al*., 2020). Endophytes are microorganisms (bacteria and fungi) living within the tissues of plants without harming the host plant (Szewczyk-Taranek *et al*., 2020). They live mostly within plant cell junctions and intercellular spaces of parenchyma cells (Reed., 2015). Most endophytic bacteria synthesize bioactive compounds, secrete chemicals that inhibit growth for pathogens, and stimulate immune response from plants amongst others. (Reed and Piyarak Tanprasert., 2020). However, in the case of tissue culture, they are considered the first sources of pollutants that must be removed for producing healthy in vitro plant growth since they initiate severe complications like browning of tissues or necrosis, etc.

(Romadanova *et al*., 2022). Endophytes can resist poor aseptic techniques and surface sterilization because they live in the interiors of plant tissue (Reed., 2015). Another main problem with endophytes is that some of them are not visible until after several rounds of subculture or after cultures have undergone stress (Romadanova *et al*., 2022).

Contamination caused by bacteria poses a problem that cannot be resolved through surface sterilization techniques alone. As a result, the use of antibiotics becomes necessary. Numerous research findings indicate that *Azadirachta indica* (neem plant) demonstrates important antimicrobial effectiveness against different bacterial strains. (Jennifer Mordue & Nisbet, 2000). Neem is a part of the plant family Meliaceae. It originated mostly from south-eastern Asia e.g., Bangladesh, Pakistan, India, and Nepal. (Francine *et al*., 2015). The neem plant's antibacterial effectiveness arises from the abundance of bioactive compounds. There are more than 140 bioactive compounds contained in neem plants encompassing biologically active chemical constituents (Ali *et al*., 2021). Azadirachtin, a terpenoid, functions as the key active component accountable for antibacterial attributes of the neem plant (Mohapatra *et al*., 2014).

Therefore, the present study was conducted to assess the antibacterial activity of *Azadirachta indica* (Neem) leaf methanolic extract in eliminating endophytic bacteria present in D. sanderiana.

MATERIALS AND METHODS

Micropropagation of *Dracaena sanderiana*

Plant material

Two varieties of healthy *D. sanderiana* plants, var. 'White' and 'Gold', were obtained from Omega Green (Pvt) Ltd., a commercial nursery. Stem nodes of *D. sanderiana* were used as the explant material for culturing. These experiments were performed in the Plant Tissue Culture Laboratory of the Department of Applied Science at the Sri Lanka Institute of Information Technology.

Surface sterilization and explant preparation Stem nodes with buds were dissected from the mother plants. Stem cuttings of 6 to 8cm in size were obtained from the upper portion of the plant. All extraneous plant parts, including leaves, were removed. Nodes were then washed by rubbing gently with soap and running tap water for several minutes. Then nodes were soaked in a 100 ml solution containing a few drops of liquid soap (Dettol™ hand wash). The soap water was decanted and then washed with 10% Clorox™ solution. The container was placed on a shaker and agitated

at 180 rpm for 10 minutes. The Clorox solution was then decanted and replaced with a fresh solution of 10% Clorox, which was agitated for another 10 minutes.

Culturing of explant

Explants with the Clorox solution was taken into the laminar airflow cabinet and was decanted, and nodes were rinsed once with absolute ethanol and three times with autoclaved distilled water. The two edges of the sterilized nodes were trimmed to remove Clorox-damaged tissue. Explants were then prepared by cutting the nodes into two cm segments which include the axillary bud. To prevent fungal contamination, the explants were dipped in a tebuconazole fungicide solution (5µl/L) for about 5 minutes. Finally, explants were cultured in tubes containing half strength Murashige and Skoog medium which was sterilized with CSUP sterilization method (Peiris *et al*., 2012).

Determination of antibacterial activity

Plant material

Fresh mature neem leaves were collected from a healthy grown mature neem tree located in the Malabe area. First the neem leaves were immersed in tap water for twenty minutes to eliminate contaminants including dust and unnecessary metals. The leaves were cut into pieces and air-dried in the dry oven at 50ºC until fully dried. Dried leaves were then ground to a fine powder.

Preparation of extracts (Solvent extraction)

The weight of 5g of dried neem leaf powder was taken and put into a centrifuge tube. A volume of 25ml methanol (96.9%) was poured into the tube representing a mass-to-solvent ratio of 1:5 (w/v). Tubes were sonicated at 350C for three hours and then centrifuged at 3000rpm for 2 minutes. The supernatant was collected into another tube and a volume of 25ml methanol was added to the same pellet and procedure was repeated twice. The extraction was transferred into clean evaporating disks and put in the water bath at 37ºC to evaporate methanol.

Isolation of endophytic bacteria

Bacterial contaminations were observed from one month of culturing *D. sanderiana* explants. These were identified as endophytes because contaminants were not on the surface of the explant or the medium but secreted from the explants to the medium (Figure.01). Those contaminants were isolated and plated on Luria Bertani Agar (LB agar) composed of 10g of tryptone, 5g of Yeast extract, and 10g of NaCl. Cultures were incubated at 37ºC for 24 hours. Colonies were chosen based on their size, shape, and color and streaked onto fresh LB agar twice to attain pure, individual colonies of each bacterial species.

Figure 01: Endophytic bacterial contaminations observed in cultured plants.

Transfer of bacteria

After incubation, a pure colony was selected with an inoculating loop and transferred to a volume of 20 mL of sterile Luria Bertani broth (LB broth) test tubes. Then the bacterial cultures were incubated for 16 hours at 370C. After incubation, bacterial concentrations were determined using a spectrophotometer.

These cell suspensions were diluted with sterile LB to provide final cell counts of about 107 CFU/ ml.

Antibacterial activity by well diffusion assay

Mueller-Hinton agar (MHA) was prepared by dissolving 3.8g of MHA powder in a hundred milliliters of distilled water. The mixture was then autoclaved at 1210C for 15 minutes at 100 kPa pressure. Once autoclaved, a volume of 15ml of the agar was poured into Petri plates to create a consistency thickness of around four millimeters. A sterile cotton swab was dipped in the microbial broth culture, subjected to rotation to ensure thorough inoculation, and then pressed against the interior surface of the tube to draw out surplus liquid. Then the face of the agar plate was streaked using the cotton swab in four overlapping strokes to achieve uniform growth. Wells with a diameter of 5mm were punched using a sterile pipette tip. Seven serial dilutions of methanolic neem leaf extraction were prepared, resulting in concentrations of 200mg/mL, 100mg/mL, 80mg/mL, 60mg/mL, 40mg/mL, 20mg/mL, and 10 mg/mL of extract, which were then added to each well. A volume of 70 µL of neem extract was added to each well and incubated at 37°C for 24 hours. The diameter of growth inhibition zones was measured in millimeters (mm). Bacterial suspensions with a turbidity of 0.5 McFarland (equivalent to 1.5×108 colony-forming units (CFU)/ml) were prepared using single colonies on agar plates that had been incubated for 18 to 24 hours. The turbidity of the bacterial suspension was measured at 600 nm. The antibiotic, Augmentin (625mg) was used as the positive control. All experiments were carried out in triplicate.

Minimum Inhibition Concentration (MIC)

The agar dilution method is carried out to determine the MIC of microorganisms against antimicrobial agents. A series of antibiotic dilutions were prepared by diluting with Dimethyl Sulphoxide (DMSO, extrapure AR Grade, 99.5%, 500ml, CAS 67-68-5). A volume of 19ml of MHA was added to each sterile container and a volume of 1ml of each plant extraction was added to the containers. Augmentin was used as the positive control. A volume of 1-2µl from the broth culture was inoculated onto the agar surface. Spots were allowed to dry before incubation. Plates were incubated at 37ºC for 24 hours.

Data collection and statistical analysis

Daily monitoring and recording of tissue-cultured plant contamination were conducted throughout the three-month period. At the conclusion of this period, contamination percentage was computed. Experiments were replicated three times, and gathered data underwent analysis of variance by IBM SPSS Statistics 29.0.1.0.

RESULTS AND DISCUSSION

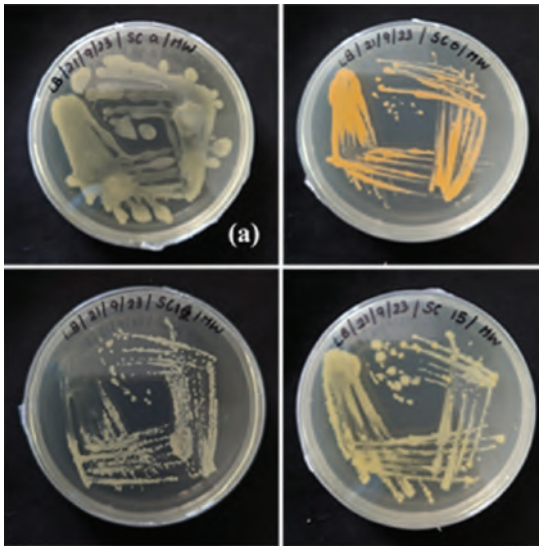
Isolation of Endophytic Bacteria

In this experiment, three establishment rounds were conducted for *D. sanderiana* explants. Within 3 days of culture initiation, contamination was observed. They were believed to be epiphytic microorganisms. The first establishment exhibited a high level of fungal contamination, which was easily identifiable by the presence of mycelia. In contrast, the second and third replications exhibited significantly lower levels of fungal contamination. This may be attributed to the use of fungicide, which was not applied to the first establishment(Figure 01).

Typically, endophytic bacterial contamination becomes detectable approximately one month after initial introduction. The process of media contamination by these endophytic bacteria is a gradual one, taking some time to become visible. Endophytic bacterial contamination appeared as a white, cloudy appearance within the growth medium. Epiphytic bacterial contaminations were observed as black and pink, oozy appearances on the surface of the medium and the explant (Szewczyk-Taranek *et al*., 2020).

Isolation and subculturing of endophytic bacteria

Eight morphologically distinct varieties were observed by plating the microbial growth from contaminated cultures. The analysis of contamination cultures revealed the presence of the two most common strains, tentatively labeled as bacterial strains 19 and 21. It is noteworthy that multiple endophytic bacterial strains can be attained from a single plant (Nxumalo *et al*., 2020). A significant observation was that these bacteria typically require more than 20 hours to grow to a visible density, and even after 24 hours, some colonies may be very small and difficult to see with the naked eye.



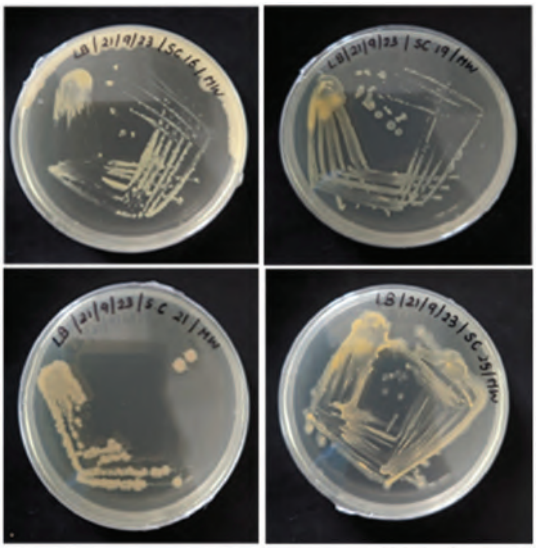


Figure 02: Subculturing of endophytic bacteria isolated from contaminated culture in LB medium. (a)Bacterial Strain a; (b)Bacterial Strain 6; (c)Bacterial strain 16; (d) Bacterial Strain 19; (e)Bacterial Strain 12; (f)Bacterial Strain 15; (g)Bacterial strain 21; (f) Bacterial Strain 25

Note: The above bacterial strain numbers serve solely for the purpose of culture identification and are not organized according to a specific protocol.

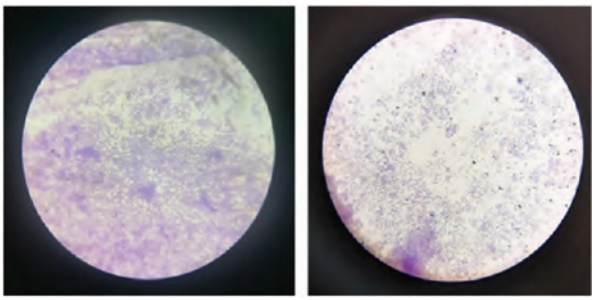


Figure 03: Results of Gram staining of isolated endophytic bacteria. (a) Gram-positive bacteria (BS19); (b) Gram-positive bacteria (BS21)

Antibacterial activity of neem extraction

Concentrations of 200mg/mL, 100mg/mL, 80mg/mL, 60mg/mL, 40mg/mL, 20mg/mL, and 10 mg/mL of neem leaf extraction were tested for antibacterial activity for the selected bacteria isolated. As depicted in the table below, the zone of inhibition for the methanol extract of neem leaves exhibited a proportional increase with rising concentrations. Since the diameter of a well was 5mm, it ensures

that even the slightest concentration of the extract exhibits the least antibacterial activity. The concentration of 200mg/ml of the plant extraction showed maximum antibacterial activity with a mean inhibition zone of 22mm. This was followed by the concentration of 100mg/ml (20mm) and 80mg/ml (16mm). Results show that Augmentin (625mg/100ml) was the most universally effective antibacterial agent in this study as all the isolates showed susceptibility. (Kamble *et al*., 2022).

Table 1: The inhibition zone value of neem extract toward bacterial strain no.19

Extract concentration (mg/ml)	N	Mean (mm)	Std. Deviation
10	3	9.33	1.155
20	3	12.67	1.155
40	3	13.00	1.000
60	3	13.67	.577
80	3	16.33	1.528
100	3	20.00	.000
200	3	22.00	1.000
Augmentin	3	24.67	.577

N= Number of samples

Table 2: The inhibition zone value of neem extract toward bacterial strain no.21

Extract concentration (mg/ml)	N	Mean (mm)	Std. Deviation
10	3	8.67	1.155
20	3	12.33	.577
40	3	14.00	.000
60	3	15.33	.577
80	3	15.33	.577
100	3	15.67	.577
200	3	18.33	.577
Augmentin	3	27.00	2.646

N= number of samples

Results of Agar dilution method (MIC)

A noticeable reduction in colony sizes was observed as the concentration of samples on agar plates increased. No antibiotic or plant extracts were added to the control plate. According to results, all five bacterial strains had grown on the control plate. Augmentin was added to the second plate. Based on outcomes, there was no visible bacterial growth on that plate. With increasing concentration of plant extraction, growth of all bacterial strains decreased. The Concentration 200mg/ml plate can be identified as the best concentration since there was no bacterial growth on that plate. The MIC for the methanolic extract of neem leaves was determined to be 25 mg/ml against the tested organisms.

Table 3: Results of agar dilution method for neem leaf extraction

Control	BS12 ++	BS15 ++	BS16 ++	BS19 ++	BS21 ++
-	For the absence of any microorganisms				
+	for mean growth of microorganisms				
++	for the significant growth of microorganisms				
BS- Bacterial strain	-	-	-	-	-
200mg/ml	-	-	-	-	

The ultimate objective of conducting this research was to integrate these extractions into tissue culture media. A wide range of antibiotics such as ampicillin, gentamicin, and amoxiclav are utilized to suppress or eradicate endophytic contaminants (Singh, 2000). However, prolonged use of these synthetic antibiotics can develop resistant bacterial strains(Yehia, 2016). Therefore, the employment of natural antibiotics will mitigate this challenge, as it is consistently advantageous to use natural products due to their intrinsic mechanisms, which are particularly effective in eliminating bacteria (Sarma *et al*., 1990). Application of a high concentration of plant extraction introduces another question, namely, whether these high concentrations may cause phytotoxicity in the plant culture. Therefore, it is important

to continue this research to systematically examine the ultimate outcomes, aligning with the primary objective of the study. Koona and Budida, (2011) reported the antibacterial effects of A. *indica* on Proteus vulgaris and Micrococcus luteus as most susceptible bacteria to the hexane, chloroform and methanol extracts. Also, Staphylococcus aureus has shown susceptibility to fresh extracts of neem leaves and bark (Francine, *et al*.,2015).

In this experimental procedure, leaves were chosen as the botanical component for the extraction procedure. This decision is based on the higher concentration of Azadirachtin, the primary secondary metabolite responsible for the antibacterial properties, which is significantly higher in leaves compared to other parts of the plant. (Francine *et al*., 2015). Precise identification of the bacterial isolates was not performed at this stage, as the primary goal was to obtain pure cultures. This is because the objective of the research is to identify a suitable antibacterial compound that can eliminate endophytic bacterial contamination.

CONCLUSION

Endophytic bacterial contamination may persist even three months after initial culture establishment due to the delayed emergence of certain endophytes. Current practices involve the use of synthetic antibiotics to eliminate both epiphytic and endophytic bacteria. However, their continued use has led to the development of antibiotic-resistant strains, rendering these treatments ineffective. The data generated in this study demonstrate that methanolic extraction of neem leaves possess robust antibacterial activity against the isolated endophytic bacteria. Most bacteria revealed inhibited growth at the highest concentration utilized in this study, specifically at 200 mg/ml.

The next phase of the research involves incorporating these neem leaf extracts into the tissue culture medium. To gain deeper insights into the characteristics of the isolated bacteria from contaminated cultures, molecular analysis, especially 16S rRNA gene sequencing, will be employed.

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Optimizing Cost-Effective Growth Medium for Enhanced *In vitro* Seed Germination and Shoot Development in Dendrobium Orchids

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ABSTRACT

Orchids are among the top ten cut flowers in the export market due to their high ornamental value. Among orchids, dendrobiums gain high popularity due to its attractive and long-lasting flowers and availability throughout the year. However, application of micropropagation to produce high quality planting material is hindered due to high cost in media preparation. Hence this study was carried out with the objective of optimizing cost effective growth medium for dendrobium orchid seed germination and shoot multiplication. A medium containing Albert solution fertilizer in different concentrations were used (2g/L, 4g/L, 6g/L, 8g/L) to prepare low-cost media where one treatment was carried out with straight fertilizers Urea, Muriate of Potash (MOP), Triple Super Phosphate (TSP) and the chemical calcium nitrate ($Ca(NO_3)_2$) with the use of ferrous (Fe) tablets. Unipower Albert's mixture was used in all treatments that were carried out with Albert's solution.All the treatments were carried out by replacing myoinositol and vitamins in Murashige & Skoog (MS) medium with Vitamin B tablet. Treatments were carried out for both orchid seeds and shoots in two experiments. Seed germination was observed through seed emergence and shoot growth was observed based on the dry weight and shoot number. Among the low-cost treatments, the highestorchid seed germination was observed in 2g/L Albert solution. The highest mean shoot number (17.66) andmean shoot growth (0.1790g) was observed in 8g/L Albert medium, however it was observed to be low in quality whereas 6g/L Albert medium showed high shoot number and shoots with high quality. The total cost for 1L MS medium was about Rs.2800 and the total cost of 1L Albert's media for all treatments done was only about Rs. 20- 40. It could be concluded that low-cost Albert solution fertilizer medium also can be used for in vitro seed germination and shoot multiplication in dendrobium orchid.

Keywords : Dendrobium, Albert's solution, Low-cost media

INTRODUCTION

The Family Orchdaceae, which includes orchids, is known for its long-lasting magnificent flowers with varied morphology. Since ancient times, floriculture has made great use of orchids. The family consists of roughly 736 genera and over 28,000 species (Chase *et al*., 2015). Orchids occupy a position as one of the top ten cut flowers and it was helped by large scale multiplication of exquisite and rare hybrids using micropropagation techniques. Orchids in the wild could reproduce asexually by vegetative propagation and sexually through seed. Embryos of Orchid seeds are typically quite small, ranging in size from 0.3 to 14 g and 0.05 to 6 mm in length, breadth, and weight (Arditti, 1967). Dendrobium is a family of epiphytic orchids that includes up to 1,500 species. Several Pacific islands, Australia, and tropical and subtropical Asia all have native dendrobium species. Many are grown for their ornamental qualities, and some are significant to the florist business. In order to improve floriculture trade use of high-quality planting materials is the key application. This goal can be achieved through micropropagation, which

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uses limited land by continuously producing and contributing plantlets of the necessary variety or genotype. (Biswas, 2012). However, micropropagation demands high input thereby production cost becomes high. A majority of commercial laboratories have a large problem with the expense of tissue culture medium components (Babbar & Jain, 2006). Production costs are highly influenced by the making criteria of the culture media being utilized for micropropagation. Hence, the necessity here is to introduce a low-cost tissue culture technology. Low-cost tissue culture technology must depend on procedures and tools, so they can reduce the cost per unit of plant production while maintaining best quality of plants.

When analyzing the composition of MS medium, basic compounds used in the media can be divided into several categories such as, Basal salts, vitamins, buffering substances and reinforcing agents. A popular fertilizer mixture Albert solution could be used for replacing the MS medium with the same nutrients, vitamins, and basic components. Albert solution is a nutrient solution that can be used in tissue culture. It is balanced with all the essential nutrients that plants need to grow. Albert solution consists of 11% N, 8% P2O5, 15% K2O, 14% MgO, Fe, Mn, B, Zn, Cu and Mo in balanced amounts. However, Albert solution doesnot contain sugar, vitamins, and hormones. Therefore, with the addition of those components, a series of experiments can be done to develop a low cost-effective basal medium to replace MS medium. The common growth regulators, benzyl amino purine (BAP) and naphthalene acetic acid (NAA) used in orchid micropropagation were replaced with coconut water in some treatments and myoinositol was replaced with Vitamin B tablets.

Orchid shoot culture

Six treatments were carried out with 6 types of media for orchid shoot culture. Full MS medium with 3% sugar, 2mg/L BAP, 0.1mg/L NAA and 100mg/L myoinositol was used as the control (T1) while other treatments were changed according to the concentration of Albert's mixture, presence of BAP and NAA hormones or coconut water and media supplied with external fertilizer-chemical mixture. Three concentrations of Unipower Albert's mixture were used as 4g/L, 6g/L, and 8g/L while another treatment was carried out with addition of fertilizer-chemical mixture to the 4g/L Albert's mixture. The required amounts of macronutrients that are deficit in Albert's mixture compared to the MS medium were calculated and added to 4g/L Albert's solution. It comprised of 3.1g/L Urea, 3.3g/L Muriate of Potash (MOP), 1.4g/L Calcium Nitrate, 0.3g/L Triple Super phosphate (TSP) and 0.14g/L Ferrous. Ferrous Sulphate tablets were crushed and put into the media as the Ferrous source. Myoinositol was replaced with 0.1g/L (1tablet per liter) Vitamin B tablet in all treatments except the MS control. 2mg/L BAP and 0.1mg/L NAA was used as the hormonal combination in all 3 concentrations of the Albert's media while one treatment was carried out by replacing BAP, NAA hormones with 150ml/L coconut water in 4g/L Albert medium. All other Albert's media concentrations were supplemented with same amounts of BAP and NAA. Subsequently, PH for all other media were adjusted 5.4 while the PH of MS was adjusted to 5.8.

250ml from each medium were prepared for all treatments. Then the required amount of agar (MS- 4.25g/L and Albert's and other media- 5g/L) was added to the medium and it was heated for 1-2 minutes at 1800C in

microwave oven until all the agar was fully dissolved. The culture jars were sterilized with CSUP technique (Peiris *et al.*, 2012) using 10% Clorox solution. Thirty milliliters of each medium were poured into jars and closed using pre-sterilized cellophanes and sealed with a rubber band. Similarly, 250ml was poured into 6 jars and the jars were kept at room temperature (25^0C) to solidify the media. Then all jars were cultured with threein vitro dendrobium shoots per jar. The number of roots per shoot was kept as 4 or 6 and the size of the shoot was 3-4cm. After completing the culturing process aseptically, cultures were placed in the growth room at 25^0C. Final data were observed after 8 weeks from culture establishment.

STATISTICAL ANALYSIS

Results were analyzed using independent sample T- test in SPSS and the significant difference was observed through p values ($p \leq 0.05$).

RESULTS AND DISCUSSION

RESULTS ON ORCHID SEED CULTURE.

Results were obtained through seed emergence from 1 to 4 months based on the color concentration of germinated seeds. Dendrobium seeds were able to germinate in all three types of medium (Table 1). Huh *et al.*, 2016 alsoreported that, the 2g/l albert's medium was the best for protocorm growth after 1/2 strength MS media.

Table 1: Seed germination performance of dendrobium orchid.

Treatment	Seed emergence results (germination)
½ MS control medium(T1)	Highest germination
2g/L Albert's medium(T2)	High germination
1g/L Albert's medium(T3)	Low germination

It gave a dark green color that was equally similar to the 1/2 MS medium while the 1g/L albert's medium gave a light green color. After 4 months of culturing, all the treatments had a positive effect on the shoot induction. Small leaf structures were beginning to arise from the shoots in ½ MS medium while it was observed to turn bright green in color. Germination of the seeds was observed in all treatments. The 2g/l albert medium also acted positive to the arising of leaf like structures while 1g/l albert medium just started its shoot induction. However, a significant color variation could be seen in all treatments. The green color concentration of the shoots was highest in MS medium and 2g/l albert medium was roughly similar but not as much as the MS medium (Figure 1). The seed coatings were torn, and the seed embryos expanded after two months in culture. After four months, they transformed into young protocorms, and meristematic domes formed at the basal regions,(Zeng *et al.*, 2014). However, the 2g/l Albert medium that replaces ½ MS medium showed better results than the 1g/l Albert medium that replaced 1/4MS medium. 1/4MS medium was replaced due to high results that occurred in a previous research (Huh *et al.*, 2016)

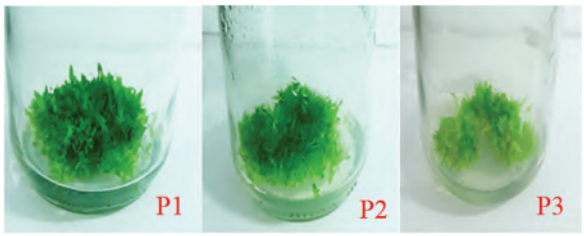

Figure 1- Seed emergence at the end of 4 months period on 3 treatments. (P1- ½ MS medium, P2- 2g/l Albert medium, P3- 1g/l Albert medium)

Orchid shoot culture

Results were observed through shoot number and dry weight. The results were taken by calculating the dry weight of 3 replicates for each treatment which had 3 explants per replicate (Table 2).

Treatment	Dry weight	Shoot number	Prescence of dead parts
	Mean dry weight	P-value	Mean shoot number
Full MS control (T1)	0.1483	-	16.6

High concentrations of Albert fertilizer medium resulted in high shoot growth, since Albert mixturelacks some macro nutrients where the 4g/L Albert medium showed less shoot number. Number of shoots per explant were observed through visual observations (E. P. Venkatasalam, 2013). The highest mean number of shoots (17.66) were observed in 8g/l Albert medium that was formulated with 2.0mg/l BAP and 0.1mg/l NAA, while 6g/l Albert medium demonstrated a similar mean shoot number (16) to the MS medium with the same hormonal composition. (16.66)

When results were compared based on the dry weights of the shoots, the dry weight increased with number of leaves, shoots, and shoot length by stimulating cell division and elongation through nutrient (Peres, Majerowicz, & Kerbauy, 2001). Fresh weight of shoots wasweighed using an electronic balanceand subsequentlyshoots were maintained at 60°C for 4-5 days, or till the dry weights for each replicate were constant. Three replications per treatment and three explants per replication were manipulated in the experiment's design. Data were gathered after aneight-week culture period. Dry weights were measured in grams and highest mean dry weight(0.1790) was observed in 8g/l Albert medium while the 6g/l Albert medium(0.1474) showed nearly similar mean dry weight to the MS control medium(0.1483) (Peres *et al*., 2001). The 8g/l Albert medium and MS medium was significantly different ($p \leq 0.05$) and there was no significant difference between 6g/l Albert medium and MS control medium. When consideringshoot number, highest mean.

Shoot number was obtained from 4g/l Albert medium (14.33) compared the 4g/l Albert + Fertilizer- Chemical mixture medium (11). This may have been caused due to toxicity or nutrient imbalance of the media (Fernando & Subasinghe, 2007). Additional fertilizers may have provided nutrients in a different proportion than the plants required.

Results revealed that number of shoots observed from treatments that used BAP and NAA as hormone combinations were higher when compared to treatments with coconut water. One of the 4g/l Albert mediums wassupplemented with coconut water for testing the efficiency of coconut water to replace hormones. Shoot number was decreased when adding coconut water as hormones.(Riva, 2016) However, some of media were contaminated with fungus and bacteria when the media was supplemented with coconut water. The main objective of this research was to find a low-cost medium to replace MS medium in Orchid Dendrobium tissue culture. Hence, it is crucial to discuss costs and compare them with MS media. However, results obtained indicated that all 5 treatments except MS control have different costs as they consisted of different types of components. But results showed that the best media to replace MS media is 6g/l medium with BAP, NAA hormones.

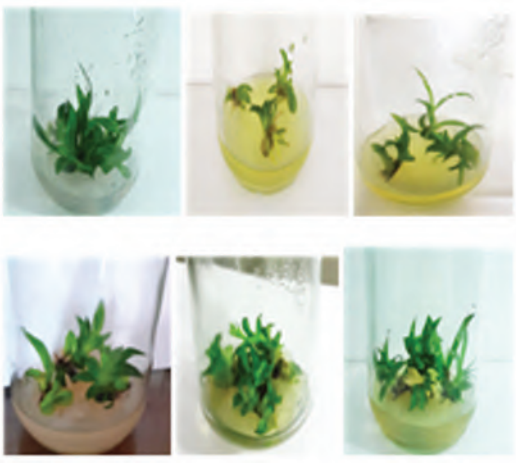

Figure 2- Shoot growth of all treatments at 8 weeks. (T1- Full MS, T2- 4g/l Albert+ Coconut water, T3- 4g/l Albert+ Hormone, T4- 4g/l Albert+ Fertilizer-chemical +Hormones, T5- 6g/l Albert. T6- 8g/l Albert media)

CONCLUSION

Since this research is based on cost reduction in tissue culture, low-cost media with different concentrations and contents were tested. Results were obtained based on the morphology, shoot number and dry weight. The highest orchid seed germination was observed on 2g/L Albert medium than 1g/L Albert medium and showed nearly similar germination to the MS media. Dry weight and shoot number in 8g/L Albert medium were higher than the MS medium while 6g/L Albert medium showed nearly similar results compared to MS medium, with high quality shoots without any deterioration. The media supplied with a Fertilizer- Chemical mixture which had BAP, NAA hormones showed a higher dry weight than 4g/l Albert medium while showing low quality and high deterioration. Hence, the 6g/l Albert medium may be considered the best among all treatments for shoot number, dry weight, and the good quality of shoots. The total cost for 1l MS medium was about Rs.2800 and the total cost of 1L Albert's media for all treatments tested was only about Rs. 20- 40. Thus, it could be concluded that MS medium could be replaced by low-cost Albert medium.

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Sterilization of *Anubias barteri* for *in-vitro* Culture

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ABSTRACT

Anubias barteri Schott is an exotic ornamental aquatic plant species native in Africa and has a high economic value. Apart from its potential economic value, there are many obstacles in the growing of Anubias. One of them is related to the propagation of Anubias plants due to their slow growth rate. The aim of this study was to develop a sterilization process for *Anubias barteri* to be propagated through in vitro culture. Plants brought in to the laboratory from their growing habitat are very vulnerable to microbial contamination from bacteria and fungi. By employing the proper sterilizing agents, those contaminants can be removed. In this study, three different concentrations of mercuric chloride ($HgCl_2$), 0.1%, 0.2%, and 0.3% were used to dip rhizome segments of *Anubias barteri* for 3 minutes and 0.05%, 0.1%, and 0.1% were used to dip leaf segments of *Anubias barteri* for 3 minutes. Percentages of explants that survived were observed in 5-day intervals up to 25 days. *Anubias barteri* rhizome segments were successfully established with $HgCl_2$ 0.2% while leaf segments of *Anubias barteri* were successfully established in $HgCl_2$ 0.1%. Keywords - Dendrobium, Albert's solution, Low-cost media.

Keywords : *Anubias barteri*, apical bud, leaf explants, survival percentage

INTRODUCTION

Anubias barteri is a popular aquatic plant that is commonly used in aquariums and aquatic landscapes. They are native to Africa and belong to the family Araceae. Anubias plants are highly valued for their attractive and durable foliage, making them a favorite choice among aquarists. Anubias plants have broad, dark green leaves that are often heart-shaped or lanceolate (Carter *et al*., 2011). The leaves are thick and leathery, which helps them withstand conditions in aquariums Anubias plants are slow-growing, which makes them suitable for low-maintenance aquarium setups. They can grow both submerged and partially emerged. Anubias plants can adapt to a wide range of lighting conditions, from low to moderate intensity. However, they should be protected from direct, intense light, which can cause algae growth on their leaves. Anubias plants prefer slightly acidic to neutral water with a pH range of 6.0 to 7.5. The water temperature should be maintained between 22°C to 28°C. They can tolerate a variety of water hardness levels. Besides, Anubias plants are best attached to rocks, driftwood, or other decorations in the aquarium. Anubias plants have relatively low nutrient demands (Christensen, 1996).They can derive nutrients from the water column and not necessarily require substrate fertilization. However, they can supplement their growth with liquid fertilizers or root tabs containing essential nutrients like iron and potassium. Anubias plants can thrive in tanks without the addition of carbon dioxide. They can obtain carbon dioxide from the atmosphere and do not heavily rely on carbon dioxide injection like some other aquatic plants. *Anubias barteri* plants are also compatible with a variety of fish and invertebrates. However, care should be taken to ensure they are not eaten or uprooted by herbivorous or digging species

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(Carter *et al*., 2011) These plants are generally low-maintenance, requiring trimming of yellowing or damaged leaves regularly to maintain their aesthetic appeal. They may also benefit from occasional cleaning to remove algae or debris from their leaves. With proper care and suitable conditions, they can add a touch of natural beauty to an aquarium or aquatic setup. Thus Anubias are considered to be a high potential plant by many aquarists plant to maintain in their setup, since the light and nutrient requirements are very low. When growing these plants, existing plant propagation methods shows less responses and takes long time. Several reports have revealed that efforts had been undertaken to reproduce *Anubias barteri* plants through micro propagation techniques. Conventional methods are not widely used. However, *Anubias barteri* plant from its existing habitat are highly exposed to microbial contamination such as bacteria and fungi.

Hence, these contaminants need to be eliminated by using suitable sterilizing agents, for the success of micro propagation techniques.

MATERIALS & METHODS

Healthy apical buds and leaf of developed plantlets of Anubias were obtained from the tank of aquariums and used as the source of explant for the study.

Experiment 1: Surface Sterilization using Sodium Hypochlorite

Rhizome segments of *Anubias barteri* were washed with tap water and dipped for 12 hours in a fungicide (1.4 g/l). Subsequently explants were washed with 70% ethanol for 30 seconds. Then they were disinfected with different concentrations of NaOCl solutions as 0.0%, 5%, 20%, 15%, 10% for 20 minutes and rinsed with sterile distilled water three times. 3-5 mm sterilized apical buds were placed in Murushige and Skooge medium.

Leaves of *Anubias barteri* were washed with tap water with 2% detergent for 20 minutes. Then the leaves were washed with 70% ethanol for 30 seconds. Subsequently they were disinfected with different concentrations of NaOCl solutions as 0.0%, 5% , 10%, 15%, 20% for 20 minutes and rinsed with sterile distilled water three times. 1cm x 1cm leaf segments were placed in Murushige and Skooge medium.

Experiment 2: Rhizome segments of Anubias barteri were washed with tap water and dipped for 12 hours in the fungicide Captan (1.4 g/l). Then the explants were washed with 70% ethanol for 30 seconds. Finally they were disinfected with different concentration of $HgCl_2$ aqueous solutions as 0%, 0.1% , 0.2% , 0.3% three time and rinsed with sterile distilled water thrice. Sterilized apical buds were placed in Murushige and Skooge medium.

Leaves of Anubias were washed with tap water and 2% detergent for 20 minutes. Subsequently leaves were washed with 70% ethanol for 30 seconds. And were then disinfected with different concentrations of $HgCl_2$ aqueous solutions as 0%, 0.05%, 0.1%, 0.15% three time and rinsed with sterile distilled water thrice. 1cmx 1cm leaf segments were placed in Murushige and Skooge medium.

The experiment was arranged in a Complete Randomized Design (CRD) with 30 replicates per treatment. Data were subjected to statistical analysis of one-way ANOVA with Minitab 17 statistical package. Numbers of contaminated explants were counted at five days interval up to 25 days after establishment of explants. The following calculation was done to obtain survival percentage of explants after five days interval up to 25 days of establishment of explants.

$$\text{Survival Percentage (\%)} = \frac{\text{Total number of survived plantlets}}{\text{Total number of plantlets}} \times 100$$

RESULTS AND DISCUSSION

All explants that were treated with different concentrations of Sodium Hypochlorite were contaminated within 10 days after they were initiated to the MS medium.

Table 01: Survival percentage of rhizome segments sterilized using Mercuric Chloride

Treat ment	Concentration on of solution of Mercuric Chloride	Survival percenta	P value
1	0%	19.3	0.000
2	0.1%	48.00	
3	0.2%	80.67	
4	0.3%	48.0	

Table 02: Survival percentage of leaf segments sterilized using Mercuric Chloride

Treat ment	Concentration on of solution of Mercuric Chloride	Survival percenta	P value
1	0%	18.67	0.000
2	0.5%	43.33	
3	0.1%	78.00	
4	0.1%	42.00	

Survival percentages of rhizome segments of Aubias was significantly different at 0.05 significant level (Table 1). 0.2% Mercuric Chloride was the most effective concentration for surface sterilization of Anubias rhizome segments. This showed the highest survival rate after one month of induction to MS media.

Survival percentage of Aubias leaf explants was significantly different at 0.05 significant levels (Table 2). 0.1% concentrated Mercuric Chloride was the most effective concentration for surface sterilization of Anubias leaf segments. This showed the highest survival rate after one month of induction to MS media.

Explant contamination in plant tissue culture can be due to the presence of unwanted microorganisms, such as bacteria, fungi, or viruses, in explants that are being cultured in a controlled environment for different purposes. Contamination can severely affect the success of tissue culture experiments and lead to compromised results or the loss of valuable explant material. Contaminations in plant tissue culture can cause a significant effect on expected results. When establishing tissue cultures, depending on the explant used, surface and endophytic microorganisms could be carried over into culture media. Explants obtained from somatic tissues such as shoot tips normally have a greater chance for contamination either by bacteria, fungi or yeasts (Dolezel *et al* .,1998)

CONCLUSION

Results of this study indicate that Mercuric Chloride can be utilized as an effective disinfectant for Anubias explants. Anubias rhizome segments can be successfully surface sterilized with the minimum contamination percentage when treated with Mercuric Chloride at 0.2% for 3 minutes and leaf explants treated with Mercuric Chloride at 0.1 % for 3 minutes. These finding suggest that Mercuric Chloride is more efficient than Sodium Hypochlorite for surface sterilization of Anubias. Therefore, future studies are needed to be conducted with different concentrations for sterilization of Anubias plants along with subsequent growth performances *in-vitro*.

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In-vitro establishment of *Microsorum pteropus*

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ABSTRACT

Java fern (*Microsorum pteropus*) is a popular aquarium plant species with many diverse geographical varieties used for aqua scaping. Large scale multiplication of high-quality M. pteropus using micropropagation will pave the way to increase production. Hence this study was conducted to develop a method for *in vitro* establishment using leaf and rhizome explants. The most effective surface sterilization method was observed in leaf explants soaked in 70% Iso propyl Alcohol (IPA) for 30 seconds and 0.01%mercuric chloride ($HgCl_2$) for 1 minute where the explants were cultured in Murashige & Skoog (MS) liquid medium without sucrose. Leaf explants were also experimented on MS medium supplemented with 0.125, 0.25, 0.5 and 1mg/L 2,4- Dichlorophenoxyacetic acid (2,4-D) combined with 0.125, 0.25, 0.5 and 1mg/L A-Naphthaleneacetic acid (NAA) as an initiation media without sucrose for regeneration. Leaf explants survived without contamination in 0.125 mg/l 2,4-D + 0.125 mg/L NAA (57.14%) and 0.25 mg/L 2,4-D and 0.25 mg/L NAA (14.28%) containing media and produced shoots. Rhizome explants were sterilized using 70% IPA for 30 seconds, 10% Clorox in 10 minutes, and 0.01%HgCl2 in 1 minute, and cultured in Murashige & Skoog medium supplemented with 6- Benzyl aminopurine (BAP), A -Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) and N^6-(2-Isopentenyl) (2IP) and 10 ml/L Myoinositol. Explants survived in media supplemented with BAP, 2IP, and myoinositol at 2mg/L, 0.5 mg/L and 10 mg/L, respectively and media containing 1.25 mg/L in each BAP, 2,4-D and NAA. After 8 weeks rhizome explants did not have considerable contaminations and survived with this surface sterilization method and initiation medium combination.

Keywords: *Microsorum pteropus*; In-vitro establishment; Aquatic fern; mercuric chloride

INTRODUCTION

Microsorum petropous is a native species of fern which has become more popular in the horticultural sector because of its attractive foliage and low maintenance requirements to improve the aesthetic appeal of freshwater aquariums. It is widely used in aqua scaping. *Microsorium petropous*, a popular aquatic plant in the Polypodiaceae family, is also referred to as Java fern. This plant species is extensively dispersed throughout tropical Southeast Asia, including the Philippines, Thailand, Malaysia, and some areas of China. It originated on the Island of Java in Indonesia. This plant grows fully or partially submerged in freshwater streams and rivers, coastal brackish areas, and tropical rainforests. M. pteropus cultivars have erratic growth terminations and accidental blade dissections in their leaves (Sripinyowanich *et al*., 2021). They are semi-aquatic and in high demand as aquarium plants and ornamentals. The plant has rhizomatous growth. The rhizome spreads its roots and leaves as it grows horizontally. Their widespread application in infection and wound healing has been supported by reports of their antioxidant and antibacterial properties. SNPs were synthesized using a plant extract from M. pteropus, and their antioxidant properties were examined *in vitro* with an eye

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toward their therapeutic use as nanoparticles (Chick *et al*., 2020).

The development of more effective procedures for micropropagation is still crucial to modern biotechnology because these methods have numerous benefits. The tissue culture method is appropriate for wider use since it can produce a large number of strong, disease and virus-free, genetically homogeneous plants. Many aquarists value the bushier growth and more adventitious shoots that tissue cultured water plant species exhibit. Few studies have been done on the *in vitro* propagation of ferns using donor explants such as leaves and rhizomes. This species can be reproduced and conserved in an environment that is regulated and repeatable by *in vitro* establishment. This method allows for the production of a large number of pathogen-free individuals from small explants, as well as the ability to regulate light and temperature, prevent seasonal variations, and alter the amount of nutrients and growth regulators used. (Phillips and Garda, 2019d).

Sterilization is necessary to prevent and minimize contamination because maintaining sterile conditions is crucial for many *in vitro* culture operations. (Gammoudi *et al*., 2022). Sterilization is crucial for maintaining sterile conditions in *in vitro* culture activities, and chemical solutions like calcium hypochlorite ($Ca(OCl)_2$), sodium hypochlorite (NaOCl), Mercuric chloride ($HgCl_2$) and hydrogen peroxide (H_2O_2) are commonly used as disinfectants. These chemicals can remove contaminations without endangering plant cells. (Mihaljevic *et al*., 2013).

Plant tissue culture is the aseptic and controlled growth and multiplication of plant cells, tissues, and organs on specified solid or liquid medium. (Kumar and Loh, 2012b) MS medium was used as basal media to assess the best sterilization process for this plant species. Major hormones employed in this experiment, at varying quantities, are 2,4-D, NAA, BAP, Myoinositol and 2IP using leaf and rhizome explants. This was done to determine the ideal circumstances for the *in vitro* development of Microsorum petropous. The goal of the current study was to create an effective and dependable *in vitro* establishment technique for Microsorum petropous that included an appropriate surface sterilization procedure.

MATERIAL AND METHODS

Plant material and explant preparation

Plant materials were collected from healthy and disease-free specimens of Microsorum petropous. Healthy mother plants were supplied by the Ruvini aqua plants Pvt.Ltd. Healthy and disease-free rhizomes were collected from mature mother plants (60 days old).

Leaves were initially washed with tap water to eliminate excess soil particles and subsequently transferred to a beaker filled with sterile distilled water. A gentle cleansing was carried out with soap and water, using a soft brush, ensuring no damage to leaves. These leaves, exhibiting a healthy green color, were obtained for initiation. Following this, a 70% alcohol rub for 30 seconds with a soft brush was performed, succeeded by another round of washing with sterile distilled water. Subsequently, the leaves were carefully cut into small explants of approximately 2-3 cm in length and transferred to a beaker with sterile distilled water. These explants were then kept for one-hour in running tap water. Further, a surface sterilization process was initiated using a 0.2% (w/v) fungicide (Mancozeb™) solution at a concentration of 20 mg/L for 15 minutes, with the cleaned explants placed in jars. In a parallel procedure, rhizome explants

were treated with an overnight immersion in the fungicide solution. They were cut into small segments of about 1-2 cm in length. The rhizome explants were then thoroughly washed with tap water to remove surface debris and soil particles, followed by transfer to a beaker with sterile distilled water. A gentle washing, with Vim™ liquid soap utilizing a soft brush, was conducted to remove excess debris including small root parts and tips. Subsequently, a 70% alcohol rub for 30 seconds was applied to the rhizome explants, followed by a thorough washing with sterile distilled water. The jars containing the explants were then exposed to running tap water for 1 hour, and finally, the explants were treated with the same 0.2% (w/v) fungicide (Mancozeb™) surface sterilization process as described earlier, with the cleaned explants added to the jars.

Sterilization of explants

In the laminar floor, leaf explants were washed with few drops of liquid soap in 100ml sterile distilled water, they were agitated vigorously by hand for 10 mins. Subsequently explants were rinsed with sterile distilled water to remove soap. This step was repeated 3 times, each for 2 minutes duration. Afterwards explants were sterilized with 70% IPA for 30 seconds and rinsed with sterilized distilled water trice for 2 minutes each. In another treatment explants were sterilized as described before and finally immersed in a 0.01% $HgCl_2$ solution for 1 minutes followed by washing with sterile distilled water trice for 2 minutes each to remove any traces of the sterilizing agent.

Rhizome explants were sterilized with 2- 3 drops of Vim™ liquid soap in 100ml of sterile distilled water for 10 minutes. Subsequently explants were washed with sterilized distilled water trice for 2 minutes each to remove excess soap. Afterwards explants were sterilized with 70% IPA for 30 seconds and rinsed with sterilized distilled water trice for 2 minutes each. Explants were then sterilized with 10% Clorox for 10 minutes. They were rinsed with sterilized distilled water again 3 time for 2 minutes each. Finally, explants were sterilized with 0.01% $HgCl_2$ for 1 minutes. Subsequently explants were washed with distilled water trice 3 for 2 minutes each.

Preparation of Culture medium

Using the successful sterilization method leaf explants were cultured using the liquid formations of full-strength MS medium, (Murashige and Skoog, 1962) with 3% sucrose and without sucrose in order to screen the appropriate conditions of MS media on the in-vitro establishment of M. petropous. After four weeks, they received further supplements containing varying amounts of plant growth regulators (PGRs). As a control, medium devoid of PGR was employed. The MS medium was supplemented with 4 concentrations (0.125,0.25,0.5, and 1 mg/L) of NAA and 2,4-D each for leaf explant initiation in liquid media formation with and without sucrose.

Rhizome explants initiation was carried out with MS medium supplemented with BAP, 2IP and Myoinositol concentrations of (2, 0.5, 10mg/L) with 3% sucrose and without sucrose. Subsequently BAP, 2,4-D and NAA were used in concentrations of 0.25,0.5, and 1.25 mg/L which created 18 treatments in MS medium (Murashige and Skoog, 1962) with 3% sucrose and without sucrose. Hence there were 20 treatments in this experiment. Medium pH was adjusted to 5.6 and 0.425% agar was used as the gelling agent. The medium was boiled using a microwave oven until agar was completely dissolved. The medium was dispensed into appropriately coded culture tubes, and then it was autoclaved for 15 minutes at 121 ^{0}C and 100 kPA of pressure. Subsequently, culture

tubes were tightly closed and maintained at room temperature until culturing of explants. A number of 7 culture tubes for leaf explants and 14 culture tubes for rhizome explants were prepared for a hormone combination (30 mL/tube).

Evaluation of cultures

Explants were transferred to a sterile culture medium under aseptic conditions. Cultures were maintained under controlled environmental conditions to promote growth and proliferation of shoots. Cultures were regularly monitored for contaminations, then contaminated and unhealthy samples were discarded. Plantlet regeneration percentage, and number of regenerated plantlets were identified for the final results.

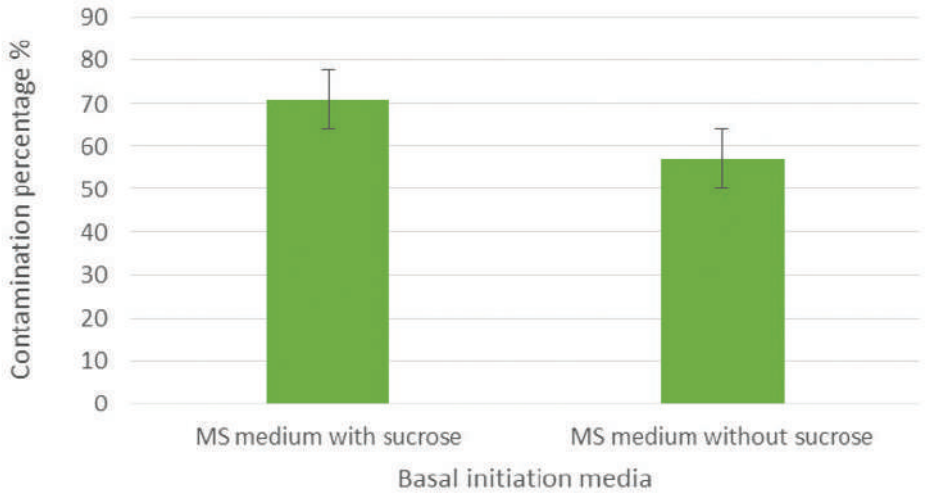
Experiment on leaf explants

The most effective surface sterilization method was observed for leaf explants soaked in 70% IPA for 30 seconds and 0.01% mercuric chloride ($HgCl_2$) for 1 minute where the explants were cultured in Murashige & Skoog (MS) liquid medium without sucrose.

Leaf explants after 4 weeks of culturing in MS medium with 3% sucrose and without sucrose revealed distinct contamination patterns, emphasizing the critical role of growth media formulations and sterilization methods. After four weeks MS medium with sucrose exhibited a contamination rate of 71%, whereas the MS medium without sucrose had a contamination rate of 57%. (Figure 1).

Figure 1: Results indicating contamination percentage of leaf explants with basal media after 4 weeks. (n=7)

RESULTS AND DISCUSSION



Leaf explants in 0.5mg/L and 1mg/L 2,4-D and NAA did not show any explant growth and had 100% contamination (Table 1), indicating challenges in tissue development under these conditions.

Explants in 0.25mg/L concentration demonstrated limited success with 14% survival rate but was hindered by a substantial contamination rate (86%). In contrast, 0.125mg/L presented a more favorable scenario with 57% explant survival and a significantly reduced contamination rate of 43%. According to these results, the medium containing 0.125mg/L of 2,4-D and 0.125 mg/L NAA is most appropriate for *in vitro* establishment. All surviving explants regenerated shoots within two months (Figures 2 to 5).

Table 1: Survival rate after eight weeks of initiation of leaf explants with 4 concentrations of NAA and 2,4-D in MS liquid medium without sucrose

Initiation media	Survival percentage (%)
1 mg/L 2,4-D+1 mg/L NAA	0
.25 mg/L 2,4-D + 0.25 mg/L NAA	14.28
0.125 mg/L 2,4-D + 0.125 mg/L NAA	57.14
.5 mg/L 2,4-D + 0.5 mg/L NAA	0

N= 7

All Treatments were not mentioned here and lowest contamination media treatment were added here with survival rates.

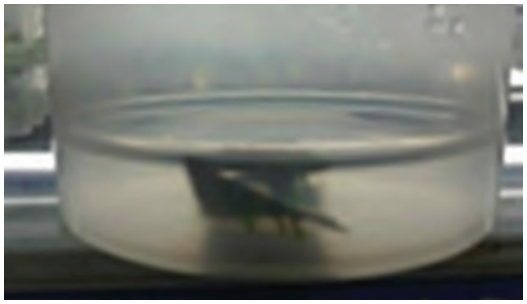


Figure 2: Leaf explant growth without contamination

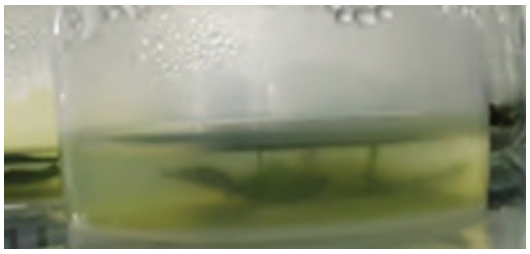


Figure 3: Leaf explant growth without contamination

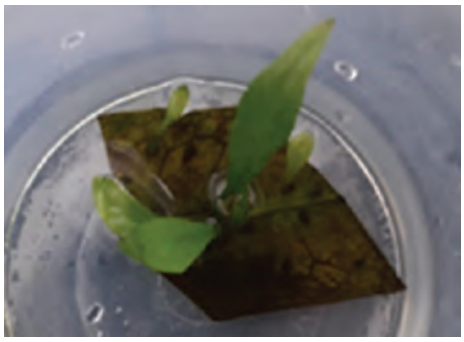


Figure4: Leaf explant growth without contamination

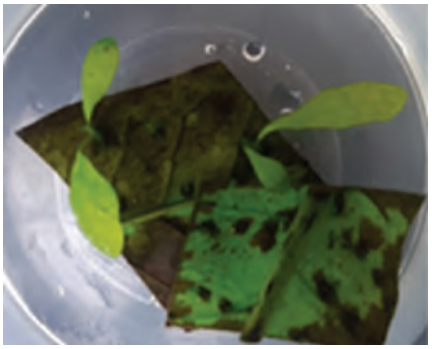


Figure 5: Leaf explant growth without contamination

Experiment on Rhizome explants

After two months of initiation, out of 20 media (treatments) tested for rhizomes only 4 media produced uncontaminated cultures. MS medium supplemented with BAP, 2IP and Myoinositol at concentrations of (2,0.5,10mg/L) with 3% sucrose had 35.72% survival rate and the same medium without sucrose showed 21.43% survival. Rhizomes showed comparatively high survival rate 42.86% in the MS medium supplemented with BAP, 2,4-D and NAA each at 1.25mg/L with sucrose in semi- solid media condition. The same medium without sucrose produced 28.58% survival rate (Table 2, Figure 2).

Table 2: Survival rate after two months of initiation of rhizome explants

Media	BAP mg/L	2,4-D mg/L	NAA mg/L	2IP mg/L	Myoinositol	Sucrose	Survival percentage (%)
Treat 1	2	-	-	0.5	10	0.03	35.72
Treat 2	2	-	-	0.5	10	-	21.43
Treat 7	1.25	1.25	1.25	-	-	0.01	42.86
Treat 8	1.25	1.25	1.25	-	-	-	28.58

N=14

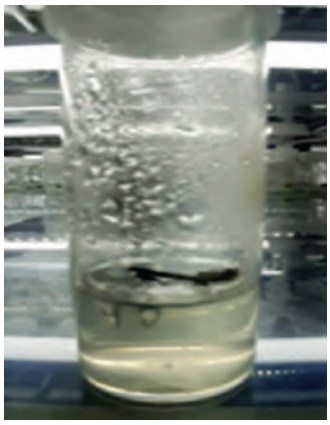


Figure 2: Rhizome explant growth without contamination after 8 week

In this research contamination and explant browning percentages as well as percentage plantlets regeneration were checked periodically to search for any changes that might have happened during incubation of two months following the start of the culture initiation. Rhizome explants become brown due to phenols in the tissue due to oxidizing. Reduction of cell division and explant regeneration potential due to browning could result in the inability to regenerate tissue cultured plantlets. Explants showed a range of growth abnormalities, including delayed needle expansion, browning of the tips, browning of the explants' sides *et al.,* as well as the medium, vitrification, and even mortality. (Permadi *et al*., 2023) The majority of these findings came from the experiment carried out with 0.01% HgCl2.

With the exception of cryptic contaminants, most fungal contaminants were apparent from the very beginning of the rhizome *in vitro* culture. These pollutants usually cause suppressed plant growth and frequently outcompete them for resources in the nutrient media. As a result, *in vitro* pollutants frequently negatively affect the survival and growth of cultures. Bacterial contamination is a serious problem that causes many plants, including leatherleaf fern, to drastically fail *in vitro* cultures (Kulkarni *et al*., 2007). Chemicals that eliminate all pathogens, including fungal spores, from plants are known as surface sterilants. Surface sterilants typically act on the explants' exteriors. They inhibit the development and spread of pathogens, which include bacteria, fungi, and other microorganisms. Plants can naturally produce growth hormones, however in tissue culture additional growth hormones must be added in order to promote greater development and enhance the synthesis of metabolites, particularly plant bioactive compounds. Auxin and cytokinin combinations are more beneficial for shoot organogenesis at equal ratios (1:1) or even lower, according to data from earlier studies. The present study aimed to test two combinations of PGRs at equal ratios of NAA,2,4-D and BAP at different absolute concentrations. The results indicated that the medium concentration (1.25 mg/L NAA+1.25 mg/L BAP+1.25 mg/L) demonstrated a much faster and more efficient rate than other concentration in rhizome explants. Auxins have been shown to have inhibitory effects on shoot organogenesis *in vitro*, owing to their role in apical dominance (George, Hall and Klerk, n.d.). In this investigation, a combination of 2,4-D and NAA in the media effectively generated Plantlets. An increase in the number of roots was seen upon the addition of auxin, NAA, or 2,4-D. Considering about the leaf explant cultures, highest contamination percentage was recorded in media with sucrose microbiological contamination and plant growth are highly influenced by sucrose in the medium for plant tissue culture. Correct growth and development depend on maintaining the right concentrations of sucrose, but too much sucrose can encourage the growth of microorganisms. (Lievens *et al*., 2014) Achieving tissue culture success requires balancing sucrose concentrations, following stringent aseptic procedures, keeping on growth, and figuring out possible

contamination sources. The conditions for tissue culture can be further optimized by modifying the medium composition according to the needs of individual plant.

CONCLUSION

Leaf explants were successfully surface sterilized with soaking in 70% IPA for 30 seconds and 0.01%mercuric chloride ($HgCl_2$) for 1 minute where the explants were cultured in Murashige & Skoog (MS) liquid medium without sucrose. Medium containing 0.125mg/L of 2,4-D and 0.125 mg/L NAA reported highest survival rate of 57.14%. All surviving explants regenerated shoots within two months. Rhizome explants did not have considerable contaminations and survived with this surface sterilization method and initiation medium combination after two months.

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In –vitro Establishment of *Versicularia dubyana*

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ABSTRACT

Versicularia dubyana, also known as java moss, is a widely used aquarium plant for aqua-scaping and a highly variable plant species with numerous geographical variations. In order to meet the demand for high quality plants micropropagation techniques can be applied if a protocol is optimized. Therefore, this study was carried out with the objective of *in-vitro* establishment of *Versicularia dubyana* as the first step to develop an optimized and standardized *in vitro* propagation protocol. Leaves from plant clumps were used as the explant in experiments for surface sterilization and regeneration. In this study Isopropyl alcohol (IPA), Clorox, and hydrogen peroxide (H_2O_2) were used as the main sterilization agents with several experiments. Murashige and Skoog (MS) medium with 3% sugar and without sugar and half -MS medium with 3% sugar and without sugar were used as the basal media. Plant growth regulators 1 –Naphthaleneacetic acid (NAA), 2, 4- Dichlorophenoxyacetic acid (2, 4-D), 6 –Benzylaminopurine (BAP) and 6 – (ɣ, ɣ -Dimethylallyl amino) Purine (2IP) were used with several combinations as treatments to find the best initiation media. Successful survival and growth were observed in comparatively high number of cultures (80%) when leaf explants were dipped in 3% hydrogen peroxide for 1 minute and cultured in full strength MS medium containing 0.5ml BAP, 0.5ml NAA, 0.5ml 2, 4-D hormones without sugar.

Keywords : *Versicularia dubyana*, Java moss, *in vitro* establishment, Micropropagation, Tissue culture

INTRODUCTION

Mosses are a kind of small, non-vascular plant that are a part of the Bryophyta division. The popular aquatic plant species known as Java moss, or *Versicularia dubyana* (*Taxiphyllum barbieri*), is in high demand in the aquarium trade. "Java moss" (*Versicularia dubyana*) was the original tropical aquarium moss. *Versicularia dubyana* can grow even in low light, but it looks best in at least medium lighting with a good supply of nutrients and CO_2.

Versicularia dubyana (*V. dubyana*) was known as an easy-going aquarium moss. Though it's slower growth can be advantageous, *V. dubyana* has a more ornamental appearance due to its finely ramified branches. It looks best tied onto driftwood or rocks, and it is easy to keep its form with regular trimming. Although it does not require much light, it does ramify better in at least moderate lighting. The spore capsules on V. dubyana's reddish-brown stems add to the stony forest moss-like habit of the plant. This moss grows in submerged cultivation as well. (Siregar *et al*., 2023)

The aseptic and controlled growth and multiplication of plant cells, tissues, and organs on a designated solid or liquid medium is known as plant tissue culture. (Harshal and Bhoite, 2014). Tissue culture and micropropagation techniques are superior to conventional methods in several ways. These advantages include the capacity to produce large numbers of plants quickly throughout the year, produce true-to-type planting

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materials, bulk up recently released varieties quickly, propagate new varieties quickly, produce uniform, healthy, stress-resistant, and pathogen-free planting materials, and stop the spread of pests and diseases. Large populations of rare plants can be produced using plant tissue culture, commonly referred to as *in-vitro* culture, a technique that preserves the natural habitat of the plants by cultivating them in aseptic conditions.

In-vitro establishment of *V. dubyana* moss offers numerous benefits, including efficient propagation, disease-free stock production, and potential genetic manipulation. Its lush, green appearance and versatile growth habit make it a sought-after choice for aquarists seeking to enhance the aesthetic appeal and ecological balance of their tanks. Traditionally, the propagation of *Versicularia* moss involves manual division and transplantation of established plants. However, *in-vitro* establishment techniques have gained increasing attention due to their potential advantages, including efficient mass propagation, disease-free stock production, and opportunities for genetic manipulation. (Yong Hu *et al*., 2023). Plant cells, tissues, or explants are grown under sterile circumstances in a controlled laboratory setting as part of *in-vitro* establishment. Versicularia moss plants are intended to grow and develop *in-vitro* by being provided with the best nutrient media, growth regulators, and ambient conditions. This method not only provides for the quick production of many uniform and healthy plantlets, but it also opens new possibilities for plant enhancement and biotechnological applications.

However, a major contributor to plant losses is contamination with microorganisms such as bacteria, fungi, viruses, and yeast. *In-vitro* culture activities require sterilization to maintain sterile conditions, and chemical solutions such as hydrogen peroxide, sodium hypochlorite, and calcium hypochlorite are frequently used for sterilization. (AM, 2014). Only the pollutant is removed, leaving the living material intact and capable of biological activity. A disinfectant solution is applied to the surfaces of the transplants at an appropriate concentration and time. Sustaining aseptic conditions is essential to avoid contamination and guarantee the experiment's success. (Pereira *et al*., 2021). In this study, chemicals such as hydrogen peroxide (H_2O_2), isopropyl alcohol (IPA), and Clorox (sodium hypochlorite solution) were frequently employed for sterilizing purposes. IPA is frequently used to sanitize instruments, containers, and work surfaces. Concentrations between 70 and 100 percent are frequently employed; 70% IPA is a well-liked option due to its superior uptake by microbiological cells (Mashiguchi *et al*., 2011). It is possible to utilize H_2O_2 in moss tissue culture as a disinfectant. Prior to being cultivated, plant materials are frequently sprayed or dipped in it. The main objective of this study was to develop optimized and standardized protocols for the *in-vitro* propagation and establishment of *Versicularia* moss.

MATERIALS AND METHOD

Preparation of Medium

Murashige & Skoog (1962) medium, supplemented with 6.5g agar, and without agar supplemented with 0.5ml 1 – Naphthaleneacetic acid (NAA), 0.5ml 2, 4-Dichlorophenoxyacetic acid (2, 4-D) 0.5ml benzyl amino purine (BAP), 5µL of the fungicide (Mancozeb) was prepared, and pH adjusted to 5.6. The most important point is that this medium was a sucrose – free medium. Then the medium was boiled using a microwave oven until agar was completely dissolved. The medium after being dispense in suitably

coded and labeled culture tubs was autoclaved for 15 min at a pressure of 100kPa and at a temperature of at 121°C. Subsequently culture tubes were tightly closed and maintained at room temperature until culturing of explants.

Surface sterilization of *Versicularia dubyana* explants

Healthy and disease-free *Versicularia* moss samples were selected for *in-vitro* propagation. Explants were washed gently under running tap water to remove dirt and debris. Damaged or infected portions of the moss were trimmed using a sterile scalpel. Well matured green leaf parts that are not discolored or faded were used as explants. Then they were agitated with distilled water (100 ml) with two drops of liquid soap. Subsequently, washed leaves were kept under running tap water for 30 minutes. They were then transferred into an autoclaved distilled water jar (100 ml) and 0.1% (W/V) fungicide (Mancozeb™) and left for 10 minutes. These leaves were divided into four portions and exposed to 3 and 5% Clorox™ for 1 minute and 3 and 5% hydrogen peroxide solutions for 1 minute separately as four treatments. This sterilization was carried out in the laminar air flow cabinet. Soon after leaves were washed with autoclaved distilled water. This was repeated six times for two minutes each. In this research 12 treatments were carried out as 12 media combinations. They were tested with factors such as sugar content (with 3% sugar and sugar free), and several plant growth regulator combinations (Figure 1),

Culturing of Explants

Sterilized moss samples were carefully transferred into the relevant liquid or semi-solid culture medium using sterilized forceps. Then culture tubes were closed and sealed with appropriate closures to maintain sterility. Inoculated culture tubes were placed in a climate-controlled growth room. Suitable lighting conditions were provided using LED lamps. A temperature of 22-26°C was maintained during the light period. Relative humidity levels of around 70-80% were ensured. The cultures were regularly monitored for contamination, and any contaminated or unhealthy samples were discarded. Finally, healthy and actively growing moss fragments were periodically sub cultured onto fresh culture medium to promote continuous growth.

RESULTS AND DISCUSSION

The media that were successful and that produced the least contaminations were all sugar free media. According to Sabovljevic *et al*, (2005) mosses were contaminated very quickly, the use of sugar-free medium for their *in-vitro* establishment process has often been successful. Moss plants in their natural habitat typically do not have access to high concentrations of sugars. Using sugar-free media can help replicate the natural conditions and microenvironment of mosses. High sugar concentrations in media can promote the growth of undesirable microorganisms. Sugar-free media may be used to reduce the risk of microbial contamination, which can be crucial for experiments involving the establishment of axenic (microbe-free) moss cultures.

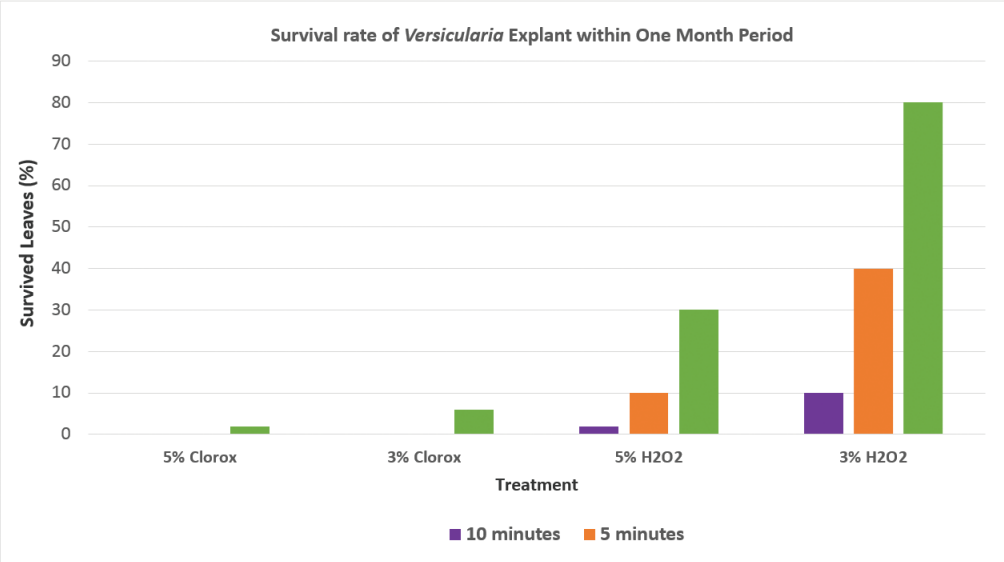

Figure 1: Survival Percentage of Versicularia Explant within One Month Period

The treatment using 3% H_2O_2 was successful in producing contamination free cultures (80%). Plant clump explants had very low survival rates in other disinfecting treatments. As in figure 1, 5% H_2O_2 was only 30% successful. Treatment with Clorox was not successful even with 1 minute soaking. In tissue culture, hydrogen peroxide is used to remove impurities; however, larger concentrations of this chemical can be harmful to the plant tissue itself. Like many other plants, mosses may be vulnerable to high hydrogen peroxide concentrations, which could result in tissue damage or growth inhibition. The susceptibility of various contaminants to hydrogen peroxide may differ. The 3% concentration may be more effective against a wider variety of contaminants without damaging moss tissue. However, 1% to 3% is a typical range for hydrogen peroxide concentration in the sterilization processes for plant tissue culture. It is important to remember that each unique situation may require empirical testing to determine the ideal concentration (Mahendran *et al*., 2021).

The main objective of this study was to develop an optimized and standardized protocols for the *In - vitro* propagation and establishment of Versicularia moss. This involves identifying the most effective growth media composition, nutrient supplementation, growth regulator application, and culture conditions to promote the growth and development of healthy plantlets (Bhatla & Dhingra-Babbar, 2019). Besides it would also include mass production, disease free stocks, acclimatization and transplantation, commercial applications also need to be studied. Surface sterilization and media preparation had a very important place in the research and 12 treatment were tested. They were tested with many factors such as sugar content (with 3% sugar and sugar free), and several plant growth regulator combinations (Figure 2), Finally, after several culture media and surface sterilization treatments, the research was successful with the medium prepared by combining BAP (0.5 ml), 2, 4-D (0.5 ml) and NAA (0.5 ml) in liquid form. Successful concentrations of disinfectants were 3% H_2O_2 and the 0.1% fungicide (Mancozeb). Findings of this study could be useful in media preparation and surface sterilization of moss varieties as well as other aquatic plants.

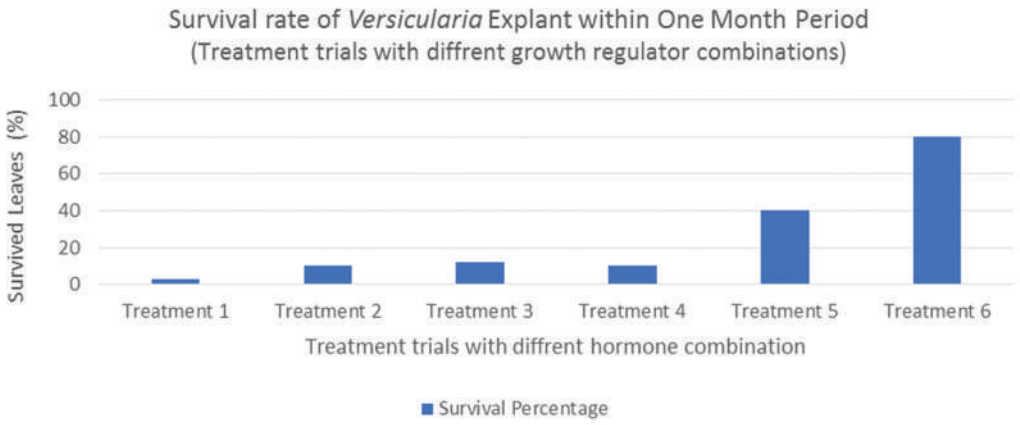

Figure 2: Treatment trials with different hormone combination and survival rate after one month period

T1- 1 mg/L BAP+0.5mg/L 2IP+10 mg/L myo inositol;T2 -0.5mg/L BAP+0.125mg/L 2IP+10 mg/L myo inositol; T3 – 0.25 mg/L NAA +0.25 mg/L 2,4-D; T4 - 0.125 mg/L NAA +0.125 mg/L 2,4-D; T5 – 0.25 mg/L BAP+0.25 mg/L NAA +0.25 mg/L 2,4-D; T6- 0.5 mg/l BAP+0.5 mg/L NAA +0.5 mg/L 2,4-D. All media contained full strength MS component with 6.5 g/L agar medium at pH 5.8

Hormone combinations were tested under several surface sterilization procedures. However, out of a number of treatments, treatment 6 showed 80% successful results. According to this study, the following points were elucidated. The occurrence of contaminations increases proportionally as the concentration of ethanol increases. Contaminations develops very quickly and very rapidly in the medium with sugar. It is important to provide low or moderate nutrition for the medium. Contaminates grow quickly as hormone concentrations increase. Contaminations can be minimized by maintaining the concentration of disinfectant between 1 - 3%. When using sterilized tools such as, Scalpel blade, tweezers, and forceps to cut or move explants in the laminar floor, their temperature should be taken care of Since these plants are very delicate and sensitive, they are quickly damaged by high temperatures.

CONCLUSION

This research was mostly based on surface sterilization and media preparation. Sterilizing method and culture medium showed that leaf explant survival was comparatively high (80%) when treated by dipping in 3% hydrogen peroxide and 0.1% (W/V) fungicide (Mancozeb). Moreover the most effective growth media composition was full strength MS agar medium containing 0.5mg/L BAP, 0.5mg/L NAA, 0.5mg/L 2, 4-D growth regulators without sugar. Therefore, this novel medium and surface sterilization method worked comprehensively well for *in-vitro* establishment of *Versicularia dubyana* leaf explants.

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Optimization of Sterilization Protocol for *in-vitro* Regeneration of *Nepenthes* spp.

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ABSTRACT

Nepenthes belongs to the group of carnivorous plants known as pitcher plants. *Nepenthes distillatoria* is the only species endemic to Sri Lanka. *Nepenthes* spp. has medicinal value. Presently, it is popular as an ornamental plant. However, the *Nepenthes* plant is still mostly propagated by seeds and stem cuttings. Yet seeds are small and seed germination is poor. Besides, *Nepenthes* seeds are very rare. Vegetative propagation through stem cuttings also has high contaminations due to various reasons. Therefore, the most effective method for large-scale propagation of the *Nepenthes* plant is *in vitro* culture technique. The best sterilization protocol needs to be developed to reduce the contamination of explants in tissue culture. In this study, leaves of the *Nepenthes* plant were used as an explant. Non-contamination percentage was the data that was collected. The observed data was analyzed using non-parametric analytic tools (the chi-square test) by IBM SPSS Statistics 25 software, and a p value of < 0.05 was considered statistically significant. All leaf explants were washed with running tap water and Teepol for 20 minutes. It was then dipped in 1% Captan (a fungicide) for 30 minutes and washed with distilled water. Subsequently explants were treated with 70% isopropanol alcohol for 30 seconds. Then explants were treated with five sterilization treatment: a) 5% Clorox for 10 minutes; b) 5% Clorox; and three different concentrations of $KMnO_4$ (23 mg/l, 24 mg/l, and 25 mg/l) for 10 minutes separately. c) 5% Clorox and 0.1% $HgCl_2$ for 2 minutes. Later, sterilized leaf explants were transferred to culture bottles that contained modified Murashige & Skoog (MS) medium with half concentration. Each treatment contained 12 replicates and 2 explants per replicate. Data were recorded weekly up to the 4th week. Results indicated that the sterilization method of 0.1% $HgCl_2$ had the highest non-contamination rate (91.7%). The second-highest non-contaminated rate (83.3%) was shown in treatment with 25 mg/l $KMnO_4$. Therefore, among the two sterilization treatments, 25 mg/l $KMnO_4$ can be recommended due to the high toxicity of $HgCl_2$.

Keywords : *Nepenthes* spp., Sterilization Method, Contamination, *In-vitro*, Explant

INTRODUCTION

Nepenthes, is commonly known as tropical pitcher plants or monkey cups. It is a diverse and intriguing group of carnivorous plants known for their unique adaptations and striking appearance. *Nepenthes* plants are primarily found in tropical and subtropical regions of Southeast Asia. They are especially abundant in lowland rainforests and cloud forests. The plant can live in the lowlands and highlands (Moran and Clarke, 2010).

The genus encompasses approximately 170 known species, along with numerous natural and hybrid varieties. Among them only one species is endemic to Sri Lanka (Anon, 2000). Borneo, Sumatra, and the Philippines have the greatest diversity, with many endemic species ("*Nepenthes*", 2023). *Nepenthes* species are in danger of extinction because of over exploitation and clear cut deforesting (D'Amato, 1998).

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The *Nepenthes* spp. has several possible uses, including use as an ornamental plant both indoors and outdoors, due to the pitcher' s many shapes and colors (Sani *et al*., 2000). Additionally, several species have medicinal value. The *Nepenthes* plant, which produces very small seeds, with a low germination rate has made an efforts to protect the plant ex situ slow (Sani *et al*., 2000). Therefore, to ensure the species existence and expansions, it is important to look for an alternative propagation technique through micro propagation.

Optimizing sterilization protocols in *in-vitro* growth is crucial to prevent contamination, ensuring aseptic conditions for plant tissue culture. Contaminants can compromise experimental results, compromise tissue health, and lead to unreliable findings. A well optimized sterilization process promotes reproducibility, accuracy, and the overall success of in-vitro experiments. The applied protocol must be suitable for the culture of interest during the micro propagation process, as it contains numerous steps that must be properly designed to avoid challenges and failures. The establishment of the explant in-vitro is one of the most important stages of micro propagation; if done correctly, it can limit the chances of contamination and excess oxidation (Caldeira *et al*., 2020).

Nepenthes is a dioecious plant with insect aided pollination, wind assistance and offspring from multiple species, resulting in impurity of seeds (Cheek and Jebb, 2001). *Nepenthes* plants produce very small seed which are known to have poor germination rate. Vegetative propagation by cutting of layering takes a long time and has low results (Sani *et al*., 2000). In most commercial and academic plant tissue culture laboratories, explant losses as a result of contamination occur at every subculture and range from 3-15% on average. The bulk of these losses are brought on by bacterial and fungal contaminants (Leifert *et al*., 1989). Therefore, a more efficient vegetative propagation alternative from the same parent is needed.

Therefore, objectives of the study was to determine a suitable protocol for *in vitro* leaf culture by determining an appropriate sterilization method.

MATERIALS AND METHOD

The research was conducted at the Tissue Culture Laboratory, Henarathgoda Botanic Gardens, Gampaha from July to October 2023. This study used leaves from *Nepenthes* species which are raised as mother plants at the Henarathgoda Botanic Gardens. Tender leaves which are 3 days after leaf bud opening (second leaf from top of the plant) were used. Surfaces of all the leaves were washed with running tap water and 5% teepol solution. Then leaves were washed thoroughly using tap water and dipped in a 1% Captan solution. Subsequently leaves were washed in a series of distilled water and brought to the laminar flow. Again leaves were sterilized with 70% Iso Propyl Alcohol (IPA) and washed with series of sterilized distilled water. Then leaves were washed from 5% Clorox and washed with series of sterilized distilled water.

There are five different surfaced sterilization protocols were used in this experiment (Table 1). Commonly treated leaves were divided into 5 groups and each group was surfaced sterilized within the laminar flow using 5 types of surface sterilization protocols as mention in below.

Table 1. Five different types of sterilization protocols

Sterilization method (STM)	Procedure
T 0	Common treatment (CT)
T 1	CT + KMnO4 23mg/l for 10 min
T 2	CT+ KMnO4 24mg/l for 10 min
T 3	CT+ KMnO4 25mg/l for 10 min
T 4	CT+ 0.1% HgCl2 for 2 min

After sterilization leaves were cut into 1cm2 size pieces. They were then inoculated into bottles containing ½ MS medium within the laminar flow cabinet under aseptic condition.

Each treatment contained 12 replicates, and 2 explants were included in each culture bottle.

Culture bottles were kept in the culture room under florescent light for 16 hour and 8 hour darkness within 24 hour. Temperature was maintained at 27°C.

Data Recording

Total number of non-contaminated bottles were takes separately. Data were recorded weekly from 4th week to 12th week and finally the grand total was taken.

Statistical Analysis

All the data were statistically analyzed using Non parametric analytical tool (Chi-square test) by using IBM SPSS Statistics 25 software ($P < 0.05$).

RESULTS AND DISCUSSION

Non-contaminated and contaminated leaf explants were calculated (Table 2). Most of the contaminated culture bottles were contaminated by fungi. It was observed that non-contamination percentages were significantly higher than contaminated percentages in 3 of the 5 methods. STM T0 and T1 both had less contaminated percentages than the non-contaminated cultures. T4 had 91.7% of the highest non-contaminated percentage. The 0.2% $HgCl_2$ solution (w/v) obtained the highest non-contamination percentage of the explant of *Nepenthes khasiana* (Devi *et al*., 2013).

Table 2. Percentages of non-contaminated and contaminated culture bottles in each of the five sterilization methods.

STM	Non-contaminated	Contaminated
T 0	12.5%	87.5%
T 1	45.8%	54.2%
T 2	66.7%	33.3%
T 3	83.3%	16.7%
T 4	91.7%	8.3%

In treatments T1, T2, and T3, the percentage of non-contamination increased as the concentration of $KMnO_4$ solution increased. When leafy vegetables were dipped in a 24 mg/l $KMnO_4$ solution for 10–15 minutes, and 97.2–99.6% of pathogenic parasites and bacteria were detached from the leafy vegetables (Ashrafi *et al*., 2006; Subramanya *et al*., 2018).

Table 3. Effect of sterilization method and contamination

Source	Probability
Sterilization method (STM)	0.003
Contamination	0.028
STM* Contamination	0.0001

Probability value < 0.05 – significantly different This study also revealed a significant difference in contamination percentage between treatment methods (Table 3). The heavy metal $HgCl_2$ used in T4 is carcinogenic (Siriwardana, Zuhry and Weerakkody, 2013). As a result, it may affect the viability of the leaves as well as pollute the environment if it comes into contact with ground water. Thus, when considering health, economic, and environmental aspects, T3, which is $KMnO_4$, can be recommended.

CONCLUSION

Results of this study indicated that KMnO4 25mg/l T3 and T4 (0.1% $HgCl_2$) are most suitable for surface sterilization of *Nepenthes* leaves among all treatments.

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Acclimatization of in vitro micro propagated *Dendrobium* cv. "Big jumbo white"

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ABSTRACT

Dendrobiums are the most popular flowers in the global and local floriculture market due to their ornamental value and high consumer demand. The slow propagation rate has drawn, plant growers to focus on developing dendrobiums through *in vitro* propagation with the best performance. Acclimatization of *in vitro* propagated plants is crucial in the process of transitioning these plants from a controlled environment to natural or plant house conditions. Orchids are particularly sensitive in this phase and proper acclimatization is essential for their survival and healthy growth. Therefore, the objective of this study was to identify the best cost-effective medium compositions for the acclimatization of *in vitro* micro propagated Dendrobium cv. "Big Jumbo White". Five different potting media containing different compositions of three components; coir dust, charcoal, bricks, topsoil, sand, coconut husk chips, and compost, were used in the acclimatization process. Each treatment was conducted in five replicates. Growth performances were assessed using growth parameters, such as plant height, leaf width, number of leaves, and root length during the period of 90 days. According to the One-way ANOVA statistical analysis, the combination of charcoal, coconut husk chips, and brick pieces in a 1:1:1 ratio was identified as the most effective potting medium for acclimatization of *in vitro* propagated Dendrobium cv. "Big jumbo white".

Keywords : Acclimatization, *Dendrobium* cv. "Big Jumbo White", Effective potting media

INTRODUCTION

Dendrobium is one of the largest genera in the Family Orchidaceae that has high consumer demand because of their ornamental and pharmaceutical uses. Among the orchids, Dendrobium is the most popular genus in the global floriculture industry (Puchooa, 2004). Plant growers tend to maintain these plants in pots for commercial purposes by providing required conditions for growth. The main challenge in the cultivation of dendrobiums is their slow growth rate in vegetative propagation (Jaime A. Teixeira da Silva *et al*., 2017). Therefore, developing an effective *in vitro* propagation method is very useful in producing a large number of plants within a short time period. Moreover, it supports producing genetically identical and disease-free plants. During *in vitro* propagation, culture media are enriched with optimal temperature, pH, aseptic conditions, high humidity, and high nutrition compositions. When plantlets are transferred to the ex-vitro media, they need to undergo a critical period of acclimatization. Adaptation of *in vitro* propagated Dendrobium cultivars to new conditions requires a period of careful maintenance and ensuring required growth conditions (Nasution *et al*., 2020). The establishment of suitable acclimatization conditions can be done using different potting media and growth performance can be measured to assess the suitability of the media to obtain healthy plants. Non-sterilized Coir dust, Charcoal, Bricks, Topsoil, Sand, Coconut husk, and compost are commonly used potting substrates. Therefore, the objective of the

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present study was to develop an effective medium for acclimatization of tissue-cultured Dendrobium cv. "Big Jumbo White"

MATERIALS AND METHODS

Collection and preparation of plant

After 45 days of culturing, initiated Protocorm-like bodies (PLBs) were transferred to basal MS medium supplemented with 0.5 mgL-1 NAA and 2mgL-1 BAP and grown for 3 months. 2-5 cm tall plants were used for the acclimatization process. Contaminated plantlets were taken after retreatment using a fungicide for 30 minutes. Healthy plantlets were also treated with a fungicide for 10 minutes for better performance. Plants were fully dipped in the fungicide solution followed by cleaning of roots and leaves with water.

Preparation of potting media

Seven materials were used for preparing five different potting media: soil, coconut husk chips, brick pieces, charcoal, coir dust, sand, and compost. Bricks and charcoal were chopped into 2.0 – 3.0cm sized pieces. Each combination contained 3 components at a 1:1:1 ratio. Then plastic pots (15.0cm in diameter) were filled to approximately three-quarters with each potting medium of different combinations. A total of five combinations were used in five replicates with a control treatment. Each pot contained three plantlets.

Acclimatization of Dendrobium cv. "Big Jumbo White"

Plantlets were transferred from culture bottles into the potting media as three plants in each pot. Pots were hung and were kept inside a room not facing direct sunlight for a period of 4 week. After one month, the pots were moved to normal environmental conditions and kept outside the room. Normal tap water was applied daily, and fertilizers were applied once a week using a sprayer. After two months, the measurements and counts of growth parameters were taken and data were analyzed.

Statistical analysis

A Completely randomized design (CRD) approach was used for the five treatments with five replications and three plantlets per replication. One-way ANOVA and Tukey's mean comparison tests were used to analyze results assisted by Minitab 17.0 software.

RESULTS

Growth performance of Dendrobium cv. "Big Jumbo White" plantlets during acclimatization in different potting media; Soil (SO), Coconut husk chips (HU), Brick pieces (BR), Charcoal (CH), Coir dust (CO), Sand (SA), Compost (CM) (Table No: 01) were measured after 90 days. Each combination contained three components at 1:1:1 ratio.

Table 01: Growth performance of *Dendrobium* cv. "Big Jumbo White" plantlets in different potting media after 90 days

Treatment No	Medium composition	Plant height (cm)	No. of leaflets	Leaf width (cm)	Root length (cm)
T_1	SO : HU : BR 1:1:1	10.26^{b}	5.20^{b}	0.46^{b}	$1.58^{b,c}$
T_2	CH : HU : BR 1:1:1	19.90^{a}	7.80^{a}	1.14^{a}	3.46^{a}
T_3	SA : CO : BR 1:1:1	9.50^{b}	$4.80^{b,c}$	0.56^{b}	1.86^{b}
T_4	SO: CO : BR 1:1:1	$8.98^{b,c}$	$4.00^{c,d}$	0.60^{b}	1.76^{b}
T_5 Control	SO : SA : CM 1:1:1	7.68^{c}	3.40^{d}	0.40^{b}	1.32^{c}

Values followed by the same letter are not significantly different as determined by Tukey's mean comparison test ($p \leq 0.05$).

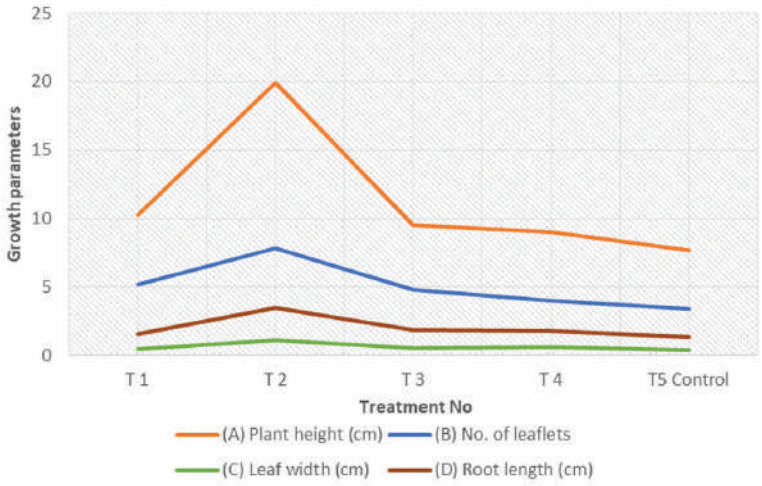

Figure 01: Growth performance of *Dendrobium* cv. "Big Jumbo White" plantlets in different potting media after 90 days. Growth parameters as, (A) Plant height (cm) (B) No. of leaflets (C) Leaf width (cm) (D) Root length (cm)

Figure 02: Growth performance of *Dendrobium* cv. "Big Jumbo White" plantlets during acclimatization with different potting media composition; (T1) SO : HU : BR (T2) CH : HU : BR (T3) SA : CO : BR, (T4) SO: CO : BR, (T5) SO : SA : CM.

DISCUSSION

This study was done to introduce an effective medium for the acclimatization of *Dendrobium* cv. "Big Jumbo White". Five different raw materials with different combinations were used by adding three types of components for each pot. According to the observations and recorded data, after 90 days of acclimatization the highest plant height, number of leaves, leaf width, and root length were observed in the medium with coconut husk chips, charcoal, and Bricks (1:1:1). The control treatment consisted of soil, compost, and sand (1:1:1) considering the common usage of these material in orchid cultivation (Pratiwi *et al*., 2020). T2 has shown best growth performance than T1 since charcoal absorbs secondary metabolites like phenolic compounds, toxins, and salts (Figure

02, T2). Similarly, in T2, coconut husk chips, bricks, and charcoal facilitate keeping the media aerated due to the ability of easy water drainage. Coir dust, soil, and compost are high in water-holding capacity therefore, the media with those components showed lower growth performances than T2. During the acclimatization phase, some plants were rotten in the control treatment. The reason may be that the media had high moisture. T1 and T3 have shown similar growth performance and in T4 considerable growth performance was noted compared to the control treatment.

All the ingredients in the potting media were used without sterilization in this study to assess the successfulness in adapting plantlets to natural conditions. When preparing the plant materials before adding to the pots all the plant materials were dipped in a fungicide (30.0g/). Fertilizers were applied once a week as foliar application. All the pots were hung, and the purpose of the hanging process was to provide epiphytic conditions for the plants. During the first four weeks pots were hung inside a room without being exposed to direct sunlight and then gradually transferred to outside conditions. This knowledge of a successful acclimatization process of *in vitro* cultured plants is essential to growers to produce healthy plants in large quantities on a commercial scale.

CONCLUSION

Coconut husk chips, charcoal, and brick pieces at a ratio of 1:1:1 was the effective potting medium composition for the successful acclimatization of *Dendrobium* cv. "Big Jumbo White".

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Effect of Substratum on Growth of *Crptocoryne parva*: An Endangered Fresh water Plant

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ABSTRACT

Cryptocoryne parva is an endangered freshwater plant with beautiful floral and leaf patterns and a popular choice for aquascaping. To meet the demand in the commercial market without endangering its natural habitats, it is crucial to find a simple and economical method for propagating and growing of *C. parva*. Thus, the purpose of this study was to identify a suitable substrate for *C. parva* which has significance for the aquatic plant sector. The experiment was conducted using four different growth media such as river sand (M1), paddy field mud (M2), paddy field mud: river sand=1:1 (M3), and paddy field mud: river sand =1:2 (M4). Healthy, vigorous, and uniformed plantlets were established in similar-sized pots and placed in a shade house. Water level was maintained in emerged condition and 1g/L Albert solution was applied weekly throughout the study period. A completely randomized design was used with ten replicates. Survival percentage (%), number of leaves, plant height (cm), and number of roots were measured twelve weeks after establishment. Data was analyzed using appropriate statistical software at a 5% significance level. Results revealed that significantly ($P \leq 0.05$) the highest survival percentage (100%), number of leaves (6.3), plant height (5.58 cm), and higher number of roots (8.8) was recorded in growth substrate, river sand. Hence, the optimal medium for *Cryptocoryne parva* to grow to its maximum potential is river sand supplemented with commercially available fertilizer at emerge condition.

Keywords: *Cryptocoryne parva*; emerge condition; growth characters; growth substrate

INTRODUCTION

Any plant/s, belonging to the kingdom Plantae, which can survive and finish their life cycle in water, on the water's surface, or on hydric soils are considered aquatic plants. Aquatic plants have a significant part in the structure, function, and supply of services within aquatic ecosystems, fulfilling a diverse array of ecological roles (O'Hare *et al*., 2018). Aside from their ecological benefits, some aquatic plants are widely employed as ornamentals in aquaria and water gardens because of their attractive foliage and flower colors, forms, and patterns. In recent decades, the aquatic plant industry has seen a notable expansion in its export capability, which has resulted in the involvement of numerous registered exporting enterprises in this market (Nissankaa et al., 2018).

Cryptocoryne is a member of the Araceae family, With over 50 distinct species spread across Southeast Asia, of the amphibious genus (Nissankaa *et al*., 2018). The majority of cryptocoryne species are found in shallow rivers and slow-moving freshwater streams. They help to preserve the ecosystem by avoiding soil erosion through the growth of rhizomes that penetrate deeply into the substratum (Wijesundara and Shantha Siri, 2004). Because of its exquisitely distinct flower shape and leaf patterns, Crptocoryne is typically seen in ponds and aquarium decorations. The three most

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significant aquarium plants in Sri Lanka are Aponogeton, Lagenandra, and Cryptocoryne, which are among the most popular ornamental plants in modern aquariums (Yapabandara & Ranasinghe, 2007).

C. parva is the smallest of all Cryptocoryne species only 3-6 cm tall. *C. parva* is notable for some of its exquisite floral and leaf characteristics. More or less glossy, green leaves. Blackish-purple throat (of spathe) that suddenly turns nearly white and is nearly closed by the obliquely horizontally oriented, dark purple limb (Yurchenko & Wu, 2016). *C. parva* is widely used for stunning arrangements in water gardens. Some stunted varieties of Cryptocoryne, including *C. parva*, are employed to produce "lawns" in aquascaping(Wijesundara and Shantha Siri, 2004). The distribution of *C. parva* species is found in the lowlands and midland rain forests of Sri Lanka in the districts of Kandy, Kegalle, and Matale, (APCTT, 2000) as well as in and around springs, streams, and rivers.

Figure 1: *Cryptocoryne parva*

Numerous vendors of aquatic plants in Sri Lanka collect wild specimens for both local and export markets, endangering the species (Bambaranda & Peiris, 2016). All species of the very fragile genus Cryptocoryne have been identified as threatened in both previous and current assessments for Red Listing, and *C. parva* is classified as an endangered freshwater plant (Weerakoon & Wijesundara, 2012). *C. parva* is already designated as a species with high threat (Yapabandara and Ranasinghe, 2007).

Since perennial aquatic plants like cryptocoryne have not been extensively studied (Herath *et al*., 2008), establishing a method of propagation and growth of *C. parva* is urgently needed to meet demand and minimize extreme pressure on its natural habitats. Attempts have been made at mass propagation via tissue culture For *C. wendtii* however, the high cost of production makes it impracticable (Bambaranda & Peiris, 2016). Consequently, the development of a simple and efficient technology for growing *C. parva* could be a good initiative for small- and medium-sized freshwater plant farmers since it would allow them to meet their demand. Additionally, this might be highly beneficial in lessening the strain on *C. parva*'s natural habitats.

MATERIALS AND METHODS

The experiment was carried out at the Royal Botanical Garden, Peradeniya, Sri Lanka. Four different media; river sand only (M1), paddy field mud (M2), paddy field mud: river sand=1:1 (M3), and paddy field mud: river sand =1:2 (M4) was filled into similar sized transparent containers (10*10*10 cm). Uniform, healthy, and vigorous *C. parva* plantlets were established in media-filled containers and kept in a shade house. A completely Randomized Design was used as the experiment design with ten replicates. Water level was maintained in emerged condition and 1g/L Albert solution was applied once a week throughout the study period. Survival percentage, number of leaves, height of the plant, and number of roots were measured twelve weeks after establishment.

Survival percentage was calculated by using the following equation. Plant height was measured by using the direct method: the distance from the growing medium to tip of the fully opened leaves. Root parameters

were taken from carefully uprooted plants. Data were analyzed using SAS (9.1 Version) software at 5% significance level and mean separation was done using Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Results revealed that, survival percentage (%), number of leaves, height of the plant and number of roots were significantly (P≤0.05) affected by the growth medium. The highest survival percentage (100%) (Figure 2-A), number of leaves (6.3) (Figure 2-B) and, plant height (5.58 cm) (Figure 2-C) was showed by plants growing in M1 (river sand) medium. Significantly (P≤0.05) the highest number of roots (11) (Figure 2-D) was showed by M3 (paddy field mud: river sand=1:1) medium. However, plants growing in the M1 media also showed comparatively higher number of roots (8) (Figure 2-D).

The lowlands and midland rain forests of Sri Lanka are home to the various Cryptocoryne species, which can be found in and near springs, streams, and rivers (Wijesundara & Siri, 2004). Most cryptocoryne species are found in slow-moving water or soil that is periodically flooded (Weerakoon & Wijesundara, 2012). Based on the results, river sand (M1) has created the perfect environment for *C. parva* to develop rapidly. It could create nearly identical ecological conditions for the growth and development of *C. parva*. Similarly, Bambaranda, and Peiris (2016) discovered that the maximum average survival percentage and mean dry weight gain were displayed by *Cryprocoryne wendtii* 'Brown' cultivated in "river sand" under "emerged condition."

Unlike free-floating species, characteristics of the growing substrate are crucial for the growth of water plants like Cryptocorynes. Substrates with finer textures are necessary for the majority of aquatic plant species (Bornette & Puijalon, 2009). Although the largest root number was recorded by plants grown in M3 for *C. parva* (Figure 2-D), the M1 medium, which has significantly larger interstitial spaces and retains oxygen, leads to the effective root function that guides maximum growth of the plant. An aquatic plant needs a well-developed root system to effectively absorb nutrients, which has an impact on plant growth (Bambaranda & Peiris, 2016). *C. parva* grown in sand medium exhibited increased water transparency, which had a direct and favorable impact on leaf characteristics such as; leaf color, leaf area and etc. Water transparency, is influenced by the color of the water (which is partially attributed to dissolved organic matter), the amount of suspended solids in the water, and the amount of plankton in the water; all tightly control the presence of macrophytes (Bornette & Puijalon, 2009).

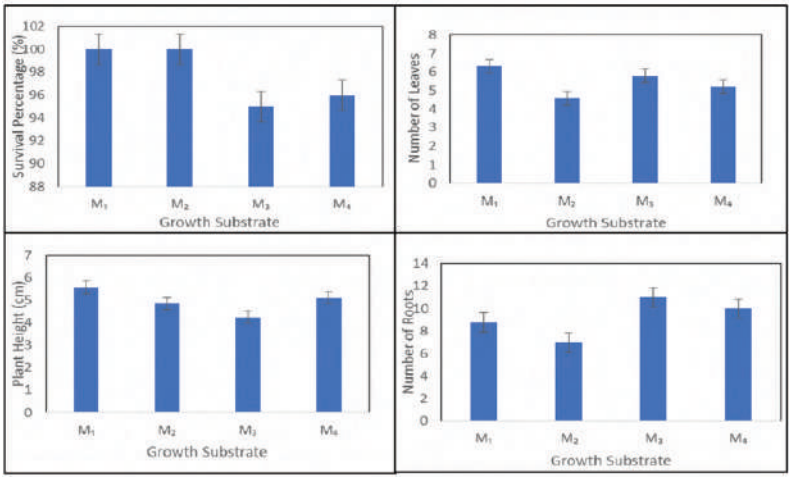

Figure 2: The effect of growth substrate on growth parameters (A-Survival %, B- Number of leaves, C-Plant height, D-Number of roots) of *C. parva*. (River sand only (M1), paddy field mud (M2), paddy field mud: river sand=1:1 (M3), and paddy field mud: river sand =1:2 (M4))

Aside from its complicated physical and chemical features, it's interesting to note that many of the benefits of growing plants in sand media were evident as compared to other media in handling growth and development. The least amount of finer soil particle deposition on leaves during turbid conditions is visible in the sand medium. Also, sand is very much easier to manage in the freshwater plant sector, and since it is widely available, small-scale farmers may even benefit from it. It's also interesting to note that, despite being an inert medium, sand has produced the perfect nutritional content for *C. parva* to grow as much as possible when combined with the lowest possible rate of commercial fertilizer (1g/L Albert).

CONCLUSION

According to the study, the highest growth was observed in *Cryprocoryne parva* grown on "river sand" under "emerged condition" when supplemented with commercially available fertilizer.

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Investigation Of the Most Suitable Growing Media Combination to Enhance Flowering Performance of Sri Lankan Lady Jane Hybrid Variety Lalani

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ABSTRACT

The study was conducted with the aim of determining the most suitable growing media combination for enhancing the growth of *Anthurium andreanum* var. 'Lalani'. Eight different pot types and growing media combinations were selected as treatments, i.e., T1M1-Plastic pot filled with leaf mold-2 parts+cattle manure-1 part+sand-1/2 part+brick pieces-1/4 part+charcoal-1/4 part of growing media, T2M1- Net plastic pot filled with leaf mold-2 parts+cattle manure-1 part+sand-1/2 part+brick pieces-1/4 part+charcoal-1/4 part of growing media, T3M1- Clay pot filled with leaf mold-2 parts+cattle manure-1 part+sand-1/2 part+brick pieces-1/4 part+charcoal-1/4 part of growing media, T4M1- Cement pot filled with leaf mold-2 parts+cattle manure -1 part+sand-1/2 part+brick pieces-1/4 part+charcoal-1/4 part of growing media, T5M2- Plastic pot filled with coconut husk-1 part+gravel-1 part of growing media, T6M2-Net plastic pot filled with coconut husk-1 part+gravel-1 part of growing media, T7M2-Clay pot filled with coconut husk-1 part+gravel-1 part of growing media, T8M2- Cement pot filled with coconut husk-1 part+gravel-1 part of growing media. Randomly selected Anthurium plantlets were planted and kept for six months in a shade house. The experiment was laid out in a Completely Randomized Design combined with two factors (pot types and growing media) and the eight treatments randomized in ten replicates and data were collected on growth and flowering parameters. Six months old Lady Jane plants grown on Net plastic pot filled with coconut husk-1 part+gravel-1 part of growing media combination showed the best performance. Based on the study findings, the Net plastic pot filled with a combined of coconut husk-1 part+gravel-1 part of growing media and the Cement pot filled with a combination of coconut husk-1 part+gravel-1 part of growing media can be recommended as the most suitable pot type and growing media combination for enhancing the growth and flowering performance of *Anthurium andreanum* var.' Lalani'.

Keywords: Anthurium hybrid Lalani, growing media, pot type, growth and flowering performance

INTRODUCTION

The "Lady Jane" type potted plant *Anthurium andreanum* named 'Lalani' is a beautiful new variety produced in 2015 by the Floriculture Research and Development Unit (FRDU), Department of National Botanic Gardens (DNBG) Peradeniya, Sri Lanka. The plant is popular commercially as an indoor plant as well as an ornamental plant in landscape gardening (Warigajeshta *et al*. 2021). However, very few experiments have been carried out for the 'Lady-Jane' varieties under local conditions (personal communications) and none have been done to assess the most suitable growing medium and fertilizer combination for the variety Lalani', since it is a new product of the Department of National Botanical Garden. This research study was implemented with

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the aim of determining the most suitable pot type and growing media combination for the growing stage of this new variety, "Lalani' under local climatic conditions. The Botanical Gardens of Sri Lanka needs to be provided with scientifically proven information that could be recommended for the best pot type & growing media combination for the commercial Anthurium growers. Moreover, finding the best pot type & growing media combination for local Anthurium varieties is very important because local varieties have a good market demand and growers can obtain better profit using the best pot type & growing media combination for local varieties of *Anthurium andreanum*. This research was carried out to assess the suitable pot type & effective growing media combination for growth of *Anthurium andreanum*. Besides no research studies have been reported on the effect of pot type and growing media combination on the growth of *Anthurium andreanum*. Therefore, present study aimed to fulfill this research gap as well.

MATERIALS AND METHODS

Experimental site

The experiment was conducted at the Floriculture Research and Development Unit of the Royal Botanical Gardens, located at Peradeniya (Mid country Wet zone of Sri Lanka). The soil group of the area belongs to the Red Yellow Podsolic (RYP). This experiment was laid out as a pot experiment under 80% shade at a plant house.

Experimental design and treatments

The experiment was laid out in a Completely Randomized Design (CRD) combined with two factors (pot types and growing media). This experimental design has eight treatments randomizing in ten replicates as T1M1-Plastic pot filled with leaf mold-2 parts + cattle manure-1 part + sand-1/2part + brick pieces-1/4 part + charcoal-1/4 part of growing media, T2M1-Net plastic pot filled with leaf mold-2 parts + cattle manure-1 part + sand-1/2 part + brick pieces-1/4 part + charcoal-1/4 part of growing media, T3M1-Clay pot filled with leaf mold-2 parts + cattle manure-1 part + sand-1/2 part + brick pieces-1/4 part + charcoal-1/4 part of growing media, T4M1-Cement pot filled with leaf mold-2 parts + cattle manure-1 part + sand-1/2 part + brick pieces-1/4 part + charcoal-1/4 part of growing media, T5M2-Plastic pot filled with coconut husk-1 part + gravel-1 part of growing media, T6M2-Net plastic pot filled with coconut husk-1 part + gravel-1 part of growing media, T7M2-Clay pot filled with coconut husk-1 part + gravel-1 part of growing media, T8M2-Cement pot filled with coconut husk-1 part + gravel-1 part of growing media.

Selection of planting materials

Three months old, tissue cultured *Anthurium andreanum*, Lalani plantlets maintained at Floriculture Research and Development Unit were selected for the study. Characteristics of Lalani – Thick, heart shaped, Orange or green spathe and Yellow white mix spadix.

Plant management and data collection

Randomly selected plantlets were planted in selected pots with relevant potting media and kept for six months in a shade house (80% shade). All cultural and management practices were done according to the recommendations given by the Floriculture Research and Development Unit, Peradeniya, Sri Lanka. Data were collected once a week on growth and flowering parameters of each plant.

Data Analysis

Data were tabulated and analyzed using analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS). Least

Significant Different (LSD) was performed to compare the differences among treatment means at p=0.05.

RESULTS AND DISCUSSION

Number of leaves

The study findings clearly revealed that T6M2 (Net plastic pot filled with coconut husk + gravel media) showed the highest number of leaves per plant (Table 1). Warigajeshta *et al*. (2021) also reported the growing media with (Coconut husk pieces) was the best medium for leaf production for variety Lalani. According to Pawar *et al*. (2006) coconut coir pieces produced a significant maximum number of leaves per plant, while the leaf sheath length and leaf area were significantly higher in coconut coir pieces+ brick piece + wooden charcoal followed by coconut coir + wooden charcoal for *Anthurium andreanum* cv. Tropical Red. Similar findings were observed in the present study as well. The growing media consisting of coconut husk pieces + gravel only produced plants with highest number of leaves.

Number of suckers

Number of suckers at growing stage was significantly different (p=0.05) among all evaluated treatments. T6M2 showed a significant difference compared to other treatments. There was no significant difference among T5M2, T3M1, T7M2, T1M1 and T2M1 during the study period. T6M2 (Net plastic pot filled with coconut husk + gravel media) showed the highest number of suckers per plant compared to other treatments. The lowest sucker content per plant was showed by T2M1 (Net plastic pot filled with leaf mould + cattle manure + sand + brick pieces + charcoal media) (Table 1).

Average number of new suckers is a good indicator for growth of plants. *Anthurium andreanum* cv. Lalani is a pot plant which is more beautiful with many suckers and flowers. Therefore, number of new suckers per plant is a very important parameter.

Plant height (cm)

Results of the study clearly revealed that treatment T8M2 which is cement pot filled with coconut husk + gravel media and T6M2 which is net plastic pot filled with coconut husk + gravel media induced the plant height growth parameter. Ajish Muraleedharan and P. Karuppaiah (2015) also indicated that maximum plant height, plant spread, number of flowers per plant, flower stalk length, spathe length and spathe breadth were recorded in (75% shade × coir pith + coconut husk). Furthermore, according to the study findings of Binas Jr *et al*., (2023), the plants applied with coconut husk + chicken manure obtained significantly higher plant height. According to these results, coconut husk growing media is a good potting media for the *Anthurium andreanum* plant growth.

Soilless media are easy to handle and may provide an excellent growing environment to plants as compared to soil (Bilderback *et al*., 2005; Mastouri *et al*., 2005) one reason being the lack of soil pests. Various locally available materials have been utilized to prepare growing media for commercial production of anthuriums, especially coconut husk, leaf humus, sand and coir dust (Umaharan and Elibox, 2011).

Length of roots and number of roots

Results of the present study indicate that, T8M2 which is cement pot filled with coconut husk + gravel media and T6M2 which is net plastic pot filled with coconut husk + gravel media showed the highest number of roots per plant for *Anthurium andreanum* var. 'Lalani' (Table 1).

According to similar findings of Tatte Sumathi *et al*., (2018), Anthurium is epiphytic in nature with creeping, climbing or arborescent stems including lots of aerial roots that aid in taping water and nourishment, whereas.

Table 1. Effect of different pot types and growing media combination on growth parameters of *Anthurium andreanum* var. Lalani

Treatment	No of leaves	No of suckers	Plant height (cm)	Length of roots (cm)	No of roots
T1M1	15.7^{b}	2.28^{bc}	24.33^{bc}	9.04^{c}	6b^{c}
T2M1	14.36^{b}	2.22^{c}	24.55^{bc}	9.16^{c}	6.2b^{c}
T3M1	14.41^{b}	2.53^{bc}	22.94^{c}	8.84^{c}	5.1^{c}
T4M1	15.52^{b}	2.68^{b}	26.49^{b}	9.33^{bc}	6.25^{bc}
T5M2	13.91^{b}	2.55^{bc}	26.85^{b}	12.06^{b}	7.9^{b}
T6M2	23.84^{a}	3.38^{a}	27.37^{ab}	12.11^{a}	8.45^{a}
T7M2	13.87^{b}	2.41^{bc}	24.7^{bc}	11.34^{bc}	7.75^{b}
T8M2	13.03^{b}	2.65^{b}	28.27^{a}	12.13^{a}	8.5^{a}

Note: Means of each category with the same letter/s are not significantly different at p=0.05

Time taken (days) for initiation of first flower bud

Results of the present study indicated that, coconut husk + gravel media was the most suited growing media for time taken for initiation of first flower bud (Table 2). However research findings Singh, *et al*., (2011) indicated, that minimum days to flowering were recorded in a growing mixture of saw dust + brick pieces + wooden charcoal + soil + sand + FYM, while soil + sand + FYM required maximum days to flowering after planting. According to Dufour & Guérin (2003), floral initiation begins approximately 90 days before emergence, indicating that each flower begins its growth almost 50 days before the anterior flower is harvested. And also, according to the results of Ajish Muraleedharan and P. Karuppaiah (2015), Days taken for flower bud initiation was early in (75% shade & coir pith + coconut husk).

Table 2. Effect of different pot types and growing media combination on time taken for initiation first flower bud of *Anthurium andreanum* var. Lalani

Treatments	Time taken for Initiation first flower buds
T6M2	98.8^{a}
T7M2	61.9^{b}
T5M2	57.3^{b}
T8M2	52^{b}
T1M1	24.1^{bc}
T4M1	16.8^{bc}
T2M1	6.9^{c}
T3M1	4.7^{c}

Note: Means of each category with the same letter/s are not significantly different at p=0.05

Number of flower buds

The present study clearly indicated that the net plastic pot filled with coconut husk + gravel media had an effect on flower bud induction of Anthurium (Figure 1). The aim of potting is to provide a confined space for roots in conditions that favour healthy growth. The interior of the pot is a microclimate and the potting material (or medium) is expected to provide a reasonable lasting combination of moisture and aeration to form a suitable microclimate. It is recommended that growing media should be well aerated (Caldari, 2004) with good porosity (Sakai, 2004) and optimum drainage, but with the ability to retain sufficient moisture and provide support to the plant (Sakai, 2004, Umaharan and Elibox, 2011). According to FL Cuquel *et al*., (2012) the best flowers were produced in plants grown in wood shavings + organic compost (1:1). Using coco husk along with the application of animal manure is a common practice in the production of Anthurium.

The combinations of media and different fertilizers can boost the performance of amthurium (Warigajeshta *et al.*, 2021) and thus contributed to the increase of cut flower production (Salachna, 2022).

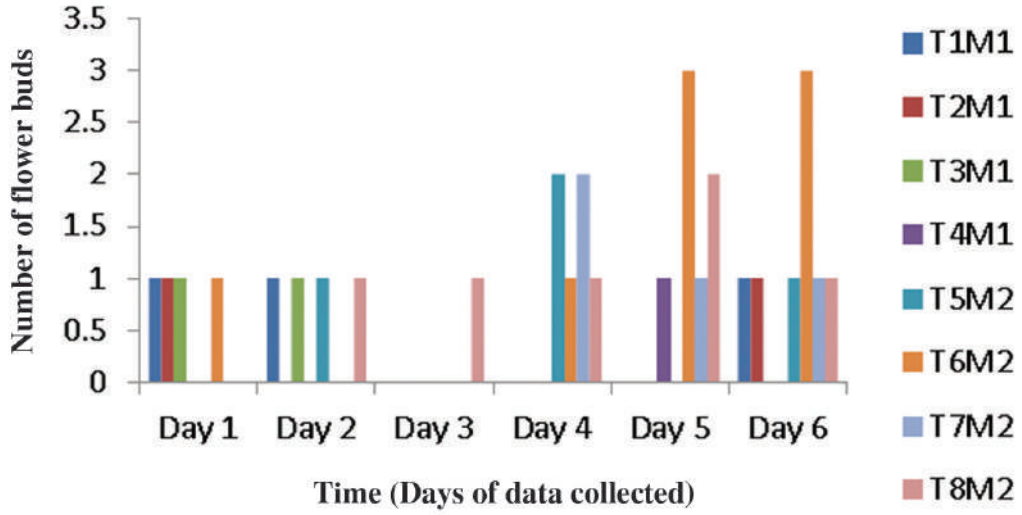

Figure 1 Effect of different pot types and growing media combination on formation of new flower buds of *Anthurium andreanum* var. Lalani

Number of flowers

In the present experiment, T6M2 (Net plastic pot filled with coconut husk + gravel media) and T8M2 (Cement pot filled with coconut husk + gravel media) showed the highest number of flowers per plant in *Anthurium andreanum* var. Lalani (Figure 2).

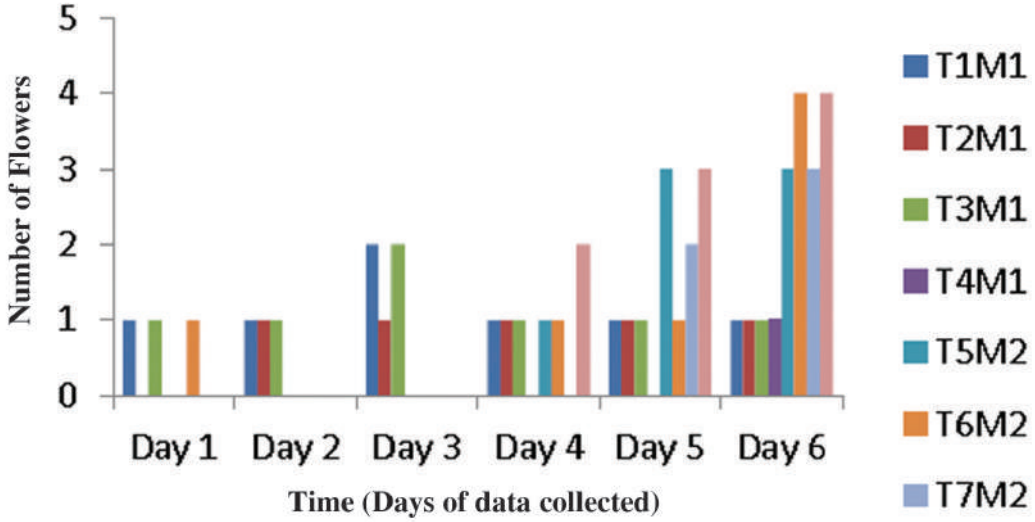

Figure 1 Effect of different pot types and growing media combination on formation of new flowers of *Anthurium andreanum* var. Lalani

Based on the results of this study the media leaf mould + cow dung + sand + brick pieces + charcoal media is not suitable or economical to all anthurium growers. Locally available low cost material is needed to replace the above medium. This experiment shows that coconut husk + gravel combined with cement pot and net plastic pot can be used for the growth of anthurium. Moreover according to findings of FL Cuquel *et al*., (2012), the growing media utilized affected the quality of anthurium flowers. In the growing media wood shavings + organic matter (1:1), the biggest length of flower spathes was produced. This finding is in agreement with Singh *et al*., (2011) who observed that growing media significantly affects the size of anthurium flowers.

Caldari-Junior (2004) agrees with this supposition reporting that porosity is one of the growing media features to be prioritized in the choice of the raw material. Besides, FL Cuquel *et al*., (2012) discovered, that wood shavings + organic matter (1:1) and the pine bark + organic matter (1:1) showed better performance in flowering frequency probably because of their lower dry density.

CONCLUSION

The overall study findings revealed that Net plastic pot filled with a combination of coconut husk-1 part + gravel-1 part of growing media and Cement pot filled with coconut husk-1 part + gravel-1 part of growing media can be considered the most suitable pot type and growing media combination that enhanced growth of *Anthurium andreanum* var. Lalani. Therefore, the Net plastic pot filled with a combination of coconut husk-1 part + gravel-1 media and Cement pot filled with coconut husk-1 part + gravel-1 part of growing media is recommended as the best pot type and growing media combination for commercial anthurium growers.

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Effect of Heat Treatment, Relative Humidity, and Shade on the Growth of Dendrobium Orchid Plants in the Acclimatization Stage

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ABSTRACT

Orchids belonging to the family Orchidaceae and represents 7% of flowering plants on earth. It is a terrestrial epiphyte with flowers in different colors, sizes and shapes, and can adapt to different climatic conditions. Dendrobium, an epiphytic orchid grows non-parasitically on plants and obtains water and nutrients from the air. The ultimate success of commercial micropropagation depends on the ability to transfer plants out of culture on a large scale, at a low cost with a high survival rate. *In vitro*-grown plantlets are acclimatized to adapt to soil substrates and natural environmental conditions under slightly modified environmental conditions. The acclimatization of *in vitro* raised plantlets plays a vital role in the mass propagation of different orchid species. The drawback of micro propagated plants is that they are tough to adapt to normal environmental conditions in the acclimatization stage, and as a result, 20-25% of plants have to be discard. However, the potting media, light intensity and moisture of the greenhouse are essential factors for successfully acclimatizing Dendrobium plantlets. In this experiment, the heat treatment, shade and relative humidity conditions were changed. Temperature, humidity and shade were controlled from 27-42 ^{0}C, 60- 80% and 50-70%, respectively. A total of 33 treatments with 15 replicates were tested with control experiments. Results show that 37 ^{0}C heat treatment, 60-75% relative humidity, and 50% shade level are preferable for the acclimatization stage.

Keywords: Orchidaceae, Dendrobium Orchid, Acclimatization, Temperature treatment, Relative humidity, Light intensity

INTRODUCTION

The two primary categories of decorative plants are ornamental flowering plants and ornamental foliage plants. A fundamental component of most gardens are flowering decorative plants, and many flower gardeners choose to grow a variety of flowers. So that the garden is perpetually in bloom throughout the spring and summer. Orchids arouse the imagination of people with their complicated shapes, brilliant colors, and alluring scents, making them highly prized by botanists, horticulturists, and flower lovers. (Jones, 2019). In addition to their visual appeal, they are appealing due to the unique reproductive strategies as they have evolved through time to facilitate effective pollination and seed dispersal. Dendrobium orchids showcase an astounding variety of flower shapes. Their blooms can range from delicate and petite to large and showy, with an assortment of hues including white, pink, purple, and yellow (Davis, 2022). Compared to certain other orchid kinds, Dendrobium orchids are typically seen to be simpler to cultivate and maintain. They are said to be hardy and flexible, making them appropriate for both seasoned and novice producers. Additionally,

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the blooming time of Dendrobium orchids is frequently prolonged, with some types producing blooms that endure for many weeks or even months. Tissue culture raised plants require extensive hardening treatments to prevent high mortality after transfer to in vivo conditions (George, Hall and De Klerk, 2008). Plantlets produced *in vitro* are unable develop resistance against minor and major microbial pathogens or other biotic and abiotic stresses caused by *in vitro* controlled conditions, which are characterized by an aseptic environment with slight temperature variations, high relative air humidity, increased availability of nutrients, low light intensity, and low carbon dioxide concentration (Pospíšilová, 2007). Acclimatization is important for ensuring the proper establishment and growth of orchids when moving them from one habitat to another, such as from a greenhouse to a home or garden setting. Acclimatization of orchids is a vital process that involves helping these delicate and sensitive plants adjust to new environmental conditions. Orchids have a diverse species and intricate adaptations which often require specific temperature, humidity, light intensity, duration, and airflow conditions to thrive. The less stress and better odds of survival enable orchids to gradually acclimate to their new environment. There is scarce information about directly assessing the response of Dendrobium to biotic stresses.

MATERIALS AND METHODS

Tissue culture Dendrobium orchid plants (480 plantlets) used to conduct the experiment. Copper sulfate ($CuSO_4$) and systemic fungicide were used to absorb moisture and sterilization respectively. propagators, transparent polythene, steel cables, shade nets (Israel 50% and 70%), tapes, UV polythene were used for the experimental setup. Steel racks were used to place propagators. Light meter were used to measure light intensity, Both Relative humidity meter (RH meter) and wet and dry bulb thermometers were used to measure Relative Humidity. potting media (0.5-inch charcoal, coconut husk and tile pieces), fertilizers (foliar applications such as 3 in 1, mugasol, grow more) 2-inch clay pots and water sprayers were used during the experiments.

Method

A total number of 480 tissue cultured sterilized plantlets were used in the acclimatization step (Figure 1). In this study, four different temperatures (T) of 27 ^{0}C (room temperature), 38 ^{0}C, 40 ^{0}C and 42 ^{0}C and four different relative humidity (R) of 60-65%, 65-70%, 70-75% and 75-80% and shade levels (S) of 50% and 70% also varied. All the parameters were maintained constant. Altogether 32 treatments were conducted with 15 plantlets. The experimental results for plant height, leaf length and width of 3rd leaf from bottom, diameter of shoot, number of roots, number of leaves and new shoots from 15 days' intervals up to 75 days were analyzed.

RESULTS AND DISCUSSION

Growth of Height

Interaction effect of heat treatment, shade level and RH conditions on plant height is not significant since the relevant p value (0.581) is higher than 0.05. (Figure 2) Also, there is not a significant interaction effect of not only heat treatment and shade level but also RH and shade level on plant height. But, the interaction effect of heat treatment and RH on plant height is significant since the relevant p value (0.013) is lower than 0.05. So, there is an interaction effect of heat treatment and RH on plant height. Consider about different heat treatments on plant height, the effect of heat treatment is significant. Because, room temperature (27 ^{0}C) has the highest plant height for most shade levels and RH conditions, and some of

them are significant. On the other hand, heat water (38 ^{0}C) has the lowest plant height for most shade levels & RH conditions, but none of them are significant. And also, there is not a huge different between plant height with heat water 40 ^{0}C and 42 ^{0}C treatments.

Growth of Leaf Length

The one-way ANOVA test shows that combinations are significant at a 0.05 significant level (Figure 3). So, a Tukey test is conducted to find the best combinations and results. It shows that leaf length under room temperature (27 ^{0}C) at 50% shade level with 60 – 65 % RH is the highest, but it does not significantly differ from the most leaf length. In contrast, the leaf length under room temperature (27 ^{0}C) at 50 % shade level with 75 – 80 % RH is the lowest, but it does not significantly differ from most leaf lengths. Consider different heat treatments on leaf length, there is not a significant difference between leaf length under each heat treatments, and the effect of heat treatment is not significant on leaf length according to the p value 0.128. Consider about different RH conditions on leaf length, effect of RH is significant on leaf length. And also, 60 – 65 % RH has the highest leaf length for most shade levels & heat treatments, and some of them are significant. On the other hand, 75 – 80 % RH has the lowest leaf length for most shade.

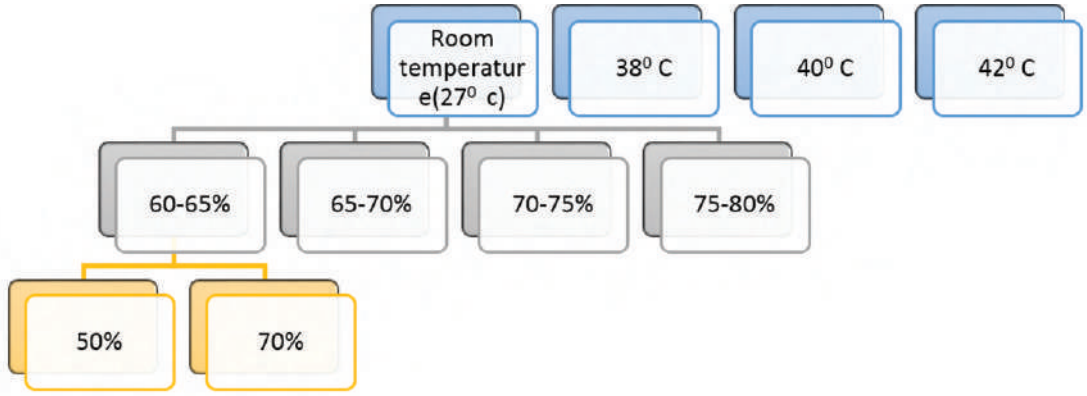

Figure 1: Experimental Procedure

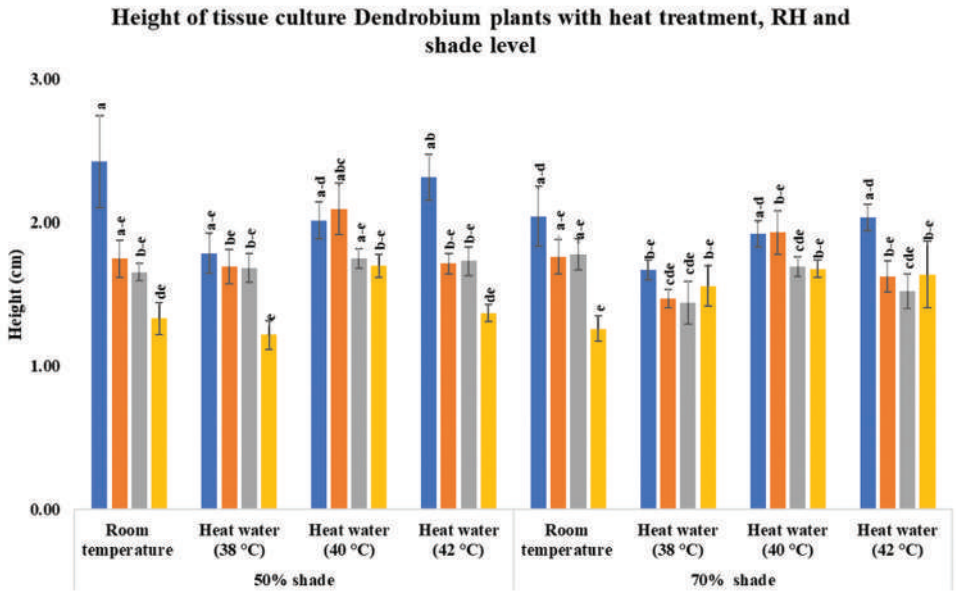

Figure 2: Plant Height with time

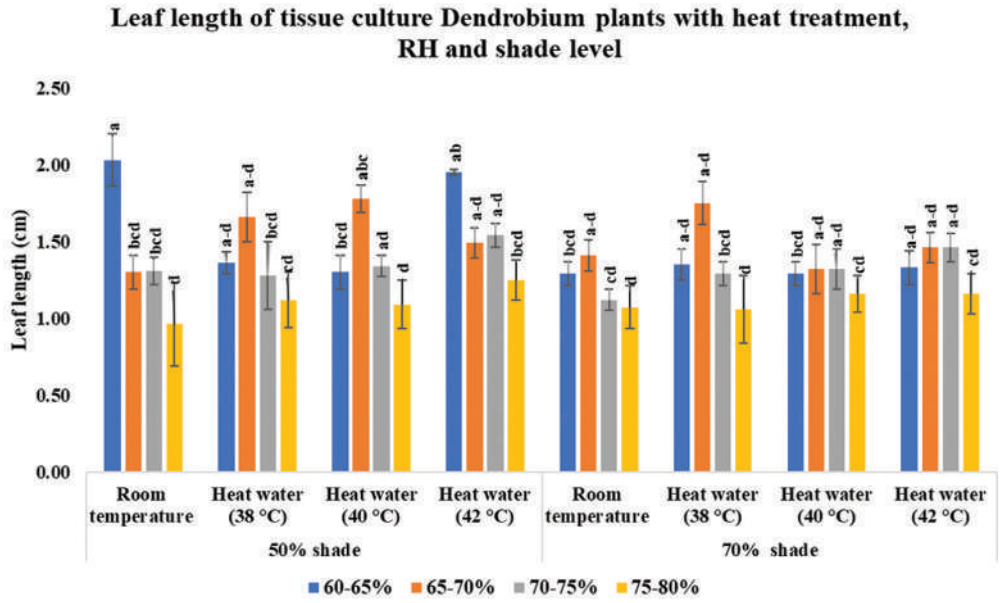

Figure 3: Leaf Length with time

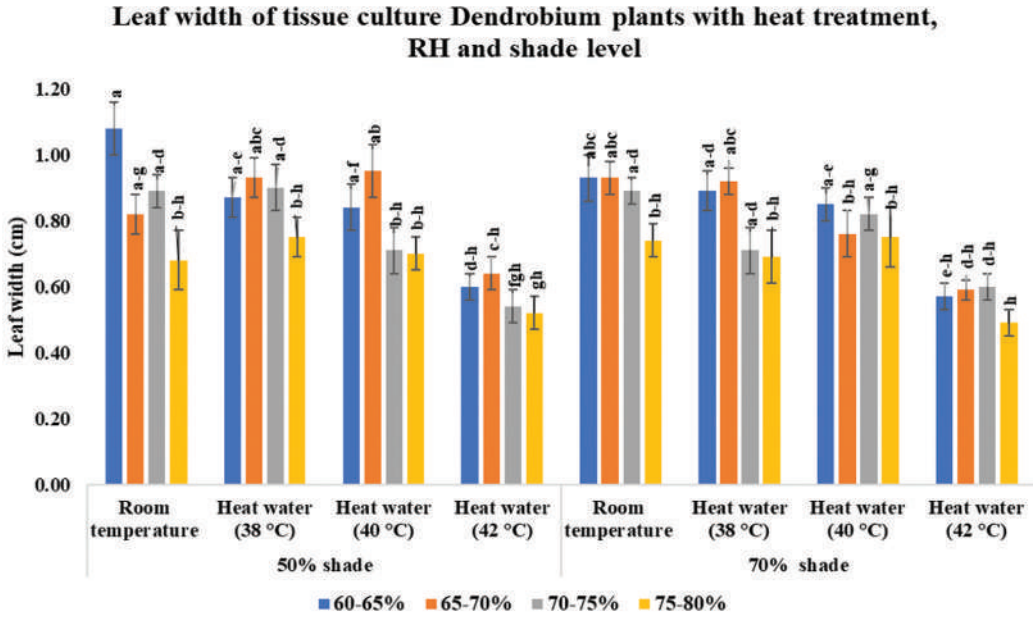

Figure 4: Leaf Width with time

Growth of Number of Leaves

The best combinations and results of that number of leaves under heat water treatment (40 ^{0}C) at 50 % shade level with 60 – 65 % RH is the highest, (Figure 6) but it does not significantly differ from the greatest number of leaves. On the other hand, the number of leaves under room temperature (27 ^{0}C) at 50 % shade level with 75 – 80 % RH is the lowest, but it does not significantly differ from the greatest number of leaves. consider different heat treatments on number of leaves, the effect of heat treatment is significant. Here, heat water at 40 ^{0}C has the highest number of leaves for most shade levels & RH conditions. On the other hand, heat water at 42 ^{0}C has the lowest number of leaves for most shade levels and RH conditions, but none of them are significant. However, there is not a huge different between number of leaves with room temperature (27 ^{0}C), heat water 38 ^{0}C and 40 ^{0}C.

Growth of Number of Roots

The interaction effect of heat treatment,

shade level and RH on number of roots is not significant since the relevant p value (0.096) is higher than 0.05. (Figure 7) Also, there is not a significant interaction effect of not only heat treatment and shade level but also RH and shade level on number of roots. Tukey test is conducted to find the best combinations and results of it shows that number of roots under heat water (40 ^{0}C) at 50% shade level with 70 – 75% RH is the highest, but it does not significantly differ from the greatest number of roots. On the other hand, the number of roots under heat water (42 ^{0}C) at 70% shade level with 65 – 70% RH is the lowest, but it does not significantly differ from the greatest number of roots.

Growth of Number of Shoots

Acceding to shows that number of shoots under heat water (42 ^{0}C) at 50% shade level with 65 – 70% RH is the highest, (Figure 8) but it does not significantly differ from the greatest number of shoots. On the other hand, the number of shoots under heat water (38 ^{0}C) at 70 % shade level with 75 – 80 % RH is the lowest. The result of this research showed that heat treatment, relative humidity (RH), and the shade level of the growth of tissue culture Dendrobium plantlets in the acclimatization stage in terms of plant height, leaf length, and width, shoot diameter, number of leaves, number of roots and shoots. The results were measured by 15 days up to 75 days. But due to easiness to gather only 75-day results were used for analysis. According to the results, plant height, leaf length, leaf width, plant diameter, number of leaves, roots, and shoots hadn't significant interaction effects of heat treatment, relative humidity, and shade level. But consider combination results have a 0.05 significant level. So, the Turkey test was conducted to find the best combinations of results. According to the analysis, the highest combined result for plant height, leaf length, leaf width, and plant diameter showed by room temperature (27 ^{0}C) heat treatment with 60-65% RH condition and 50% shade.

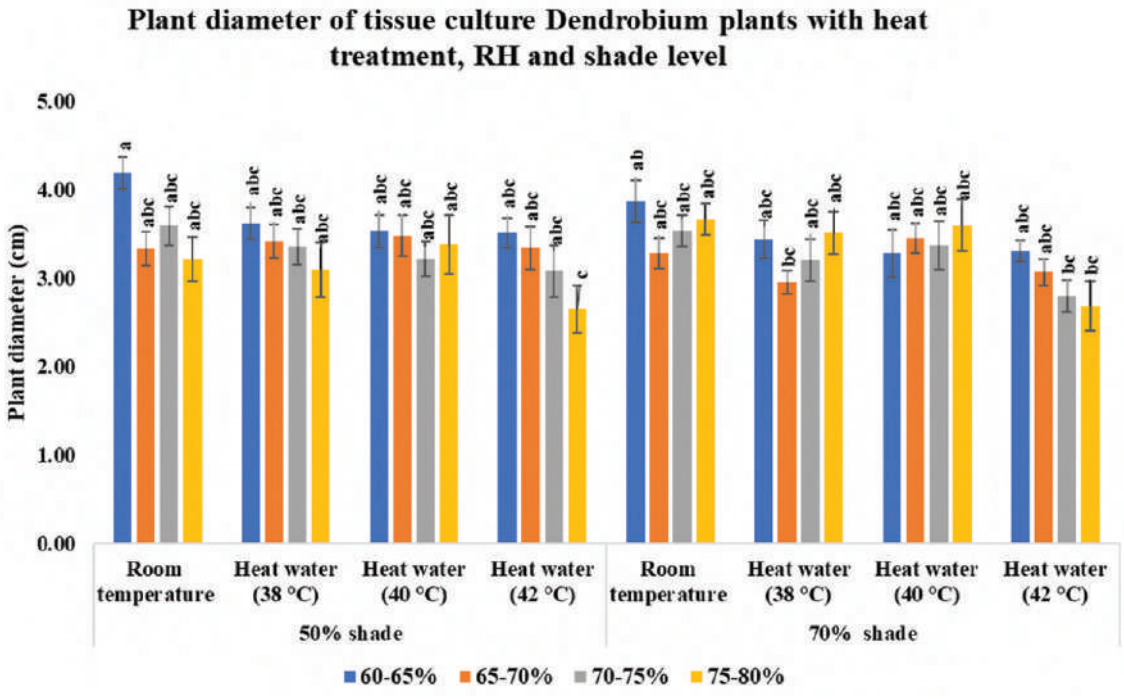

Figure 5: Plant Diameter with time

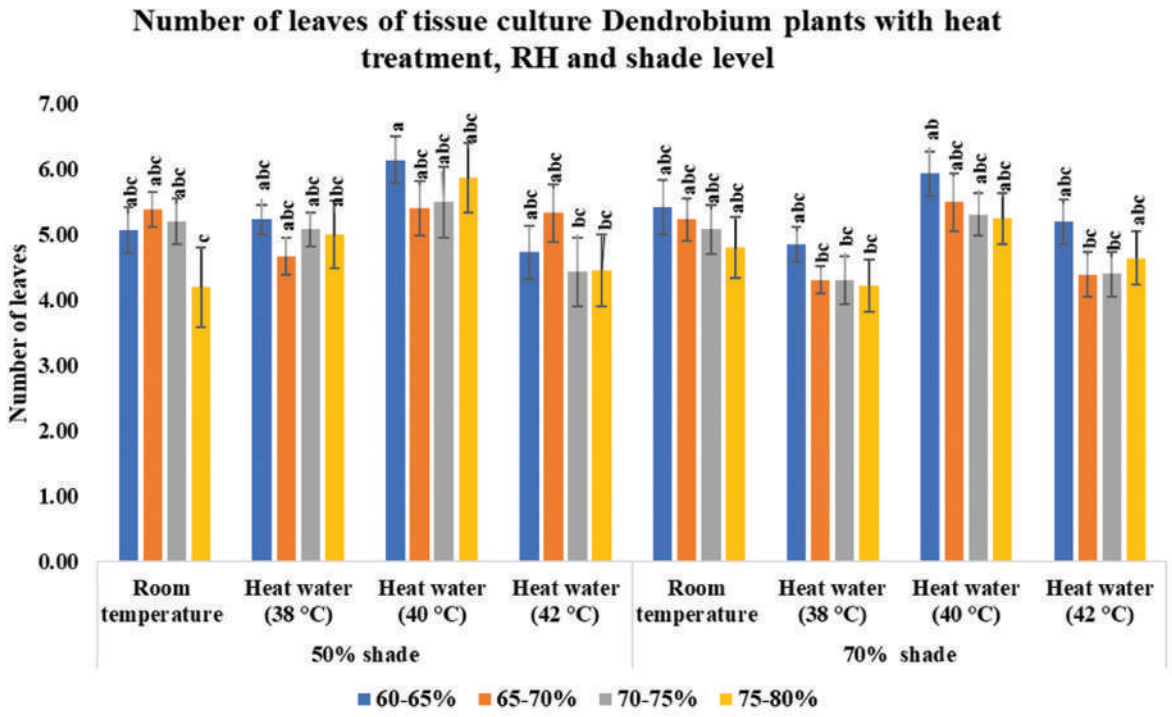

Figure 6: Number of leaves with time

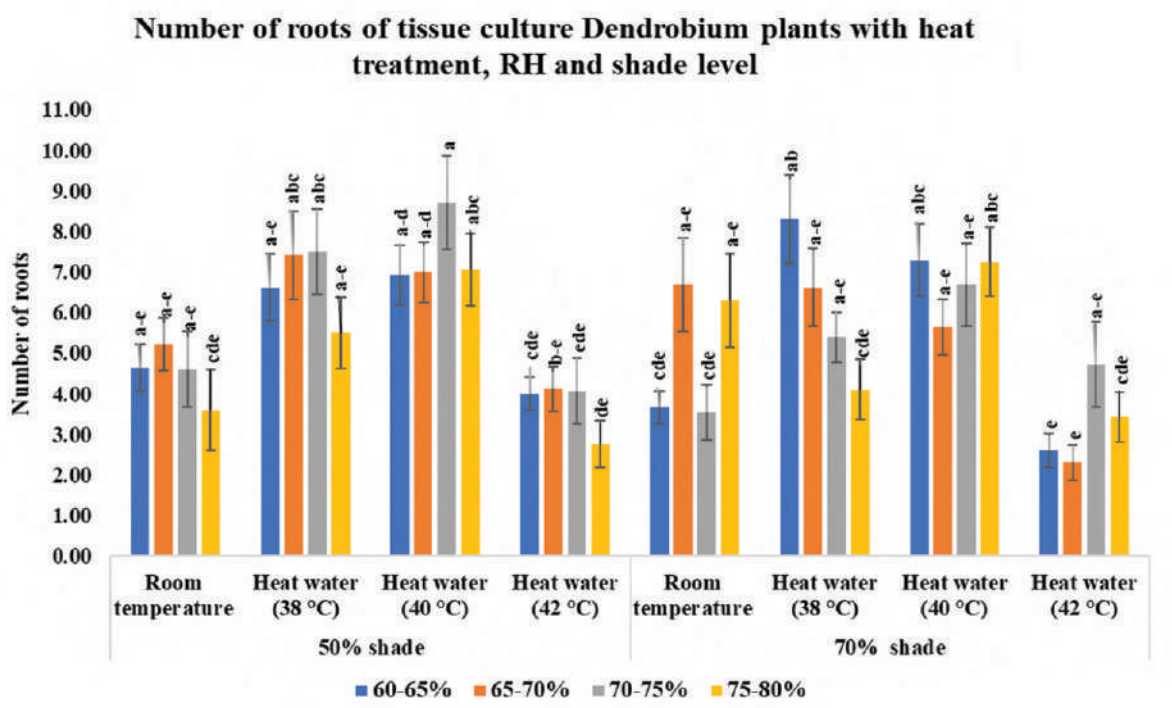

Figure 7: Number of Roots with time

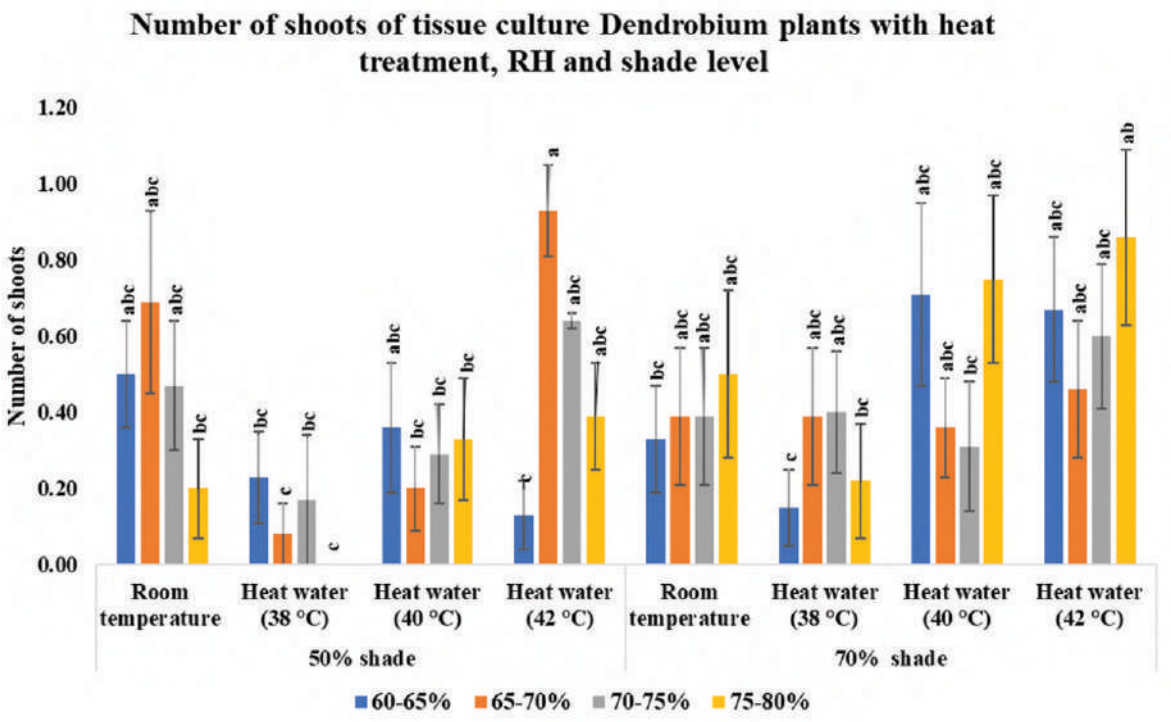

Figure 8: Number of Shoots with time

CONCLUSION

This study determined the optimum conditions for tissue-cultured dendrobium orchids during acclimatization. According to the results, the heat treatment didn't show the performance to the acclimatization. The significance RH condition performed between 65-75% range. The 50% shade level showed higher results. As a conclusion the highest result showed by 37^0C heat treatment with 60-65% RH conditions and 50% shade level.

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Propagation of Lipstick plant (*Aeschynanthus radicans*) from vegetative and seed in different potting mixtures to enhance the growth

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ABSTRACT

The Lipstick plant (*Aeschynanthus radicans*) is an ornamental flowering plant with a high economic value as a flowering hanging pot plant for both indoor and outdoor decoration. Thise experiment was conducted to study the effect of the potting media on the propagation of the lipstick plant from vegetative parts and seeds at the Royal Botanic Garden Peradeniya. The study had three experiments with four treatments each and was laid out in a Complete Randomized Design. Cuttings, leaves, and seeds were used as planting material while leaf mold: partly burnt rice husk: sand in 1:1:1, 2:2:1, 1:2:1, and 2:1:1 ratio was used for each propagation methods. The mixture of leaf mold, partly burnt rice husk, and sand at a ratio of 2:2:1 increased number of shoots, and leaves, as well as root length on lipstick plant compared to other media for cuttings propagation. The media leaf mold, partly burnt rice husk, and sand in a 1:1:1 ratio increased seed germination, number of leaves, plant height, and number of roots, root length, and fresh weight when compared to other media for seed germination. There was no significant difference among the media for propagation by leaves. The significance of this finding is that the partially burnt paddy husk can be used as a growing medium in the future for the floriculture industry.

Keywords: Partially burnt paddy husk, Lipstick plant, Propagation

INTRODUCTION

Aeschynanthus radicans, also called "Lipstick Plant," is a species of the genus Aeschynanthus and family Gesneriaceae (Mendum *et al*., 2001). Aeschynanthus consists of 160 species, from India and Sri Lanka through southern China and Southeast Asia to New Guinea and the Solomon Islands (Middleton, 2016). Due to its slow growth habit and genetic variation, there is little use of seeds for the production of plantlets in the floriculture sector. Even though seedlings grow slowly and take a while to recuperate, they have a great chance of producing many different daughter plants through seed propagation. This study revealed the effect of different selected potting media for propagation of the A.radicans seed, cuttings and leaves. The objective of this study was to identify a quick and easy method of propagation of A.radicans from cuttings, seeds and leaves while assessing a suitable propagation media for each of the planting materials. This research had six specific objectives, to determine the best way to propagate the lipstick plant, to develop an effective growing medium for propagation of lipstick plant by cuttings, to develop an effective growing medium for seed propagation of lipstick plant, to develop an effective growing medium for the leaf propagation, to measure and evaluate plant growth parameters of the new media, and to introduce partially burnt rice husk as a nursery media to the floriculture sector.

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MATERIALS AND METHODOLOGY

Aeschynanthus radicans seed pods, leaves and cuttings were collected from the A. radicans plant collection in the Floriculture Research Unit of Royal Botanical Gardens, Peradeniya. The potting mixtures were prepared using leaf mold, partly burnt rice husk and sand in different ratios of 1:1:1, 2:2:1, 2:1:1 and 1:2:1 for each planting material and the research study was conducted separately for each planting materials. The mixtures were sieved through 2.5mm sieve and sterilized with steam sterilization for 45 minutes and treated with a fungicide (Captan). All pots were kept under a single propagator covered by 250-gauge cellophane and placed under the 50% shade condition. The experiment was conducted in a completely randomized design (CRD) and each treatment included 5 replicates. Subsequently, all agronomic practices were conducted and measurements were taken at weekly intervals starting from one month after planting. Collected data were statistically analyzed using the statistical software Minitab, and mean separation within treatments was performed by Tukey's at a 5% significant level.

RESULTS AND DISCUSSION

Experiment- 01 (Propagation by cuttings)

Results of number of shoots and leaves at the 9th week after planting (WAP), indicated that the highest mean value was observed in Leaf Mold: Partly Burnt Rice Husk (PBRH): Sand in a 2:2:1 ratio.

The result of shoot height showed an increasing trend from the 4th week up to 9 weeks. Plant height on the 4th week up to 9th week showed significant differences ($p<0.05$) among the treatments. Significantly the highest plant height was observed in treatment Leaf Mold: PBRH: Sand- 2:1:1 (T4) and 2:2:1 (T2) (Figure 1). The result might be obtained because leaf mold has more nutrition content.

At the 9th week after planting, the high mean value for the root length was observed in Leaf Mold: PBRH: Sand in a 40 2:2:1 ratio (2.4cm).

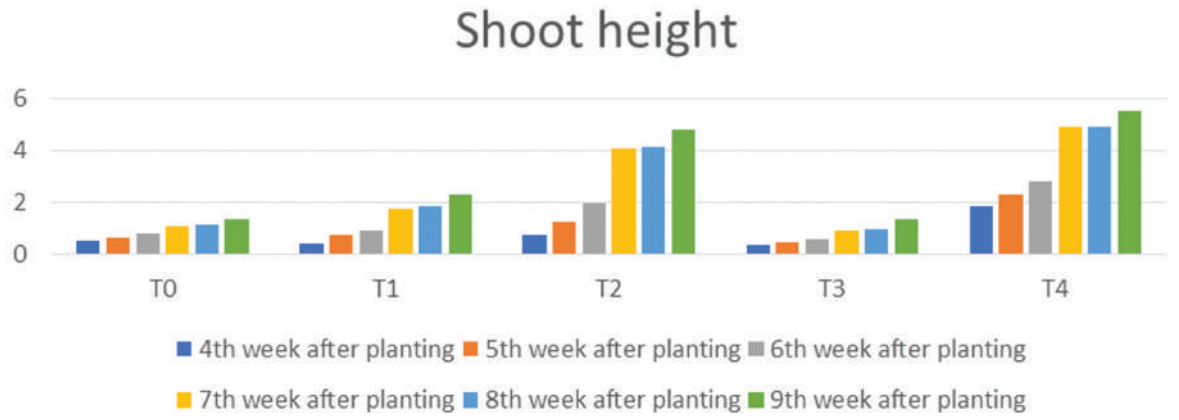

Figure 1 - The effect of different potting media on the plant height of *Aeschynanthus radicans* at 9 WAP

Figure 2 - Development of cuttings

Experiment- 02 (Leaves propagation)

In leaf propagation, there was not a significant difference ($p>0.05$) in the number of roots and root length. Surprisingly, no differences were found in root development across all treatments.

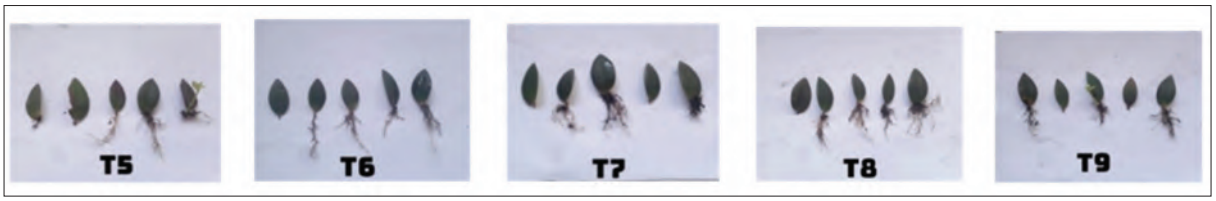

Figure 3 – Root formation of leaves

Experiment - 03 (Seeds propagation)

In seed germination, there was a significant difference ($p<0.05$). The highest mean value for the seed germination was observed in Leaf Mold: PBRH: Sand- 1:1:1 (88%). It might to be due to the water holding capacity of the partly burnt rice husk.

At the 9th week after planting, the highest mean value for number of leaves was observed in Leaf Mold: PBRH: Sand- 1:1:1. Results and previous findings showed that leaf mold played a significant role in growing maximum number of leaves because it contained decaying plant residues and contained more organic matter and humus.

The impact of different selected potting media on fresh weight of *Aeschynanthus radicans* is described in following Table 01. There was a significant difference ($p<0.05$) in fresh weight on the 9th week after planting, highest mean value for fresh weight (0.1522g) was observed in Leaf Mold: PBRH: Sand- 1:1:1.

Result of number of roots and root length at the 9th week after planting (WAP), the highest value was observed in in Leaf Mold: PBRH: Sand in 1:1:1.

Table 01 - The effect of different potting media on the fresh weight of *Aeschynanthus radicans* at 9 WAP

Treatment	Fresh weight (g)
Leaf Mold: Coir Dust: Sand: Charcoal (Control)- 1:1:1:1/2	0.0640 ± 0.00440^{b}
Leaf Mold: PBRH: Sand- 1:1:1	0.1522 ± 0.01180^{a}
Leaf Mold: PBRH: Sand- 2:2:1	0.0476 ± 0.00797^{b}
Leaf Mold: PBRH: Sand- 1:2:1	0.0712 ± 0.02190^{b}
Leaf Mold: PBRH: Sand- 2:1:1	0.0286 ± 0.00836^{b}

Plant height showed an increasing trend from the 4th week up to 9th weeks. Plant height on the 4th week shows no significant differences among all the treatments and after 9 weeks, there is a significant difference among the treatments. The highest plant height was observed in Leaf Mold: PBRH:

Sand- 1:1:1 (1.34 cm) (Figure 4). It might be due to fact that both partly burnt rice husk and leaf mold have good water holding capacity, nutrition retention and more nutrition content in compost.

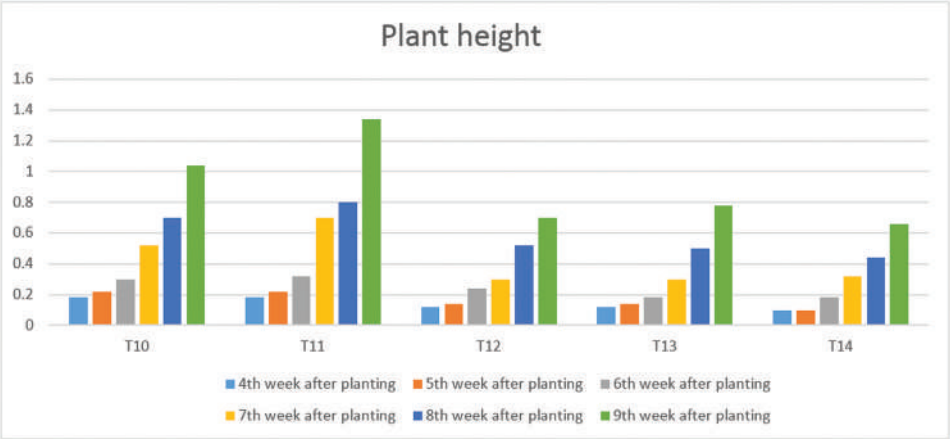

Figure 4 – Development of seed plants

Figure 5 – Development of seed plants

CONCLUSION

The present study was designed to determine the effect of selected propagation media for cuttings, leaves, and seed propagation and to identify the best way to propagate lipstick plant (*Aeschynanthus radicans*). According to this study, the media of leaf mold, partly burnt rice husk, and sand in a 2:2:1 and 2:1:1 ratio showed better growth of lipstick plant cuttings. The media leaf mold, partly burnt rice husk, and sand in a 1:1:1 ratio showed best growth of lipstick plant seeds. However, different ratios of leaf mold, partly burnt rice husk, and sand did not significantly affect leaf propagation. The contribution of this study has been to confirm that both cuttings and seed propagation methods are better and more effective for lipstick plant propagation. Seed propagation is especially important for production of new varieties of lipstick plants.

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Propagation of Syzygium companalatum Korth through stem cuttings as affected by maturity stage of cuttings and different bio fertilizers

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ABSTRACT

Syzygium campanulatum Korth (Christina) belongs to the family Myrtaceae and is an evergreen shrub rich in phenolics, flavonoid antioxidants, and betulinic acid. The plant has been found to contain high levels of antioxidants, which may help to protect cells from damage by harmful molecules called free radicals. Besides this plant has been traditionally used as a remedy for heart ailments. Currently the plant is propagated by air layering and it takes longer duration for rooting. It is also not practicable for mass propagation. Thus, the present study was conducted to find out the suitability of stem cuttings as method of mass propagation. Three types of stem cuttings (Top, Semi-Hard Wood and Hard Wood) with four types of bio fertilizers (jeewamruthum, vermi wash, fish tonic and Indo Chinese traditional microbial culture) were used for the experiment. It was arranged as Complete Randomize Design with ten replicates per treatment at a Floriculture Growers Field, in Kaluthara. Bio Charcoal & River Sand in 1:1 ratio was used as the potting medium. Growth parameters were collected weekly up to eleven weeks after planting. Data was analyzed using Mini Tab 17 statistical package. Results revealed that the top cutting with indo chinese microbial culture significantly increased the plant height, cumulative number of new leaves, cumulative number of new buds and the root volume. Therefore, it can be concluded that top cuttings planted in Bio Char & River sand 1:1 potting media treated with Indo Chinese microbial culture could be used for propagation of *Syzygium campanulatum* Korth .

Keywords: *Syzygium campanulatum* Korth, Jeewamruthum, Vermiwash, Fish Tonic, Indo Chinese Microbial Culture

INTRODUCTION

Christina (*Syzygium campanulatum* Korth) belongs to the family Myrtaceae and is a landscape crop, with a native range that extends from Africa and Madagascar through southern Asia east through the Pacific. Its highest levels of diversity occur from Malaysia to northeastern Australia, where many species are very poorly known and many more have not been described taxonomically. This plant is also known in names Brush Cherries and Lillipillies. Its dense leaves, colorful pinkish-red shades from young shoots and pyramid-shaped canopy makes it a choice plant for Narrow Avenue planting. As well as planted along waterways, parks and home gardens. It is commonly grown as a hedge and provides extensive landscape when sculptured into attractive topiaries. It is a small tree but is rarely grown to be one. Most species are evergreen, shrubs. They are also planted as ornamental plants for their attractive reddish flowers, glossy foliage and edible fruit that are eaten fresh or used in jams and jellies. In Sri Lanka Christina is mostly used as one of the major landscaping crops in Sri Lanka for in landscape gardening as an excellent specimen for avenues, pathways and also used

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for ornamental purposes in home gardens for exterior decorations since the red flush of younger leaves gives a spectacular attractive view. *Syzygium campanulatum* Korth. is a perennial semi-deciduous vigorous woody plant that can reach a height of about 10- 15 feet in gardens if not pruned properly. The plant should be pruned and trimmed at a desirable height and length to improve and maintain the red flush as well as plant height. There is a huge demand for S. companalatum Korth in the local market among the landscape designers, but supply is not matched by growers. This plant is currently propagated through layering and by using stem cuttings like in many fruit plants such as pomegranate (Bhagwa *et al*., 2017). However most of the local growers show a disinterest for propagation of S. companalatum Korth since it is time consuming and less number of plants are produced from layering. Furthermore, stem cuttings take more than 45 days for rooting. Therefore, the main objective of this study of S. companalatum Korth was to evaluate the most suitable maturity stage of cuttings of S. companalatum Korth for rooting as affected by different bio fertilizers that can be prepared locally and easily available for growers.

MATERIALS AND METHODOLOGY

Stem cuttings of Syzygium companalatum Korth were collected from vigorous mother plants maintained at the Floricultural Grower Field- Kaluthara, Sri Lanka. Three different types of stem cuttings (top cutting, semi-hard wood cutting & hard wood cutting) were used in this study. All cuttings were of the same length with four leaves, with half of the leaf blade removed and having 3-4 active buds. Before planting stem cuttings in to the potting medium of Bio Char and River Sand in a 1:1 ratio, they were treated with 3 ml each of different bio fertilizers viz., Jeewamruthum, Fish Tonic, Vermy Wash and Indo Chinese Traditional Microbial Culture.

The Jeewamruthum, Fish Tonic, Vermy Wash were in liquid phase and the Indo Chinese Microbial Culture was in semi-solid phase. All three types of the stem cutting were planted in the inert medium with about 3cm inserted inside the inert medium to maintain a constant height. All the stem cuttings were of the same height (15cm). Before planting in the potting medium they were treated with 3ml of Jeewamruthum, Fish Tonic and Vermy Wash by drenching the medium and mixing it well. 03mg of Indo Chinese Microbial Culture was mixed with the potting medium. Then pots were irrigated properly and carefully. Subsequent to irrigation, planted pots were introduced to a Plant Propagator. After placing all the planting pots inside the plant propagator it was closed for better maintenance of temperature and the relative humidity. One week after planting, they were treated again according to the treatment schedule. 03ml of Jeewamruthum, Fish Tonic and Vermy Wash were added to the medium without disturbing to the stem cutting. While 03g of the Indo- Chinese Traditional Culture was mixed with 3ml of de-choronized water and in-cooperate to the medium. The same was applied two and three weeks after planting stem cuttings.

Treatment scheduled was as T 1- Top Cutting with Jeewamruthum; T2- Top Cutting with Vermi Wash; T3- Top Cutting with Fish Tonic; T4- Top Cutting with Indo- Chinese Traditional Microbial Culture; T5- Semi-Hard Wood Cutting with Jeewamruthum; T 6- Semi-Hard Wood Cutting with Vermi Wash; T 7- Semi-Hard Wood Cutting with Fish Tonic; T 8- Semi-Hard Wood Cutting with Indo- Chinese Traditional Microbial Culture; T 9- Hard Wood Cutting with Jeewamruthum; T 10- Hard Wood Cutting with Vermi Wash; T 11- Hard Wood Cutting with Fish Tonic; T 12- Hard Wood Cutting with Indo- Chinese Traditional Microbial

Culture; T 13- Top Cutting with Rooting Hormone; T 14- Semi-Hard Wood Cutting with Rooting Hormone (Control) and T 15- Hard Wood Cutting with Rooting Hormone. So, total number of treatment combination=15; Number of replication (cuttings taken) in each treatment=10 and thereby total number of cuttings used in the experiment=150. The experimental design was two factor Complete Randamozised Design. Cuttings were tested separately weekly up to 12 weeks inside the plant propagator.

Just after establishment of plants in pots, they were watered well prior to introducing the bio fertilizers and kept in the plant propagator. Data was collected weekly up to 11 weeks. Cumulative plant height, cumulative number of new leaves per cutting, cumulative number of new buds per cutting, root length and the root volume were measured as growth parameters. Statistical analysis was performed using ANOVA in Mini Tab 17. Grouping was done to determine the significance among clusters.

RESULTS AND DISCUSSION

Results revealed that, the significantly highest cumulative plant height was recorded in top cuttings treated with indo Chinese traditional microbial culture, followed by top cuttings with jeewamurthum medium and semi hardwood cutting treated with Indo Chinese traditional microbial culture at 2 weeks after planting (Figure 1). The same result was recorded in 4weeks after planting as well as (Figure 2) 11 weeks after planting (Figure 3).

Result indicated that the excretions secreted by the microbes may have induced plant growth. Results reported by Rini *et al*. (2014) on Piper nigrum L. indicated that maximum increase in plant height was recorded by the bio fertilizers used for the experiment. Similar observations were reported by Kiran *et al*. (2012). According to the findings of Devakumar *et al*. (2014) the higher number of bacteria, different fungi and N-fixers clearly indicate that jeevamruthum enriched the consortia of native soil microorganisms. Due to the higher beneficial microbial load more plant nutrients will be mobilized and provide plant growth promoting substances and other micro nutrients required by the plants. Result of the research on gherkin cultivation of Devapriya and Yapa (2017) again proved these results that newly introduced bio fertilizers-earthworm cast treated with Jeewamruthum + compost, Indo Chinese bio- fertilizer are the most suitable fertilizers.

Number of new leaves per cutting - a significantly highest cumulative number of leaves was recorded in top cutting treated with Indo Chinese traditional microbial culture and semi hardwood cutting with Indo Chinese traditional microbial culture among all treatments 5 weeks after planting (Figure 4). Top cutting with Indo Chinese traditional microbial culture, and with jeewamurthum as well as semi hardwood cutting with Indo Chinese traditional microbial culture showed a significant effect for cumulative number of leaves 10 weeks after planting (Figure 5).

Substances that are secreting by the microbes, may have induced the growth of leaves of the *Syzygium campanulatum* stem cuttings. The most significant growth of leaves or the cumulative number of new leaves was recorded by top cuttings treated with Indo Chinese traditional microbial culture. Bio fertilizers were found very effective on plant growth especially on healthy leaf production. Sadanshu *et al*. (2009) reported that bio fertilizers are considered to be a panacea for the prosperity of agriculture. The effect of bio fertilizers on growth improvement was suggested by Muhammed (2010).

Number of New Buds per Cutting - Results emphasized that, the significantly highest cumulative number of new buds was recorded in top cutting with Indo Chinese traditional microbial culture, and with jeewamurthum as well as semi hardwood cutting with Indo Chinese traditional microbial culture among all treatments 5 weeks after planting (Figure 6). Although 10 weeks after planting a significantly highest cumulative number of the new buds was recorded for top cuttings treated with Indo Chinese traditional microbial culture, top cuttings with jeewamurthum, semi hardwood cutting with Indo Chinese traditional microbial culture and hard wood cuttings treated with Indo Chinese microbial culture among all treatments (Figure 7).

Sladky and Tichy (1959) compared the effects of foliar or nutrient solution application of humic substances on shoots. Young leaves responded to a greater extent than older ones. Previous studies reported that bio fertilizers had improved soil productivity, fertility and the propagation, which improved the yield and quality in the floricultural crops. (Dinesh *et al*., 2010) Application of foliar bio fertilizer spray on begonia plants (Sladky, 1959) yielded similar results as recorded.

Root Length - The significantly highest length of the roots was recorded in top cutting treated with Indo Chinese traditional microbial culture, top cuttings with jeewamurthum, hardwood cutting with Indo Chinese traditional microbial culture, top cutting with rooting hormone, semi hard wood cutting with rooting hormone and hard wood cutting with rooting hormone among all treatments 11 weeks after planting (Figure 8).

Similar results had reported by Ramya (2014) in the *Piper nigrum* L. cuttings that were propagated using bio fertilizers. It may be assumed that chemically synthesized rooting hormones can react with plant physiology in various ways and can induce root growth than other solutions used in this experiment.

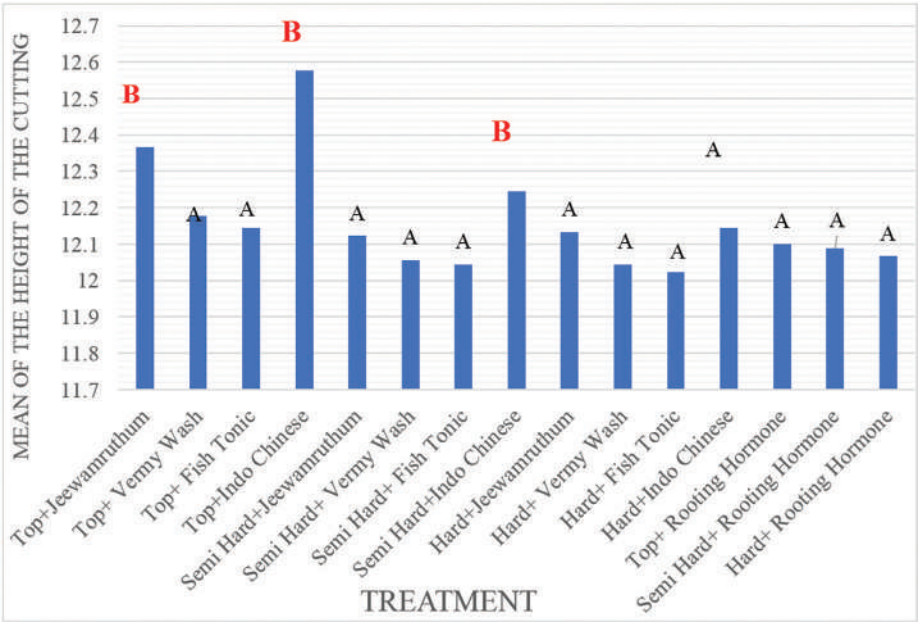

Figure 1: Effect of cutting type and different bio-fertilizers on mean shoot height of *Syzygium campanulatum* Korth , 2 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

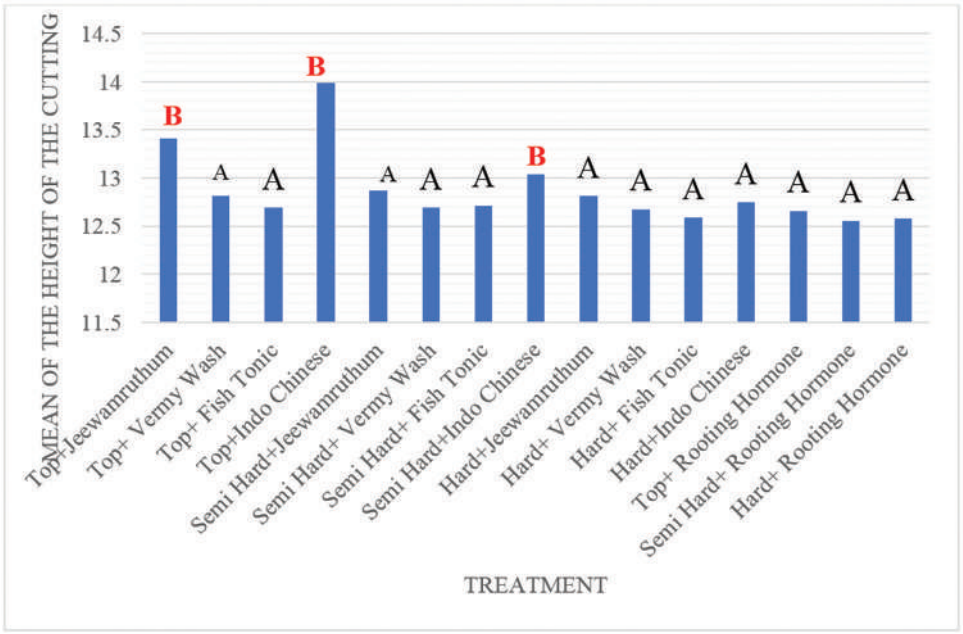

Figure 2: Effect of cutting type and different bio-fertilizers on mean shoot height of *Syzygium campanulatum* Korth, 4 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

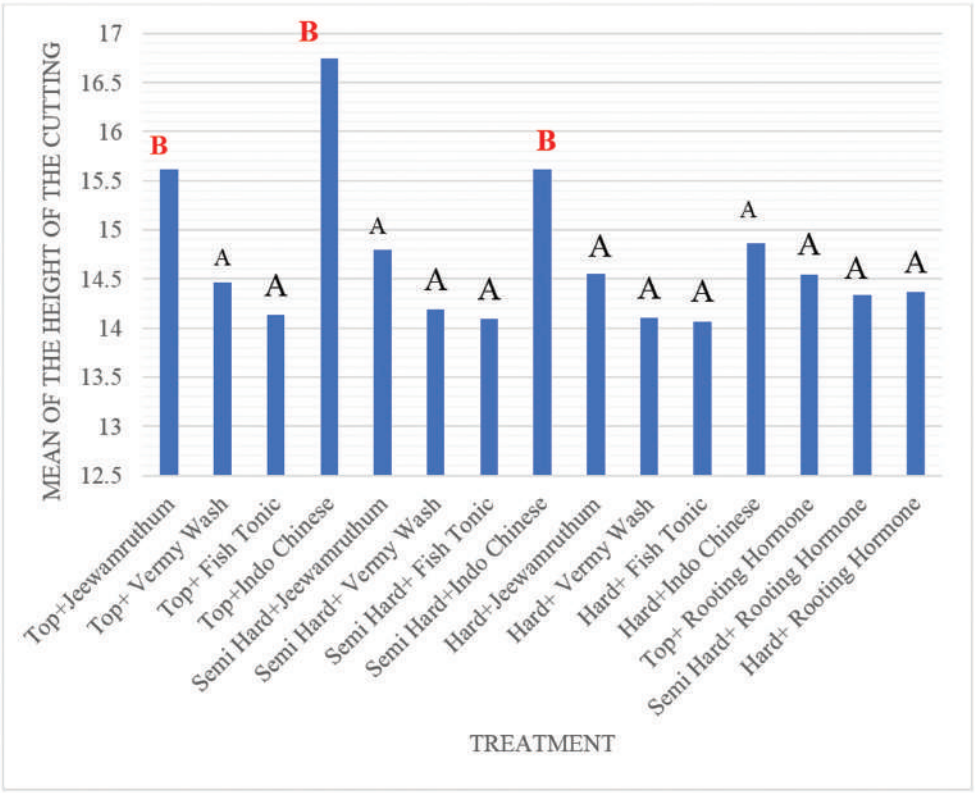

Figure 3: Effect of cutting type and different bio-fertilizers on mean shoot height of *Syzygium campanulatum* Korth, 11 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

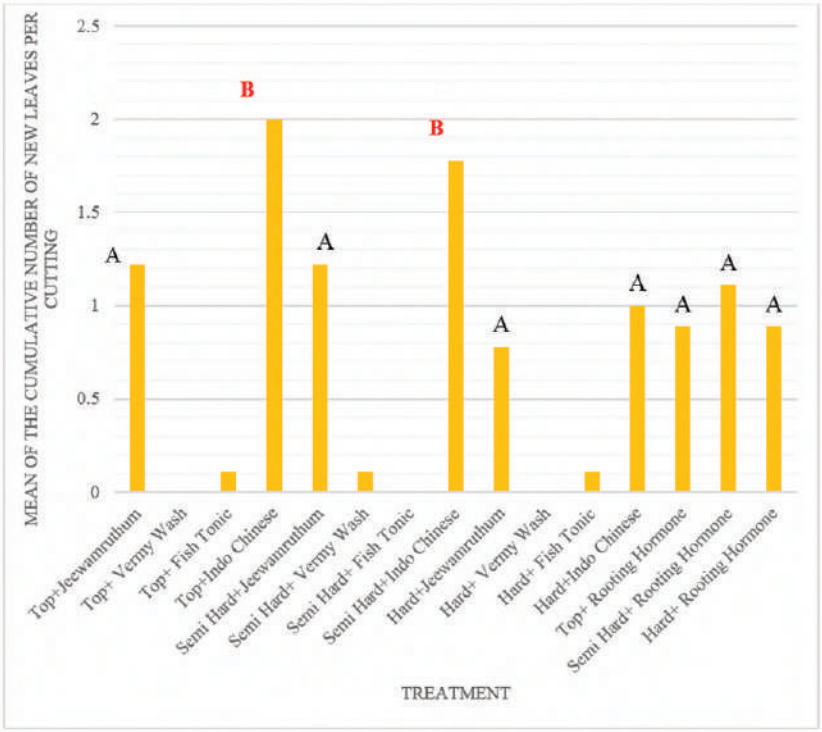

Figure 4: Effect of cutting type and different bio-fertilizers on mean number of leaves in *Syzygium campanulatum* Korth, 4 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

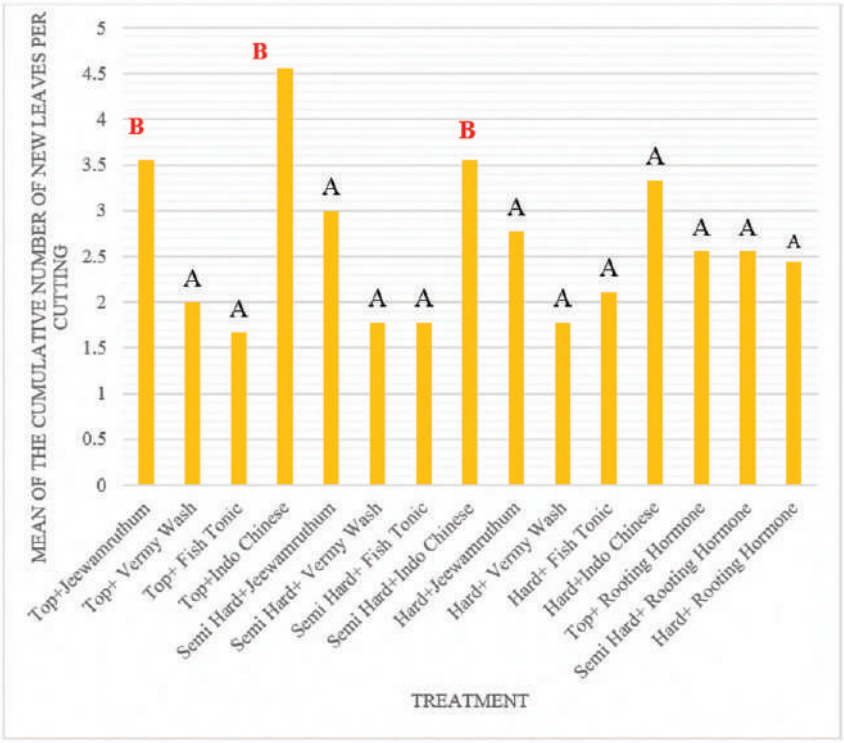

Figure 5: Effect of cutting type and different bio- fertilizers on mean number of leaves in *Syzygium campanulatum* Korth, 10 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

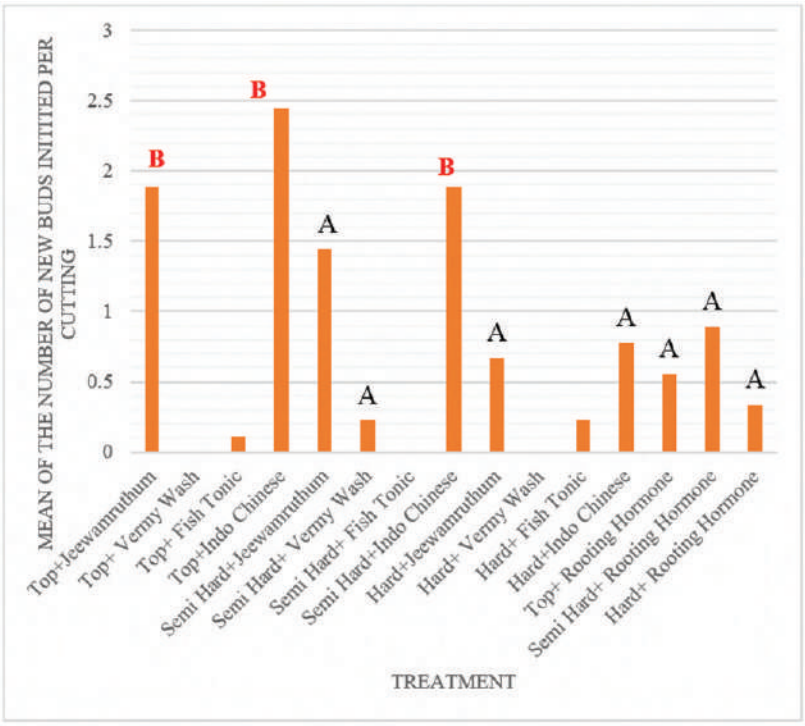

Figure 6: Effect of cutting type and different bio-fertilizers on mean number of new buds in *Syzygium campanulatum* Korth, 5 weeks after planting. Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

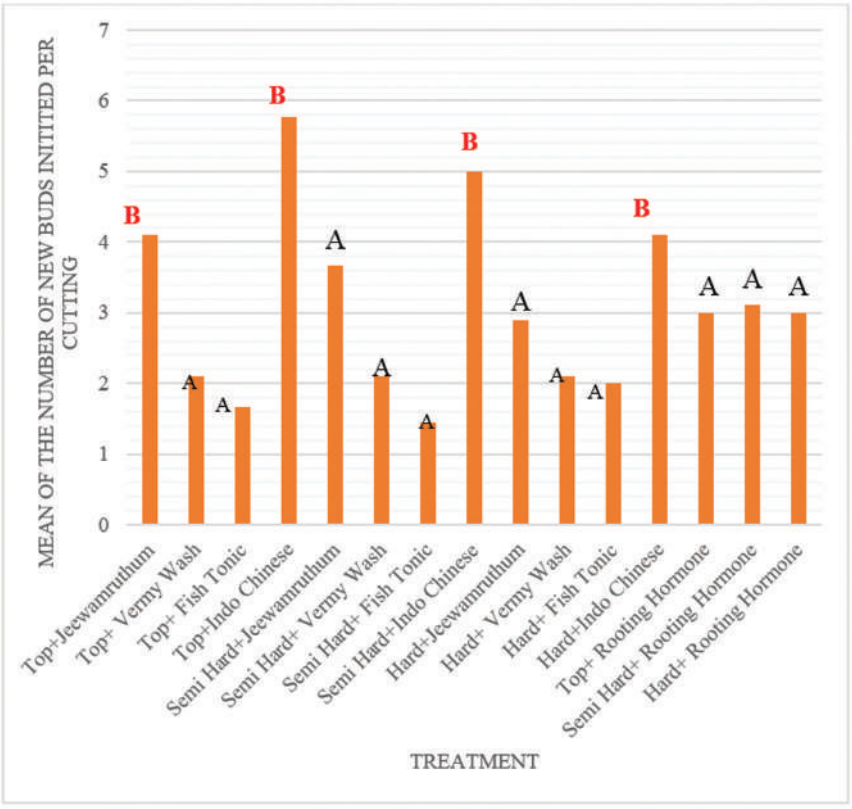

Figure 7: Effect of cutting type and different bio- fertilizers on mean number of new buds in *Syzygium campanulatum* Korth, 10 weeks after planting. Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

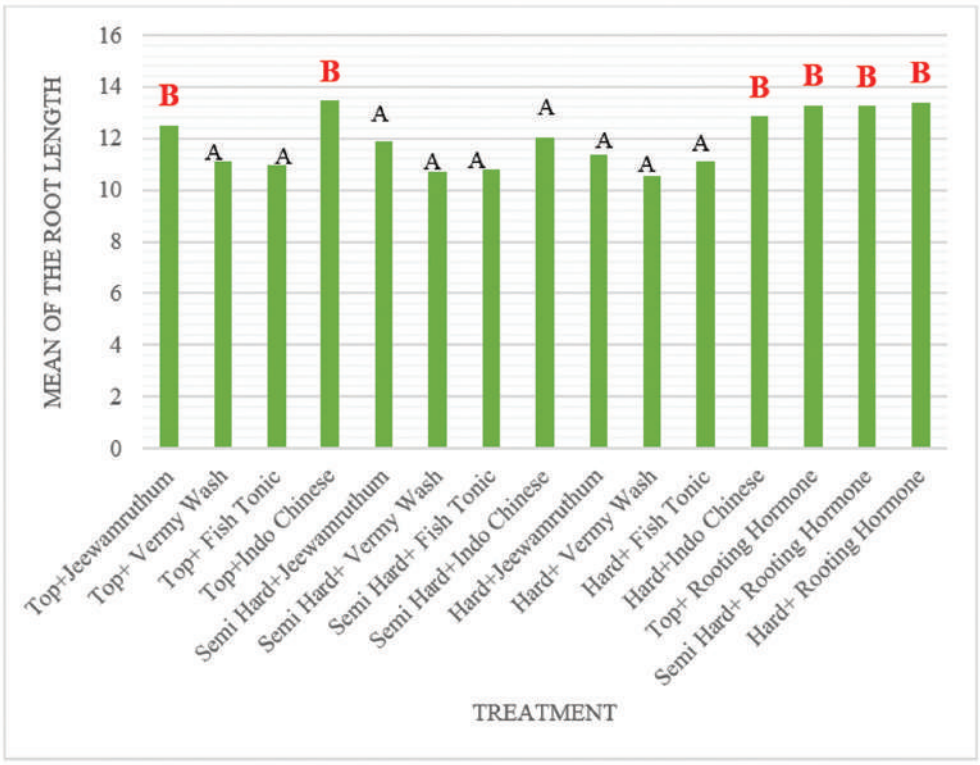

Figure 8: Effect of cutting type and different bio-fertilizers on mean root length in *Syzygium campanulatum* Korth, 11 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

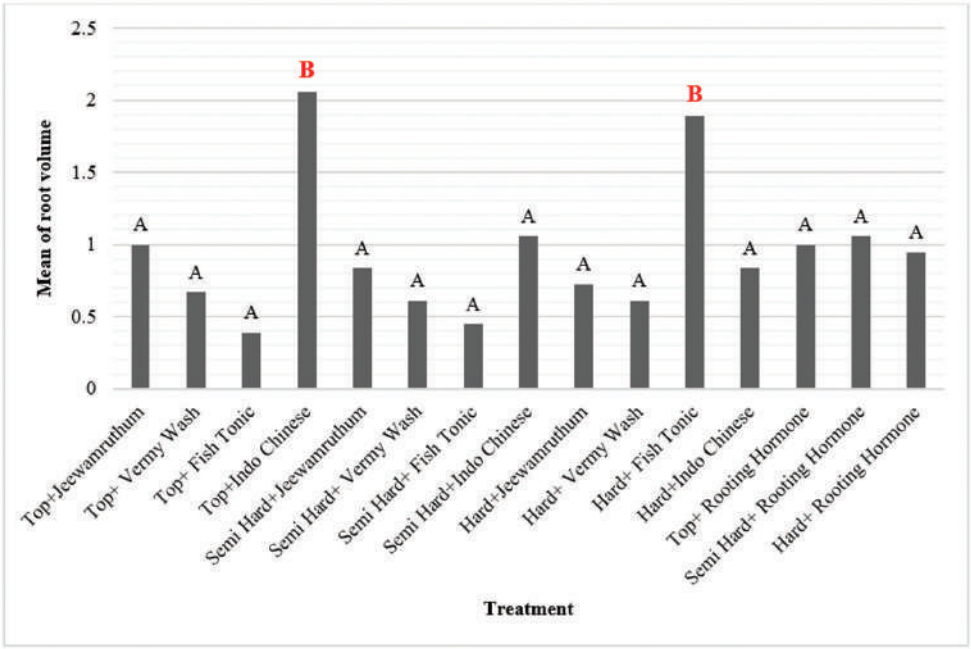

Figure 9: Effect of cutting type and different bio-fertilizers on mean root volume in *Syzygium campanulatum* Korth, 11 weeks after planting.

Means on the bars represent the same letter are not significantly different at P d™ 0.05 probability level.

Root Volume - Results had shown that, top cutting with Indo Chinese traditional microbial culture and hard wood cutting with the fish tonic were significantly affect for the amount of the root volume among the all treatments 11 weeks after planting (Figure 9).

Most of the reports on the usage of bio fertilizers emphasize the efficacy of bio control agents in enhancing plant growth, root growth and root volume in addition to their ability in increasing yield. Results of the present study are in agreement with Manoranjitham *et al*. (2000) and Manomohandas (2001). Plant growth regulators like gibberllins, cytokinins and indole acetic acid (IAA) induced by microbial strains might have contributed for better plant growth and development (Dubeikovsky *et al*., 1993).

Growth observations like plant height and number of leaves were also maximum in treatment T4 when compared to other treatment combinations. This might be

due to the cumulative effect of all organic bio fertilizers such as jeewamruthum, vermy wash, fish tonic and indo Chinese traditional microbial culture, due to the good water holding capacity, high porosity, increased surface area that provides many micro-sites for microbial activity and strong retention of nutrients. Previous studies reported that organic bio fertilizers improved soil productivity and fertility, which improved the propagation (Hossain and Ishimine, 2007, Velamurugan *et al*, 2007, Mohaopatra and Das, 2009 and Dinesh *et al*, 2010).

CONCLUSION

It may be concluded that *Syzygium campanulatum* Korth can be easily propagated by stem cuttings compared to using of air layering for the mass production at commercial scale in the floricultural industry in Sri Lanka. Results highlighted that type of stem cuttings for propagation were top cuttings and the best type of bio fertilizer was Indo Chinese Traditional Microbial Culture.

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Potting Media and the Propagation Methods Improve the Yield and Growth of *Rhynchoglossum gardneri*

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ABSTRACT

The effects of potting media and the propagation methods were studied of the *Rhynchoglossum gardneri*. Three potting media (Leaf mold + Topsoil + Sand 1:1:1, Coir pith + Sand 1:1 and Clay) were used to investigate the best potting media for both stem cutting and leaf cutting of R. gardneri plant. The result indicated that the highest growth performance was recorded in coir pith + sand 1:1 for the stem cuttings (experiment 01) and leaf mold + top soil + sand 1:1:1 for leaf cuttings (experiment 02).

Keywords: *Rhynchoglossum gardneri*, Potting media, Propagation, Growth performance, stem cuttings, leaf cuttings

INTRODUCTION

Rhynchoglossum gardneri belongs to the family Gesneriaceae and the genus Rhynchoglossum(Abdulrokhman, 2013). In this study using different potting media and the artificial propagation methods such as stem cutting and leaf cutting to improve the yield and growth of R. gardneri plant. The different growing media are providing factors to the growth of the plants and different cutting parts are enhancing the plant vegetative features.

MATERIALS AND MEHODS

The study was conducted as the two experiments, one for the stem cutting and two for the leaf cutting. The mother plants of *Rhynchoglossum gardneri* were collected from the Marathugoda, Kandy, Sri Lanka. The samples of plant were prepared to get the authentication from Royal Botanic Gardens-Peradeniya. The selected area was prepared and provided 80% shade nets because the *Rhynchoglossum gardneri* is the shade loving plants. There are three different potting media were prepared includes Leaf mold, Topsoil, and Sand ratio of 1:1:1, Coir pith and Sand ratio of 1:1 and clay. The media were sterilized (steam sterilization) and filled into the 5" inch plastic pots. The stem cuttings were prepared with 6-8cm with 2 nodes and leaf cuttings 2 mm length petiole. The both stem and leaf cuttings were established and applied the single propagators for each pot. CRD method was used for this experiment. There were four treatments with five replications. Analysis of variance and pair compression among treatment means were tested by Mini-Tab software. Number of leaves, Number of axillary branches, and the average height of plants per week of the plant were recorded.

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Table 1: Treatments used in these experiments 01 and 02

Treatments	Potting mixture	Ratio
T_0 (Control)	Leaf mold + Sand	1:1
T_1	Leaf mold + Topsoil + Sand	1:1:1
T_2	Coir pith + Sand	1:1
T_3	Clay	

RESULTS AND DISCUSSION

According to the experiment 01; the vegetative characteristics were significantly (P< 0.05) suppressed when compared with the Leaf mold + Sand (1:1) (control) (Table 2). The highest vegetative characteristics' were observed in stem cuttings planted on coir pith + sand (1:1) medium because of the plant potting media Coir pith + sand (1:1) were proved superior in all the vegetative features of the growth parameters studied such as the number of leaves, number of axillary branches, the average height of shoot per number of shoots, number of roots, the average length of roots, number of the root, and fresh weight because coir pith + sand (1;1) medium has good water and air retention ability (Mullaivananathan, Sathish and Kalaiselvi, 2017) then facilitate the rooting. The lowest vegetative characteristics' were observed in stem cuttings planted on clay medium when clay alone was used as the potting media; as they might be deficient which supports plant growth via, physical or chemical features of media and it contains limiting nutrients (Owen *et al*., 2007).

According to the experiment 02; the vegetative characteristics were significantly (P< 0.05) suppressed when compared with the Leaf mold + Sand (1:1) (control) (Table 3). The highest number of leaves was observed in leaf cuttings planted on leaf mold + top soil + sand (1:1:1) medium because of good water retention ability, consistent with rich nutrients which are enhancing the plant vegetative growth(Mehmood *et al*., 2013)and the lowest number of leaves was observed in leaf cuttings planted on coir pith + sand (1:1) medium. The highest fresh weight of the plant was observed in leaf cuttings planted on coir pith + sand (1:1) medium and the lowest fresh weight of the plant was observed in leaf cuttings planted on clay medium. The highest number of axillary branches, the average height of plants per week, number of roots and the average length of roots of the plant were observed in leaf cuttings planted on leaf mold+ top soil + sand (1:1:1) medium and the lowest number of axillary branches, the average height of plants per week, number of roots and the average length of roots were observed in leaf cuttings planted on clay medium.

Table 2: Effects of different potting media on the *Rhynchoglossum gardneri* stem cutting on after 6th week from the removing of propagators

Parameters	After 6th week from the removing of propagators			
	T_0	T_1	T_2	T_3
Number of leaves	16.200 ± 2.850^{a}	15.000 ± 1.260^{a}	21.400 ± 0.980^{a}	7.200 ± 0.970^{b}
Number of axillary branches	10.200 ± 1.280^{a}	5.400 ± 0.872^{b}	11.800 ± 1.070^{a}	3.200 ± 0.583^{b}
The average height of plants per week	7.220 ± 1.060^{b}	9.080 ± 1.270^{ab}	12.020 ± 0.819^{a}	5.700 ± 0.918
Number of roots	10.800 ± 2.350^{b}	9.600 ± 2.040^{b}	19.200 ± 1.590^{a}	4.400 ± 1.030^{b}
The average length of roots	7.460 ± 1.060^{ab}	7.920 ± 1.470^{ab}	10.833 ± 0.932^{a}	3.710 ± 1.450^{a}
Fresh weight of the plant	9.400 ± 3.060^{ab}	11.000 ± 2.450^{ab}	17.000 ± 3.390^{a}	2.200 ± 0.583^{b}

Mean values in a column having dissimilar letters/letters indicate significant differences at a 0.05% level of significance by Tukey's studentized test.

Table 3: Effects of different potting media on the *Rhynchoglossum gardneri* leaf cutting on after 6th week from the removing of propagators.

Parameters	After 6th week from the removing of propagators			
	T_0	T_1	T_2	T_3
Number of leaves	17.800 ± 1.770^{b}	23.800 ± 1.240^{a}	4.400 ± 0.678^{d}	10.800± 1.770^{c}
Number of axillary branches	8.400 ± 0.812^{b}	15.000 ± 1.000^{b}	3.000 ± 0.447^{c}	5.800 ± 0.860^{bc}
The average height of plants per week	8.570 ± 1.280^{a}	7.790 ± 1.120^{ab}	3.240 ± 1.130^{bc}	3.100 ± 1.030^{c}
Number of roots	14.800 ± 0.970^{b}	23.400 ± 1.660^{a}	14.600 ± 2.230^{b}	13.000± 0.894^{b}

Mean values in a column having dissimilar letters/letters indicate significant differences at a 0.05% level of significance by Tukey'sstudentized test.

Experiment 01 (Stem cutting plants)

Treatment 01

Treatment 02

Treatment 03

Treatment 04

Experiment 02 (Leaf cutting plants)

Treatment 01

Treatment 02

Treatment 03

Treatment 04

CONCLUSION

According to the both experiment 01 and 02 ; results; coir dust + sand (1:1) medium was best for enhance the vegetative characteristics performances in stem cuttings 6cm-8cm length with two nodes and leaf mold + top soil + sand (1:1:1) medium was best for enhance the vegetative characteristics performances in leaf cuttings, leaf with 2mm petiole of R. gardneri.

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