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INVESTIGATION OF SUITABLE PROTOCOL FOR *IN-VITRO* PROPAGATION OF *Kendrickia walkeri*

M. A. T. NIROSHINI [a], H. K. L. K. GUNASEKERA [a*], S. A. KRISHNARAJAH [b] AND M. M. D. J. SENARATNA [b]

[a] Department of Agricultural and Plantation Engineering, Faculty of Engineering Technology, The Open University of Sri Lanka, Sri Lanka.

[b] National Botanical Gardens, Peradeniya, Sri Lanka.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Kendrickia walkeri, is an epiphytic climbing shrub that belongs to the Melastomataceae family and it is vulnerable and included to the National Red List 2012 in Sri Lanka. Hence present study was aimed to find out suitable potting media for vegetative propagation and to develop suitable protocol for in-*vitro* propagation of *Kendrickia walkeri*. The pot experiment was laid out in a Completely Randomized Design (CRD) with three treatments randomized in seven replicates. Treatments were three different potting media i.e. coir dust only, coir dust: sand and coir dust: leaf mold: sand. Semi-wood stem cuttings were planted and each pot were kept in single propagators for twenty one days. After that, the data were obtained on stem height, number of new leaves, number of shoots and number of roots with two weeks intervals. Data were tabulated and analyzed using Analysis of Variance (ANOVA) procedure of Statistical Analyzing System (SAS). Duncan's New Multiple Range Test (DNMRT) was performed to compare the differences among treatment means at p=0.05. Among different treatments the highest stem height, number of leaves/shoots as well as roots per plant was recorded in coir dust, leaf mold and sand medium. The laboratory experiments was laid out in a Completely Randomized Design (CRD) with six treatments randomized in four replicates. Treatments were different protocols i.e. 1% $HgCl_2$ (3 minutes), 1% $HgCl_2$ (4 minutes), 2.5 % Clorox + 1% $HgCl_2$ (4 minutes), 5 % Clorox + 1% $HgCl_2$ (4 minutes), 7.5 % Clorox + 1% $HgCl_2$ (4 minutes) and 2.5 % Clorox + 1% $HgCl_2$ (5 minutes). Nodes were treated according to the protocols and inoculated in Murashige and Skoog (MS) medium supplemented with 1.5 mg/ L benzyl amino purine (BAP). The data of number of survived explants were taken with three days intervals. The highest number of survived explants were recorded under, 2.5 % Clorox + 1% $HgCl_2$ (5 minutes) protocol. Hence, coir dust, leaf mold and sand medium can be considered as a successful potting media for vegetative propagation of *K. walkeri* by nodal stem cuttings and *K. walkeri* exposed to 70 % ethanol for 15 seconds, 2.5% Clorox for 5 minutes and 1% $HgCl_2$ for 5 minutes duration, can be recommended for direct plantlet regeneration from nodal explant of *Kendrickia walkeri* and further studies for micro propagation technique.

Keywords: *Kendrickia walkeri*; vegetative propagation; *in-vitro* protocol; red list.

**Corresponding author: Email: hkgun@ou.ac.lk;*

1. INTRODUCTION

Kendrickia walkeri, Hook, is an epiphytic climbing shrub that belongs to the Melastomataceae family and climbs by adventitious roots. This climbing shrub is vulnerable in Sri Lankan nature and it is included to The National Red List of Flora and Fauna of Sri Lanka, 2012. *Kendrickia walkeri* is seen growing only in the mountain forest area in Kandy, Nuwara Eliya, Badulla, and Ratnapura districts. Kandy district- Rangala-knuckles, Maskeliya-Balangoda, Kotiyagala Mountain, Adam'sPeak, Hantane. Nuwara Eliya district- Matata, Rmboda pass. Badulla District – Namunukula. Ratnapura district –Pinnawala, Rajakanda Kellie, Sinharaja forest, weddagala entrance [1]. Research conducted in the past on propagation methods of *Kendrickia walkeri* in Sri Lanka is minimal [2]. "Diverse climatic conditions, which resulted due to high geographical heterogeneity as well as due to its positioning in the Sri Lankan terrain, have led the Knuckles massif to possess a wide range of rainfall and temperature regimes" [3,4]. "Plant biotechnology provide new options for collection, multiplication and short- to long-term conservation of plant biodiversity, using in vitro culture techniques. Significant progress has been made for conserving endangered, rare, crop ornamental, medicinal and forest species, especially for non-orthodox seed and vegetatively propagated plants of temperate and tropical origin. Cell and tissue culture techniques ensure the rapid multiplication and production of plant material under aseptic conditions" [5]. Most of the flora in family Melastomataceae including *Kendrickia walkeri* have attractive characteristics and values suitable for the floriculture industry. Conservation of this *Kendrickia walkeri* is very important to secure the genus and the species. Hence, this study is aimed to find out proper conservation methods of *KW* by using modern technology.

2. MATERIALS AND METHODS

2.1 Experimental Procedure

This study was conducted as two experiments, i.e. pot experiment and laboratory experiment. The pot experiment was aimed to evaluate suitable potting media for vegetative propagation by semi-wood stem cuttings and the laboratory experiment was carried out to develop suitable *in-vitro* protocol for nodal explants of *Kendrickia walkeri.*

2.2 Location

The pot experiment one was arranged in a net house, covered with 75% black shade net and the laboratory experiment was conducted in the Tissue Culture Research Laboratory of Floriculture and Research Development Unit of the Department of National Botanic Gardens, Peradeniya, during the period from June to December 2018. The experimental site is located in the Mid Country (IM3a) of Sri Lanka. The climate condition is characterized by annual rainfall of 170 mm – 2250mm and average temperature of 24.6^0C.

2.3 Pot Experiment

2.3.1 Experimental design

The experiment was conducted as a Complete Randomized Design (CRD) with three treatments randomized in seven replicates. Treatments were three different potting media, consisting of sand, coir dust and leaf mold used as given below.

T1 - Coir dust.
T2 - Coir dust + Sand.
T3 - Coir dust + Leaf mold + Sand.

2.3.2 Planting materials

Semi-wood stem cuttings were taken from the existing *Kendrickia walkeri* climber in the Arboretum Nursery at Department of National Botanic Gardens, Peradeniya including 3-4 nodes for each stem cutting.

2.3.3 Preparation of potting media

Coir dust and sand were sieved using a 2 mm mesh and three types of media i.e. Coir dust only, Coir dust + sand (5:1) and coir dust + leaf mold + sand (8:2:1) were prepared and sterilized at 121 ^{0}C, 15 psi for 20 minutes. 4.5-inch diameter black plastic pots were dipped overnight in commercial fungicide (Mancozeb) 2 g/ L concentrated solution and sterilized potting mixtures were filled. A hormone powder (Rapid Root) water mixture was applied to the bottom cutting edge of each semi-wood stem cutting before planting. After planting each pot was kept in a single propagator prepared with cellophane at 16-inch length and kept in the net house for 21 days. After twenty one days plants were gradually transferred to the environment.

2.3.4 Data collection

The height of stem cuttings, number of leaves, number of shoots and number of roots were obtained after twenty one days at two weeks intervals and four readings were collected.

2.3.5 Statistical analysis

The data were tabulated and analyzed subjected to the Analysis of Variance (ANOVA) procedure of the

Statistical Analysis System (SAS). Duncan's New Multiple Range Test (DNMRT) was performed to compare the differences among treatment means at p=0.05.

2.4 Laboratory Experiment

2.4.1 Experimental design

The experiment was carried out as a Completely Randomized Design (CRD) with six treatments randomized in four replicates. Each replicate contains three explants and twelve explants were used per treatment. The different concentrations of commercial bleaching solution (Clorox) and time duration of 1% $HgCl_2$ were changed within the treatments as given below. Six preliminary test (PT) were done prior to treatments T1, T2, T3, T4, T5, and T6 due to minimal *in-vitro* protocol developed for this variety.

T1 - 1% $HgCl_2$ (3 minutes).
T2 - 1% $HgCl_2$ (4 minutes).
T3 - 2.5 % Clorox + 1% $HgCl_2$ (4 minutes).
T4 - 5 % Clorox + 1% $HgCl_2$ (4 minutes).
T5 - 7.5 % Clorox + 1% $HgCl_2$ (4 minutes).
T6 - 2.5 % Clorox + 1% $HgCl_2$ (5 minutes).

Preliminary Tests:

PT1 - Distilled water.
PT2 - 5 % Clorox + 5 % Clorox.
PT3 - 10 % Clorox + 10 % Clorox.
PT4 - 15 % Clorox + 15 % Clorox.
PT5 - 20 % Clorox + 20 % Clorox.
PT6 - 25 % Clorox + 25 % Clorox.

2.4.2 Planting materials

Nodal cuttings of 1 - 1.5 cm were obtained from the existing *Kendrickia walkeri* climber in the Arboretum Nursery of Department of National Botanic Gardens, Peradeniya.

2.4.3 Stock preparation

Chemicals were weighed for stock A, B, C, micro elements, iron and vitamin solutions using a precision balance. (Table 3) They were dissolved separately in distilled water and one liter was prepared of each solution. 50 ml of 1 mg/ 1 ml 6-benzylaminopurine (BAP) stock solution was prepared by dissolving 50 mg of BAP in 2.5ml of 1N HCl and using distilled water.

2.4.4 Media preparation

1.5 L of full strength Murashige and Skoog's medium (MS medium, 1962) supplemented with 2 mg/l 6-benzylaminopurine (BAP) was prepared.3% w/v of sucrose was added to the medium and pH of the medium was adjusted to 5.6 before adding 0.8% w/v agar. The medium was boiled and dispensed as 20 ml per 125ml jam jar. Jars were covered with 5x5 inch cellophane and rubber bands and sterilized at 121 ^{0}C, 15 psi for 20 minutes.

2.4.5 Explant sterilization

Explants i.e. 1-1.5 cm nodes were collected from the existing *Kendrickia walkeri* climber and put into water. Explants were taken into the laboratory, thoroughly washed under tap water with liquid soap and rinsed twice. 2 drops of commercial detergent (Teepol) were added and swirled for 10 minutes. Explants were kept in running tap water for 20 minutes. Washed nodes were taken into the laminar flow hood and immersed in 70% Ethanol for 15 seconds and rinsed twice with sterilized distilled water. Explants were treated with 0, 5, 10, 15, 20 and 25 % commercial Clorox solutions for 10 minutes and repeated with the same concentrations of Clorox by reducing time for 10, 10, 10, 5 and 5 minutes. These treatments were done as preliminary test for the protocol and thereafter due to considerable contaminations percentage of explants $HgCl_2$ was included to the protocol. Explants were washed with 0, 2.5, 5, and 7.5 % Clorox solution for 5 minutes, rinsed three times using sterilized distilled water and subsequently explants were treated with 0.1 % $HgCl_2$ solution by gradually increasing time for 3 to 5 minutes.

2.4.6 Culture condition

Sterilized nodes were trimmed and place in MS + BAP medium, each culture vessel (125 ml jam jar) contained three explants. Cultures were incubated under controlled-environment growth room at 25±2 0C with 16-h photoperiod under cool white light for shoot initiation.

2.4.7 Data collection

Data on number of explants that survived and remained healthy were obtained at three days interval from the date of culture and seven readings were obtained.

2.4.8 Statistical analysis

Data were tabulated and analyzed subjected to Analysis of Variance (ANOVA) procedure of the Statistical Analysis System (SAS). Duncan's New Multiple Range Test (DNMRT) was performed to compare the differences among treatment means at p=0.05. The strength of the relationships between

growth parameters of *Kendrickia walkeri* was estimated by linear correlation analysis.

3. RESULTS AND DISCUSSION

3.1 The Pot Experiment

Stem Height (cm): Stem heights (cm) had significant differences at ($p<0.05$) among different treatments tested. The highest stem height was recorded from treatment three, i.e. coir dust, leaf mold and sand used in a ratio of 8:2:1 while the lowest from treatment one, i.e. control with coir dust only. Treatment two, i.e. consisting of coir dust and sand did not show significant difference with the control treatment (Table 1).

Table 1. Effect of different media on stem height (cm) of *Kendrickia walkeri*

Treatment	Stem height (cm)
T1	8.07^{b}
T2	8.17^{b}
T3	8.50^{a}

Note: Means with same letters along the column are not significantly different at p<0.05. Measurements are the means of seven replicates

Number of Leaves: Among different treatments tested the highest number of leaves per stem was recorded from treatment three, i.e. coir dust, leaf mold and sand and the lowest was from treatment one, i.e. control treatment. However, treatment one i.e. coir dust only and treatment two, i.e. coir dust and sand medium did not show significant difference at $p<0.05$ (Table 2). Coir dust, leaf mold and sand medium was recorded the best performance on number of leaves than other two types of media, it might be the availability micro nutrients and the soil properties that influenced by leaf mold on the growing medium. The higher leaf number was shown of Pothos (*Epipremnum aureum*) in the media containing 3:1 leaf-mold/coco peat mixture [6].

Table 2. Effect of different media on number of leaves of *Kendrickia walkeri*

Treatment	Number of leaves
T1	7.64^{b}
T2	8.68^{b}
T3	10.75^{a}

Note: Means with same letters along the column are not significantly different at p<0.05. Measurements are the means of seven replicates

Number of shoots: Number of shoots is a very important parameter when considering a plant for introduction to the floriculture industry. There was significantly different at $p<0.05$ between all treatments, i.e. coir dust, coir dust + sand and coir dust + leaf mold + sand media. Treatment one, i.e. control, was shown lowest number of shoots and Treatment three, i.e. coir dust, leaf mold and sand medium was recorded the highest number of shoots. According to the results it might be leaf mold was increased the availability of micro nutrient and porosity, soil aeration. "Shoot number was higher of Pothos (*Epipremnum aureum*) in the medium containing equal leaf mold : sand mixture compared to the other media" [7].

Table 3. Effect of different media on number of shoots of *Kendrickia walkeri*

Treatment	Number of shoots
T1	0.54^{c}
T2	1.71^{b}
T3	2.54^{a}

Note: Means with same letters along the column are not significantly different at p<0.05. Measurements are the means of seven replicates

Number of roots: Number of roots per stem showed significant different at $p<0.05$ between treatment three (i.e. coir dust, leaf mold and sand) and treatment two (i.e. coir dust and sand). However, there was no significant difference between treatment two and treatment one (i.e. coir dust only medium) at $p<0.05$ (Table 4). "Mean number of roots per stem cuttings planted on compost: coir dust medium at the fourth month differed significantly from sand medium and the compost: sand medium. Compost: coir dust medium produced the highest number of roots of *Osbeckia octandra"* [8]. According to the results root formation showed that, "semi hard wood produced higher number of roots in media one (Coir dust + sand + compost), two (Coir dust + sand) and three (Coir dust only) respectively. The lowest number of roots was observed in soft wood cuttings planted in rooting medium one (Coir dust + sand + compost) and soft wood cuttings produced less number of roots in all media" [9].

Table 4. Effect of different media on growth of number of roots of *Kendrickia walkeri*

Treatment	Number of roots
T1	5.57^{b}
T2	7.39^{b}
T3	9.79^{a}

Note: Means with same letters along the column are not significantly different at p<0.05. Measurements are the means of seven replicates

Furthermore, Coir dust has less porosity and higher water holding capacity while sand has a larger particle size and less water holding capacity , However when added together the medium has a balanced water

holding capacity and moisture that is most suitable for growth of shoots and rooting of cuttings. In this experiment, higher results was shown on leaf mold contained media than other two types of media it might be micro elements that derived from leaf mold and improving soil porosity and soil aeration. Leaf mold involves fungal breakdown and it is cool, slow process. *Kendrickia walkeri* is a climbing shrub on a tree trunks and showing well growth on a natural layer of leaf mold in the forests.

3.2 The Laboratory Experiment

3.2.1 Number of survived explants

Number of explants that survived was not significantly different ($p<0.05$) among treatments T6, T5 and T4 [i.e. T6, 2.5 % Clorox + 1% HgCl2 (5 minutes), T5, i.e. 7.5 % Clorox + 1% HgCl2 (4 minutes), T4, i.e. 5 % Clorox + 1% HgCl2 (4 minutes)]. Overall results showed that T6, i.e. 2.5 % Clorox + 1% HgCl2 (5 minutes) was the most effective treatment for sterilization of *Kendrickia walkeri* nodal explants for micro propagation. "*In vitro* culture produced phenolic exudates from cut ends and the surrounding medium browned severely during the first week. Sodium hypochlorite has turned out to be a better sterilant than calcium hypochlorite due to bleaching effects of the later, amongst the two sterilants i.e. NaOCl and HgCl2, NaOCl was found to be better for controlling infection and it did not have any adverse effect on explants even after long duration of exposure" [6,10].

Table 5. Effect of different protocols on sterilization of *Kendrickia walkeri* nodal explants

Treatment	Number of survived explants
T1	0.17^{b}
T2	0.25^{b}
T3	0.25^{b}
T4	0.41^{ab}
T5	0.50^{ab}
T6	0.75^{a}

Note: Means with same letters along the column are not significantly different at p<0.05. Measurements are the means of four replicates

3.3 Correlation Analysis

When linear correlation analysis was performed for the growth parameters of *Kendrickia walkeri,* a highly significant positive correlation ($p<0.0001$) was observed between number of leaves and number of shoots formation during the study period (Table 6).

Table 6. Linear correlation coefficient between growth parameters of *Kendrickia walkeri*

	SH	NLE	NSH	NRO
SH	-			
NLE	0.69045**	-		
	0.0005			
NSH	0.55975**	0.75065***	-	
	0.0083	0.0001		
NRO	0.16386^{ns}	0.58094**	0.47838	-
	0.4779	0.0058	0.0283*	

*Note: ns – non significant at p=0.05, * - significant at p<0.05, ** -significant at p<0.01, *** - significant at p<0.0001, SH-Stem height, NLE-Number of leaves, NSH-Number of shoots, NRO-Number of roots*

4. CONCLUSION

A significantly higher ($p<0.05$) stem length, number of , new leaves/ shoots as well as roots were manifested from plants potted in coir dust + leaf mold + sand medium. This is a successful potting media for vegetative propagation of *Kendrickia walkeri* by nodal stem cuttings [11,12]. *K. walkeri* exposed to 70 % ethanol for 15 seconds, 2.5% Clorox for 5 minutes and 1% $HgCl_2$ for 5 minutes duration produced highest percentage (75%) of contamination free cultures in *in-vitro* establishment. In addition, this *in-vitro* protocol for direct plantlet regeneration from nodal explant of *Kendrickia walkeri* will be useful to understand the potential of micro propagation of this rare ornamental climber for further studies as well as conservation purposes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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