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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P.L.M.M. Perera¹, K.M.G.G. Jayasuriya¹, J.W. Damunupola¹,², A.M.T.A. Gunaratne¹,² and M.G.M. Prasanna³

¹Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka.  
²Department of Botany, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka.  
³Ministry of Environment and Wildlife Resources, 416/C/1, Robert Gunawardana MW, Baltharamulla, Sri Lanka.

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Abstract: Considering the environmental and specific socio-economic 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 for 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; Prassanna 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 Amarasingshe, 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...
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,
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. littorea* 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 pre-treatment. Air layering experiments performed during the research are summarized in Table 1. 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 ~ 1 cm 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).

### 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
Table 2: Treatments of stem cuttings of *L. littorea* in different trials.

| Trial No: | Treatment                        | No: of replicates | Potting medium                      |
|-----------|----------------------------------|-------------------|------------------------------------|
|           |                                  | Hard wood | Soft wood                           |
| 1         | None                             | 11        | 10                                  | Sand:top soil:organic matter = 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                                   |                                     |

IBA – Indole-3-butyric acid, NAA – Naphthalene acetic acid

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.
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 ~ 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 (Eganathan 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 ± 17.9 mg/g stem tissue in methanic 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 ± 6.0 mg/g) lower than that of the *Rhizophora stylosa* (72.5 ± 3.2 mg/g) and *Sonneratia alba* (72.4 ± 4.9 mg/g) however higher than that of the methanol extracts (123.9 ± 17.9 mg/g) of *Rhizophora stylosa* (85.5 ± 5.2 mg/g) and *Sonneratia alba* (80.8 ± 5.4 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 difficult-to-root species even wounding does not induce adventitious root formation of stem cuttings. Difficult-to-root species either lack a rooting morphogen 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 juvenile 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 layering experiments give better results when performed in the monsoon season due to high activity of hydrolytic enzymes engaged in mobilizing rooting related food reserves. Both stem cuttings and air layering technique are less expensive practices in mangrove afforestation (Eganathan et al., 2000).

**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 root formation in air layering under experimental conditions. 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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