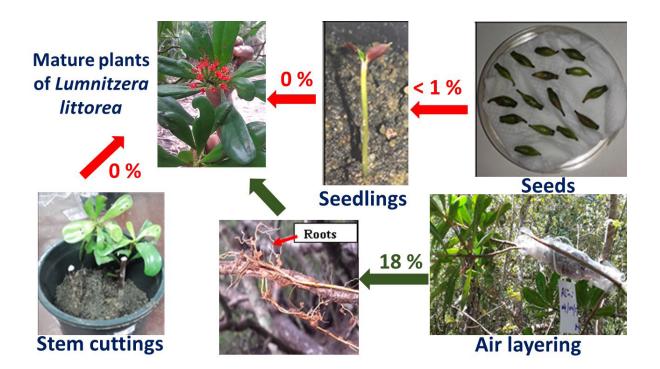
RESEARCH ARTICLE

Vegetative propagation of critically endangered mangrove *Lumnitzera littorea* (Jack) Voigt in Madu Ganga RAMSAR site of Sri Lanka, towards its conservation

P.L.M.M. Perera*, K.M.G.G. Jayasuriya, J.W. Damunupola, A.M.T.A. Gunaratne and M.G.M. Prasanna



Highlights

- About 17.5% of air layered branches of *Lumnitzera littorea* produced roots, root initials or callus within 4-26 weeks.
- A high phenol concentration was recorded in the stems.
- An uninterrupted vascular connection with the mother plant is important for the adventitious root formation of the species.

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Vegetative propagation of critically endangered mangrove *Lumnitzera littorea* (Jack) Voigt in Madu Ganga RAMSAR site of Sri Lanka, towards its conservation

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Abstract: Considering the environmental and specific socioeconomic significance of the critically endangered mangrove L. littorea in Sri Lanka, this study was conducted to prepare a vegetative propagation protocol for this species with air layering and stem cuttings. Air layering was conducted with coir dust moistened with distilled water or 0.3% Indole-3-butyric acid (IBA) solution. Stem cuttings were treated with different concentrations of IBA, Naphthalene Acetic Acid (NAA) or Albert solution prior to be transplanted in the potting media. Approximately, 17.5% of the air layered branches produced roots, root initials or callus within 4-26 weeks whereas stem cuttings did not produce adventitious roots within the study period. It is essential to maintain an uninterrupted vascular connection between the area of rooting and the mother plant for adventitious root formation. Although a high phenol concentration was determined in stems, no structural barriers for adventitious root formation were identified in stems. In future research, it is recommended to apply a broader range of rooting hormones and combinations of hormones for stem cuttings to induce the formation of adventitious roots. Air layering was recommended to propagate this critically endangered species as it was the only successful method and is a cost-effective simple technology.

Keywords: Lumnitzera littorea, vegetative propagation, air layering, stem cuttings, conservation.

INTRODUCTION

Lumnitzera littorea (Jack) Voigt (E: Red Teruntum, S: Rathamilla) of Family Combretaceae is a true mangrove, indigenous to Sri Lanka (Dassanayake *et al.*, 1995) (Figure 1). Although this species has a wide distribution in tropical Asia, locally it was known only from few locations (Jayatissa *et al.*, 2002; de Silva and de Silva, 2006; Prassanna and Ranawana, 2014). At present mangrove vegetation located in the Pathamulla area of the lower reaches of Madu Ganga wetland is the only habitat of this species remaining in Sri Lanka (Bambaradeniya *et al.*, 2002; Jayatissa *et al.*, 2002; Prasanna and Ranawana, 2014). Further, this remaining plant population was restricted to few trees and the mangrove ecosystem of the location was under immense pressure due to clearing for developmental purposes (Perera *et al.*, 2019). It is an aged population with

a low regeneration potential (Perera *et al.*, 2019). Although it is only used by the local villagers for medicinal purposes (personal communication with the villagers), in other countries it is being used for several purposes. In Thailand and Singapore, *L. littorea* is grown as an ornamental tree due its conspicuous red flowers (Ellison *et al.*, 2010). Especially it is planted along banks of ponds (Ellison *et al.*, 2010). Wood of this species is used for boat building and other construction purposes and also as fuel wood (Ellison *et al.*, 2010).

Mangroves have a little capacity for vegetative propagation and therefore dependent on seedlings for mangrove forest maintenance and spread (Tomlinson, 1986). Mangroves can be propagated via seeds and fruits easily. Stem cuttings and air layering are the major methods of vegetative propagation of mangroves as these are low cost, less time consuming, simple technologies (Clough, 1993; de Silva and Amarasinghe, 2010; Wetlands International, n.d.). Further, propagule cuttings are also used for propagation of viviparous species.

Although the fruit set is high, in *L. littorea*, a high percentage of mature fruits were empty (Tomlinson, 1994) due to the predation by a caterpillar of a moth belonging to family Gelechiidae (Perera *et al.*, 2019). Further, mature seeds have deep physiological dormancy and thus, germination of seeds is significantly low (Yong *et al.* 2004; Perera *et al.*, 2019). Thus, it is important to study alternative ways of propagation such as vegetative propagation to conserve this valuable species for future.

Certain attempts to propagate *L. littorea* by vegetative propagation methods in Sri Lanka were successful. Hettiarachchi *et al.* (2002) claimed that about 80% air layered branches formed well-developed roots during their study. Further, ~30% rooting had been observed in girdle cuttings, which were immersed in distilled water. However, they have not given much details about their air-layering procedure. Furthermore, Eganathan and Rao (2001) and Wetlands International (n.d.) have reported that mangroves such as *Lumnitzera* spp. can be propagated through stem cuttings and air layering. According to Wetlands International, n.d., stem cuttings of 12-15 cm



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Figure 1: Some important characters of L. littorea plant; A) red conspicuous flowers and B) fruits.

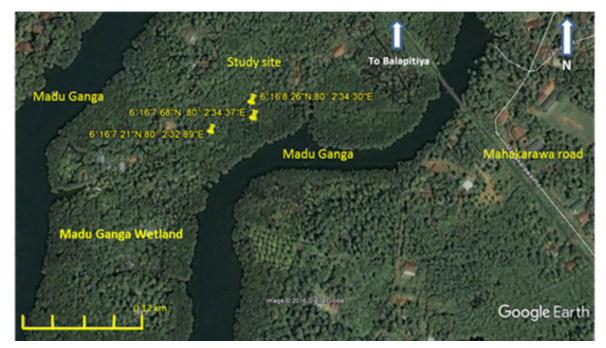


Figure 2: Study site in the Madu Ganga wetland (source: www.google.lk; downloaded on 14/04/2017).

length obtained from healthy branches should be planted in polythene bags with the application of plant hormones such as Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) to promote rooting. They recommend using rooting hormones in air layering to get successful results. However, it is not clear whether these recommendations could be used specifically to propagate *L. littorea*.

Lack of scientific evidence for success of vegetative propagation made us to prepare a vegetative propagation protocol for *L. littorea* with air layering and stem cuttings. Further, considering its ecological and socio economic significance, restricted distribution to Madu Ganga

wetland and very low seed germination of the remaining aged population in Sri Lanka, this study was aimed at the conservation of *L. littorea*.

MATERIALS AND METHODS

Study site and collection of plant material

The study site was the only existing location of *L. littorea* in Sri Lanka, a private land in Pathamulla, Balapitiya located in the Madu Ganga Ramsar site and sanctuary (06.26896° N, 080.04286° E and 06.26867° N, 080.04247° E) (Bambaradeniya *et al.*, 2002; Carder, 2001; Munasinghe,

Table 1: Air layering experiments performed for L. littorea plants

Treatment	Rooting medium	No: of replicates
Tap water	Coir dust	4
Distilled water	Pre-treated coir dust	6
Brackish water	Pre-treated coir dust	6
Tap water + IBA	Coir dust	6
Brackish water + IBA	Coir dust	6
Distilled water + IBA	Pre-treated coir dust	6
Brackish water + IBA	Pre-treated coir dust	6

IBA-Indole-3-butyric acid

2010; Silva *et al.*, 2013) (Figure 2). There were only 18 trees of *L. littorea* in the site (Perera *et al.*, 2019). True mangroves *Excoecaria agallocha* (Euphorbiaceae) and *Heritiera littoralis* (Sterculiaceae) and mangrove associates *Dolichandrone spathacea* (Bignoniaceae) and *Cerbera odollam* (Apocynaceae) were present in association with *L. littorea* in the study site (Perera *et al.*, 2019). No saplings of *L. littirea* were found in the site and only two seedlings

were observed soon after the fruiting season.

Vegetative propagation through air layering

For air layering, healthy branches with ~ 1 cm diameter, attached to the L. littorea mother plants were selected. A strip of bark of about 1 cm width was removed retaining a bridge of bark between the wounded portion and mother plant. Pretreated and fresh coir dust moistened with distilled water, tap water or brackish water was placed around the wounded portion as the rooting medium and wrapped with polythene tight enough to hold the coir dust and prevent seeping of water inside. Fresh coir dust moistened with distilled water was used as the control experiment. Commercial rooting hormone powder (0.3% IBA) was applied before wrapping along the wounded portion of some of the samples using a fine brush, as a rooting hormone treatment (modified from Eganathan et al., 2000; Eganathan and Rao, 2001). Coir-dust was water soaked for 24 hrs before the experiment as the pretreatment. Air layering experiments performed during the research are summarized in Table 1.

Vegetative propagation through stem cuttings

Following Eganathan et al. (2000) and Eganathan and Rao (2001) procedure, two types of stem cuttings; hardwood and softwood each about 15 cm long were used in the stem cutting experiment. Hardwood cuttings were ~ 1 cm in diameter, while soft wood cuttings were ~ 0.5 cm diameter. All cuttings were obtained from the mother plant under water. Leaves of the cuttings were either partially or completely removed. Stem cuttings were subjected to different treatments in three different trials. Cuttings were placed in the propagators after treatments. Propagators were prepared by filling plastic pots with the potting medium and pots were completely covered with polythene after placing stem cuttings. About one third of the cutting was placed below the potting medium. Propagators were placed at ambient plant house temperature (~ 27 ± 2 °C) and natural light conditions.

Another set of stem cuttings were kept immersed in test tubes containing Albert's solution. A strip of bark of cuttings was removed under water near the lower cut end. In the first, second and third trials stem cuttings were observed for root emergence after 3, 6 and 13 weeks respectively. In second and third trials cuttings were treated to remove phenolic compounds using the procedure mentioned by Eganathan and Rao (2001). Vaseline was applied on the exposed upper cut end of stem cuttings after potting. Different treatments performed for the stem cuttings in three trials are summarized in Table 2. Experiments were repeated at least once.

Identification of structural barriers for rooting of stem cuttings

As none of the stem cuttings were rooted in the above stem cutting experiment, anatomical observations were made on the cutting side of the stem cuttings. Hand sections of hardwood and softwood stems of *L. littorea* were made and stained with safranin. Stained sections were observed under the light microscope (CX21FX1, Olympus cooperation, Tokyo, Japan). Possible structural barriers for rooting were observed.

Phytochemical screening of the stem extracts of *Lumnitzera littorea* for phenols

Stem cuttings of ~ 1cm length were oven dried at 40 °C for 24 hrs and powdered using a grinder. Weighed 5 g of stem powder was dissolved in 50 ml of methanol and kept undisturbed at room temperature for 24 hrs. Filtrate was treated with 5% ferric chloride and observed for the colour change (Solomon *et al*, 2013).

Determination of phenol concentration of the stems of *Lumnitzera littorea*

Five grams of stem tissue powder was extracted by stirring in 200 ml of distilled water at 65 °C and methanol at 25 °C at 150 rpm separately. Extracts were centrifuged at 5000 rpm for 5 minutes. Extracts were filtered and kept in the dark at 4 °C for further analysis. To determine the phenol concentration, a 0.1 ml of the extract (water and methanol extracts separately) was mixed with 2.5 ml of Folin-Ciocalteu (FC) reagent and 1 ml of 7.5% Na₂CO₃ and diluted with 8 ml distilled water and left to stand at 65 °C for 20 min. The blue colour of the reaction was measured using a UV spectrophotometer at 765 nm (SHIMADZU UV 1800, Shimadzu Scientific Instruments

Trial No:	Treatment	No: of replicates		Potting medium
		Hard wood	Soft wood	
1	None	11	10	Sand:top soil:organic matte = 1:1:1
2	Distilled water	5	5	Mangrove soil
	IBA 1000 ppm	5	5	
	IBA 1500 ppm	5	5	
	IBA 2000 ppm	5	5	
	IBA 2500 ppm	5	5	
	NAA 1000 ppm	5	5	
	NAA 1500 ppm	5	5	
	NAA 2000 ppm	5	5	
	NAA 2500 ppm	5	5	
3	Sterile distilled water	5	5	Sand:top soil:compost=1:1:1
	IBA 500 ppm	5	5	
	IBA 1000 ppm	5	5	
	IBA 1500 ppm	5	5	
	IBA 2000 ppm	5	5	
	IBA 2500 ppm	5	5	
	NAA 500 ppm	5	5	
	NAA 1000 ppm	5	5	
	NAA 1500 ppm	5	5	
	NAA 2000 ppm	5	5	
	NAA 2500 ppm	5	5	
	IBA powder	5	5	
	NAA powder	5	5	
	Albert solution full strength	5	5	
	Albert solution half strength	5	5	
	Albert solution quarter strength	5	5	

ANOVA procedure in the MINITAB (Version 14.1) statistical software was used in data analysis.



Figure 3: Observations of air layering experiments; A) root initials or callus and B) Roots. RI-root initials; RO-roots.

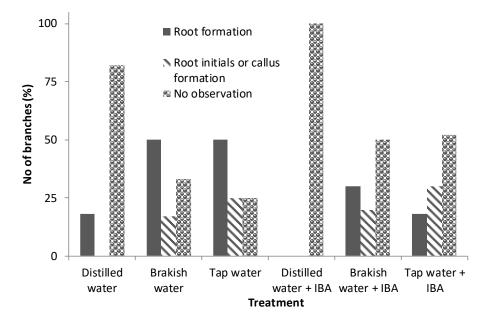


Figure 4: Percentage of *L. littorea* branches with root initials and callus or roots after 30 days from air layering with different rooting media.

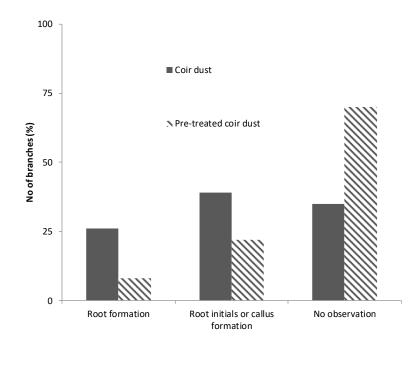




Figure 5: Percentage of *L. littorea* branches with roots, root initials or callus observed after 30 days from air layering with pretreated or non-treated coir dust rooting media.

Inc., Columbia, MD, USA). Gallic acid was used as the standard. Two-sample t-test was performed at 0.05 level of significance to determine whether there is a significant difference in the mean phenol concentration between methanol and water extracts (Capannesi *et al.*, 2000; Suh *et al.*, 2014). Phenolic content data were analyzed with one-way ANOVA procedure in the MINITAB (Version 14.1) statistical software was used in data analysis.

RESULTS

Propagation via air layering

Approximately 17.5% of air-layered branches of *L. littorea* produced roots, root initials or callus within 4 - 26 weeks (Figure 3A and 3B). Figure 4 presents the percentage of air-layered branches with root initials, callus or roots.

Compared to branches air layered with coir dust moistened with distilled water, those moistened with brackish water and tap water, had more root initials or callus. Distilled water + IBA treatment was not successful in initiating roots within the period of study. Compared to brackish water + IBA treatment, tap water + IBA treatment was successful in producing roots. Twenty five percent of the branches produced roots in untreated coir dust medium, and 37.5% of these branches had root initials or callus formed (Figure 5). However, only 21% of the air-layered branches with pretreated coir dust had callus and/or root initials, while only 8.3% of them had roots. In four of the air-layered branches, roots formed directly from the debarked area, while in other two instances roots developed from either root initials or callus. When callus or root initials did not develop into roots they disappeared with time.

Structural barriers of stems of *Lumnitzera littorea* for rooting

Hard structures that could act as barriers for adventitious root emergence such as rings of sclerenchyma fibers or stone cells were not observed in the hardwood or softwood stem cross sections of *L. littorea*. Instead the general anatomical structure of a dicotyledonous plant was observed. Cortex was consisted with chlorenchyma cells. Hypodermis and epidermis were the outermost layers and consisted of multiple layers. Cellular deposits were observed in parenchyma cells of the cortex and of the phloem.

Presence of phenols and phenol concentration of the stems of *Lumnitzera littorea*

Stem extracts formed a deep blue colour solution when treated with 5% Ferric Chloride solution indicating the presence of phenols. For water extracts and methanol extracts, mean phenol concentrations were 33.90 ± 6.01 mg/g and 123.94 ± 17.91 mg/g stem tissue, respectively. There was no significant difference in the mean phenol concentration between water and methanol extracts (p = 0.092).

DISCUSSION

During the study, root initials or callus and roots were observed only in $\sim 17.5\%$ of the air-layered branches of *L. littorea.* However, none of the stem cuttings gave rise to root initials even in the presence of rooting hormone or Albert solution. Therefore, air layering is the only possible method of vegetative propagation for *L. littorea.* In air layering experiments, coir dust moistened with tap water was the most successful which resulted higher percentage of roots and root initials or callus formed branches. Although, root initial or callus formation percentage decreased with IBA application for both brackish and tap water moistened coir dust treatments, percentage of roots formation was increased with IBA application compared to distilled water control.

Coir dust moistened with tap water was used in the first trial of air layering to check whether air layering is a possible method of vegetative propagation and adventitious roots could be produced from L. *littorea*. IBA was incorporated

to the same rooting medium to induce adventitious root formation. Since air layering was successful, in the subsequent two trials coir dust was moistened with distilled water and brackish water to determine the effect of salt concentration of water on rooting.

The most possible reason for the success of air layering experiments and failure of stem cuttings to produce adventitious roots is maintenance of continuous vascular supply between the mother plant and the area of rooting in air layering. It is essential for the area of rooting to maintain a connection with mother plant for undisturbed vascular supply of water and nutrients. Once a stem cutting is separated from the mother plant, its growth and survival completely depend on water and nutrient supply of the rooting medium. In contrast, in air layering the wounded portion continues the connection with mother plant via xylem, which transports water and nutrients from roots. Also, carbohydrates and auxins are transported form upper plant parts and accumulate in wounded portion. Accumulated auxins induce adventitious root formation while the branch is maintained alive by water and nutrient supply from mother plant (Hartmann and Kester, 2010).

Phenolic compounds are present in the bark and wood of almost all the mangrove species since they are essential to survive in extreme environmental conditions by regulating growth and other physiological functions (Eganathan and Rao, 2001; Hartmann and Kester, 2010). Our experiment revealed the same, as the stem extracts of both soft and hard wood cuttings of *L. littorea* had phenolics. However, phenolics act as endogenous rooting and shooting inhibitors and undesirable in vegetative propagation (Fganathan and Rao, 2001). Therefore, phenolics needed to be removed from the stem cuttings before they are planted. Stem cuttings of the first and second trials of vegetative propagation were obtained during the dry season. Dry season water stress causes an increase in phenols and tannins in some plants (Furlan et al., 2011). Dark brown colour phenolic exudates were observed in the Albert solutions, which used as the growth medium in one of the stem cutting trials. High phenol concentration present in the stems (e.g. 123.9 \pm 17.9 mg/g stem tissue in methanolic extracts) together with the absence of vascular connection with the mother plant would have inhibited adventitious root formation in stem cuttings. Phenolic compound removal treatments might not have been efficient in removing the inhibitory concentration of phenols. Also, the phenol concentration determined in water extracts of L. littorea stems was (33.9 \pm 6.0 mg/g) lower than that of the *Rhizophora stylosa* $(72.5 \pm 3.2 \text{ mg/g})$ and Sonneratia alba $(72.4 \pm 4.9 \text{ mg/g})$ however higher than that of the methanol extracts (123.9 \pm 17.9 mg/g) of *Rhizophora stylosa* (85.5 \pm 5.2 mg/g) and Sonneratia alba ($80.8 \pm 5.4 \text{ mg/g}$) as reported by Suk Suh et al. (2014).

Coir dust was presoaked in the third trial of air layering to remove phenolic compounds. Phenolic compound removed coir dust potting medium resulted in lower percentage of roots and root initials or callus and increased the instances without observations, hence phenolic compounds in coir dust seems not to have affected negatively on rooting of air

layered branches.

No structural barriers for adventitious root formation have been observed in hardwood and softwood stem cross sections of L. littorea. However, according to Tilney (2002) the outer cortex of the young stem of L. racemosa is collenchymatous and consists of stone cells and druse crystals. According to the observations, L. littorea could be categorized as a difficult-to-root or most difficult-to root species. In most difficult-to-root species, stem structure does not interfere with the rooting potential (Hartmann and Kester, 2010). Auxin treatment can break the continuous schlerenchymatous ring. However, in some most difficultto root species even wounding does not induce adventitious root formation of stem cuttings. Difficult-to-root species either lack a rooting morophogen such as auxin or lack the sensitivity to respond to the morphogen. Therefore, external auxin application gives little or no rooting response.

It is suitable to propagate cuttings of difficult-to-root species when optimum conditions are available (Hartmann and Kester, 2010). Dehydration from evapotranspiration water loss is possible in obtaining stem cuttings and stems from stock plants. Quality of the stock plants used is also questionable since the population of *L. littorea* is aged. In difficult to root plant species, ease of adventitious root formation declines with the age of the plant (Hartmann and Kester, 2010). Cuttings taken from young seedlings root readily, compared to those taken from old trees following juvenity factor. However, there is no option in case of *L. littorea* as individuals in the existing population are aged.

Poor nutrient availability could be another factor that affected root formation of L. littorea since optimum nutrient content is important for adventitious root formation (Hartmann and Kester, 2010). Stems of the second trial were obtained during the flowering season. Flowering is a complex phenomenon and a complete sink of metabolites need to root thereby detrimental for rooting (Hartmann and Kester, 2010). Stem cuttings were wounded to stimulate the synthesis and release of catabolic enzymes. Breakdown products or wounding related products enhance rooting when applied with low Auxin concentrations (Hartmann and Kester, 2010). If there are sclerenchymatous fibers in the cortex external to the point of adventitious root formation and if the shallow cut penetrates through this cell layer it promotes the emergence of newly formed adventitious roots. Wounding also increases the contact surface between stem and propagation medium thus enhances water and nutrient uptake. Wounding facilitates the transport of auxin up to cambium and promotes rooting. Leaves of the softwood cuttings were removed completely or partially to minimize evapotranspiration water loss until roots are formed in and water uptake is reestablished. For difficult-to-root species, softwood cuttings will be the only method of commercial propagation (Hartmann and Kester, 2010).

Air layered plants being performed in the field are subjected to many biotic and abiotic stresses such as microbial infections and fluctuating environmental conditions. Therefore, the produced seedlings are more adaptable in the field conditions. Both stem cuttings and air

CONCLUSIONS

In vegetative propagation of *L. littorea*, air layering was the only successful method and none of the stem cutting treatments were successful. Most probably, this may be due to lack of continuous supply of water and nutrients with uninterrupted vascular connections with the mother plant for adventitious root formation in stem cuttings. IBA incorporated coir dust rooting medium moistened with tap water could be identified as the most appropriate for adventitious. In future research, it is recommended to apply a broader range of combinations of rooting hormones to induce adventitious root formation in stem cuttings.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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