ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 3, pp: (117-124), Month: July - September 2018, Available at: www.researchpublish.com

Propagation and mycorrhizal effects on *Millettia puguensis* (Fabaceae, Papilionoideae) a liana endemic to Pugu Forest Reserve, Tanzania

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Abstract: ***Milletia puguensis*** **is a distinctive liana found only in Pugu forest in Tanzania. The liana has difficulties in flowering and seeds setting, limiting possibilities of it being propagated through seeds. A vegetative method for its propagation is presented in this work.** ***M. puguensis*** **has been propagated through rooting of its single-node leafy stem cuttings in a non-mist plant propagator. While 54% of the cuttings rooted, 30% died and 16% were still alive but not rooted after 51 days in the propagator. Nodes 3 to 5 of the shoots showed very good rooting ability compared to other nodes. Rooted cuttings inoculated with live mycorrhizas, had significantly higher height, stem diameter and leaf area, after 133 days growth with the live mycorrhizal inoculum. In addition, there was a positive correlation between percent mycorrhizal infection, with cutting height, stem diameter and leaf area. From this work it can be concluded that, rooting of single-node leafy stem cuttings and mycorrhizal infections, can be effectively employed in propagation of the endangered liana** ***M. puguensis*** **for conservation purposes.**

Keywords: ***Milletia puguensis*****, Stem cuttings, rooting, non-mist propagator, mycorrhizas.**

I. INTRODUCTION

The Pugu forest sometimes referred to, as one of the oldest forests of the world is a patch of coastal forest that stretches along the East African Coast [1], [2]. The Pugu forest reserve is located in Kisarawe District, Coast Region in Tanzania, approximately 23 kilometres south-west of Dar es Salaam [3], [4] and has an area of 11 Km^2 [2]. The forest is found approximately between latitudes 6°52' and 6°55'S and longitudes 39°04' and 39°07'E [5]. Pugu forest was gazetted as a forest reserve in 1947 [2].

The Coastal forests of Tanzania are known to have many endemic plant species that are of global conservation significance [6], [7]. Indeed, Pugu forest reserve so far is known to have 14 plants that are strictly localised and endemic to it. One such plant is *Millettia puguensis*, a distinctive liana found only in the Pugu forest [8], [2], [9], [5], [10]. *M. puguensis* is said to be in danger of extinction and needs urgent steps for its protection and conservation [11].

While studying the population dynamics of *Millettia puguensis* Mulibo [12] revealed that, this plant has a very high rate of flower abortion, with only 25% of the flowers setting mature fruits. For fowers that managed to set fruits, these were observed to contain only a few seeds per pod. Furthermore, Mulibo [12] showed that, at different seasons and soil depths, *M. puguensis* was completely absent in the seed bank of Pugu Forest Reserve. In addition, Mulibo [12] observed that, a majority of the young *M. puguensis* in Pugu Forest Reserve were vegetatively produced (ramets) from basal stem buds or root buds. As such, the population dynamics and characteristics of *M. puguensis* make vegetative propagation to be the only viable method of propagating this plant for conservation purposes.

The association between roots of healthy plants and one or more of some mycorrhizal fungal species is termed mycorhizae. In nature, mycorrhizal condition is the rule and non-mycorrhizal plants are unusual [13]. Mycorrhizal associations positively affect plant establishment, growth and health. They are beneficial and in some instances essential [13]. The best known benefits are enhanced uptake of water and mineral nutrients, especially phosphorus and nitrogen [14]. Different plants in the Family Leguminosae (Fabaceae) to which *M. puguensis* belong, are known to form either arbuscular mycorrhizas or ectomycorrhizas or both [15]. So far, it is not known if mycorrhizal associations in *M. puguensis* has any effects on its growth.

This work was aimed at propagating *M. puguensis* vegetatively, through rooting its single-node leafy stem cuttings in a non-mist propagator, and studying effects of mycorrhizas on the growth of the rooted cuttings.

II. MATERIALS AND METHODS

Plant material for propagation

Coppice shoots of *M. puguensis* were brought to the laboratory from Pugu forest reserve for the propagation work. The coppice shoots had been induced to be formed on some stems of *M. puguensis* after their top portions were cut off at 1.0 meter from ground during mid-rain season in March. The remaining lower stumps of the plants were left in the months of April and May to produce the coppice shoots for this work. During the month of July, coppice shoots were obtained from the coppicing stumps of *M. puguensis* by cutting them at their base with sharp secateurs. Coppice shoots from one stump were all placed together in a well tied, airtight plastic bag labelled with a clone number. Each bag had 100 ml of tap water to create moist conditions inside the plastic bags, to avoid water loss from the excised shoots during transportation to the laboratory.

Plant material for observing mycorrhizal associations in *M. puguensis*

During mid rain season, fine terminal feeder roots of *M. puguensis* were carefully dug out from 6 different plants that were more than 15 meters apart. Roots from one plant were kept together in a well-tied airtight plastic bag having 20 ml water to keep them from drying. On reaching the laboratory, each lot of roots was separately soaked in 300 ml tap water overnight in a 500-ml beaker. The roots were then washed free of adhering debris using running tap water [13]. The roots were then observed under the microscope for presence of mycorrhizas and differentiation of the mycorrhizas into either vesicular-arbuscular mycorrhizas or ectomycorrhizas [16], [17], [18].

Rooting of single-node leafy stem cuttings of *M. puguensis*

Single node leafy cuttings were taken from each coppice shoot. The length of each cutting was measured using a ruler and the diameter was also measured using a calliper. The number of leaflets on each cutting was trimmed to one, two or three depending on their size, and the length of each leaflet was measured along the midrib using a ruler. The basal end of each cutting was dipped in the commercial rooting powder seradix-2 (Rhône-Poulenc Agriculture Ltd. Essex, England) containing 0.3 w/w 4-indol-3yl-butyric acid. Excess powder was tapped off before inserting a cutting in a non-mist propagator [19]. The bottom of the propagator was filled with quartz grit (5 - 15 mm) rooting medium. Cuttings from each coppice shoot were held in node order in the non-mist propagator, with the cutting from the tip node being cutting number 1. The number of cuttings from each coppice shoot varied depending on the length of the shoot. The non-mist propagators were all placed inside a fenced area, under an open shed roofed with transparent corrugated plastic sheets.

Twice a week, each cutting was individually assessed in terms of being alive, rooted or dead. A cutting was considered rooted if it had at least one root whose length was 1.0 mm or more.

From some remaining portions of the coppice shoots, 50 randomly chosen leaflets had their lengths measured using a ruler along the midrib and their areas determined after tracing them on a graph paper. A regression plot of leaf area (cm^2) against length (cm) was done and, the resulting regression equation ($Y = 0.2063x + 4.276$; $r^2 = 0.5273$) was used to convert the length of each leaflet on a cutting to area. The areas of all leaflets on each cutting were added together to give the total leaf area per cutting.

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Effect of mycorrhizas on the growth of the rooted *M. puguensis* cuttings

A total of 45 rooted cuttings from 15 *M. puguensis* shoots were used. Each of the 15 shoots had 3 of its rooted cuttings set for this work. Two of the three rooted cuttings from the same seedling were randomly assigned to one of the treatments: live mycorrhizal inoculum, or sterilized (dead) mycorrhizal inoculum. The remaining rooted cutting from each of the 15 *M. puguensis* cutting was used for potting day data. This work was repeated three times and data averaged.

The live inoculum consisted of a mixture of fresh *M. puguensis* fine roots together with well mixed soils taken from the top 10 cm soil around the rhizosphere of 6 different *M. puguensis* plants. To make the sterilized (dead) mycorrhizal inoculum, the mixture of *M. puguensis* fine roots and the rhizosphere soils were sterilized in an autoclave at 121 $^{\circ}$C for two 1-hour periods, with a 24 hour period between the autoclaving.

The two treatments above were applied to the cuttings on potting day. Cuttings for the live inoculum treatment received 1 gram fresh weight of the fresh fine roots of *M. puguensis* plus 10 g of the fresh rhizosphere soil. On the other hand, cuttings for sterilized inoculum treatment, received 1 gram of the sterilized fine roots of *M. puguensis* plus 10 g of the sterilized rhizosphere soil.

To inoculate the rooted cuttings, new, clean plastic pots 1.0 litre in size, were first three-quarter filled with sterile growth soil obtained from Pugu forest. This was followed by placing a layer of either live or sterilized inoculum for the experimental treatments. After this, the rooted cuttings were placed on top of the inoculum, prior to filling the pots with the sterile growth soil. All the experimental as well as control pots with their cuttings were placed under ambient conditions on cement bricks inside a fenced area, and thoroughly wetted using filtered tap water. On potting day, the length (height) and diameter at pot level were measured for each cutting. For potting day, the 15 remaining rooted cuttings were placed in separate paper bags labelled with their clone and cutting (node) number and dried in an oven at 70 $^{\circ}$C for 14 days to obtain potting day dry weights. The cuttings dried for potting day dry weights data, were from the same shoots whose two other rooted cuttings had been used in the two treatments above.

The potted cuttings were incubated for 19 weeks under ambient conditions. During the incubation period, each pot was watered twice a week with 150 ml filter-sterilized tap water.

Harvesting of plants and assessment of mycorrhizal infections

After 19 weeks of incubation, the plants had their height and diameter at pot level measured again. In addition, the numbers of leaflets on each plant were counted, and length of each leaflet along the midrib was measured. The midrib leaf lengths were converted to leaf area using the regression equation obtained before.

Each plant was then separately harvested. To do this, the whole soil-plant root lump was removed from the pot intact. Each lump was then separately soaked in tap water in a plastic basin for one hour, after which the root system was placed on a 2 mm nylon sieve and washed with running tap water over a 2 mm nylon sieve. The root system of each plant was harvested and stained separately according to Wilcox [20] for observing vesicular-arbuscular mycorrrhizae, while each shoot part was placed in a separate paper bag labelled with its clone and cutting (node) number. Observations for ectomycorrhizas were done according to Ingleby [18]. After the mycorrhizal assessment, the roots were placed in a paper bag having their appropriate shoot part. The plants in their separate bags were then dried in an oven at 70 $^{\circ}$C. After 14 days in the oven, they were weighed to obtain dry weights of the cuttings roots, shoots and leaves.

III. RESULTS

After 51 days of incubation in the non-mist plant propagator, 54% of the *M. puguensis* cuttings rooted, while 30 % of the cutting died. On the other hand, 16% of the cuttings were still alive in the propagator but not rooted (Fig. 1) on termination of the incubation. Nodes 3 to 5 of the *M. puguensis* coppice shoots showed very good rooting ability (Fig. 2), compared to the other nodes of the coppice shoots.

Mycorrhizas infections on the roots of the cuttings had positive effects in terms of height (Fig. 3), diameter (Fig. 4) and leaf area (Fig. 5). Alternatively, the results are indicating that, the more the mycorrhizal infections the cutting had, the greater the height, diameter and leaf area (Figs. 3, 4, 5). Similarly, *M. puguensis* cuttings that had mycorrhizal infections, had significantly higher dry weights that those without (Fig. 6).

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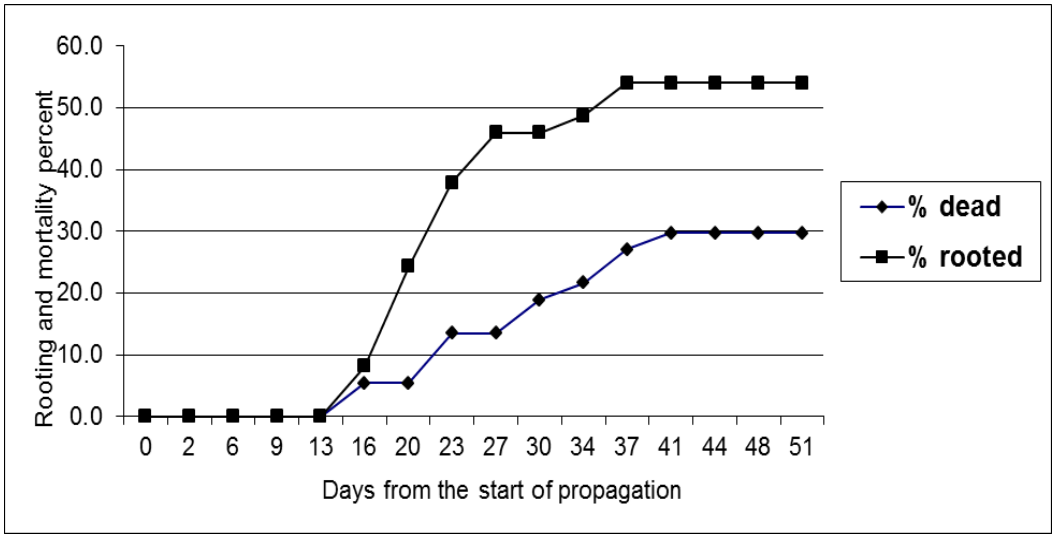

Fig. 1: Rooting and mortality of single node stem cuttings of *Milletia puguensis*

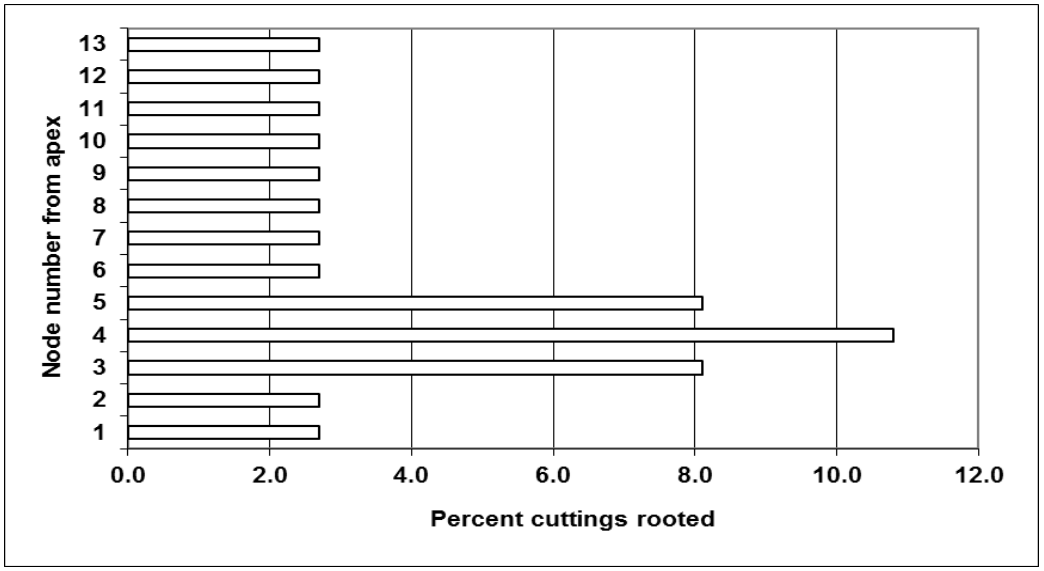

Fig. 2: Rooting percentages of single-node cuttings harvested sequentially down from 15 *Milletia puguensis* coppice shoots

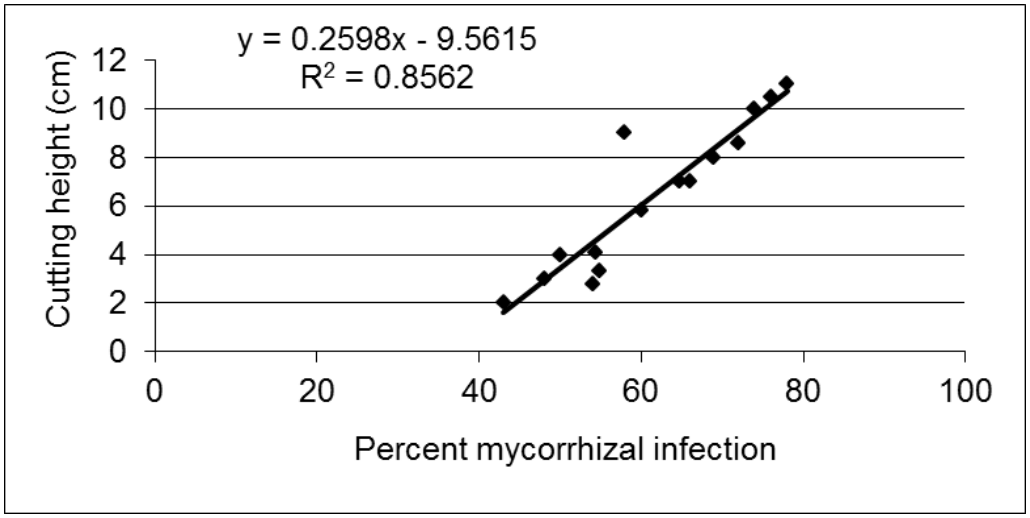

Fig. 3: Correlation between percent mycorrhizal infection and *Milletia puguensis* cutting height

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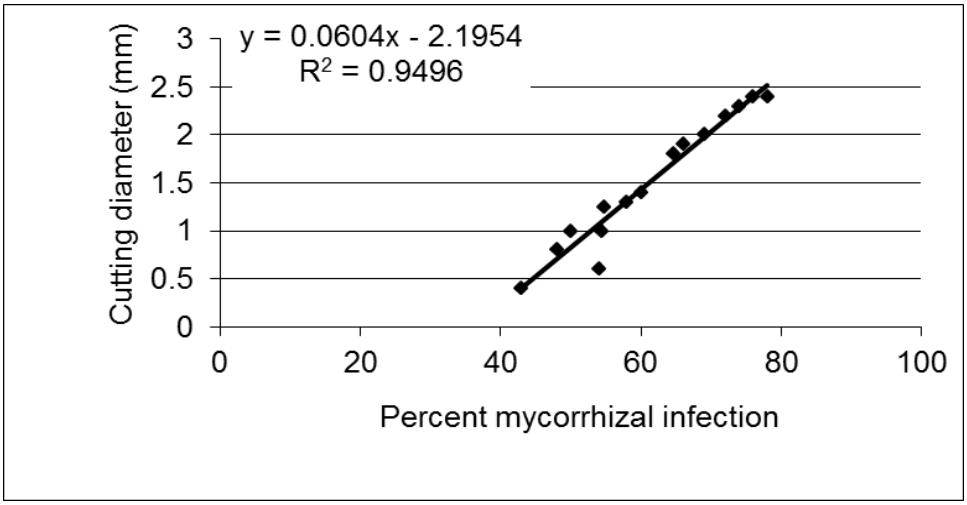

Fig. 4: Correlation between percent mycorrhizal infection and *Milletia puguensis* cutting diameter

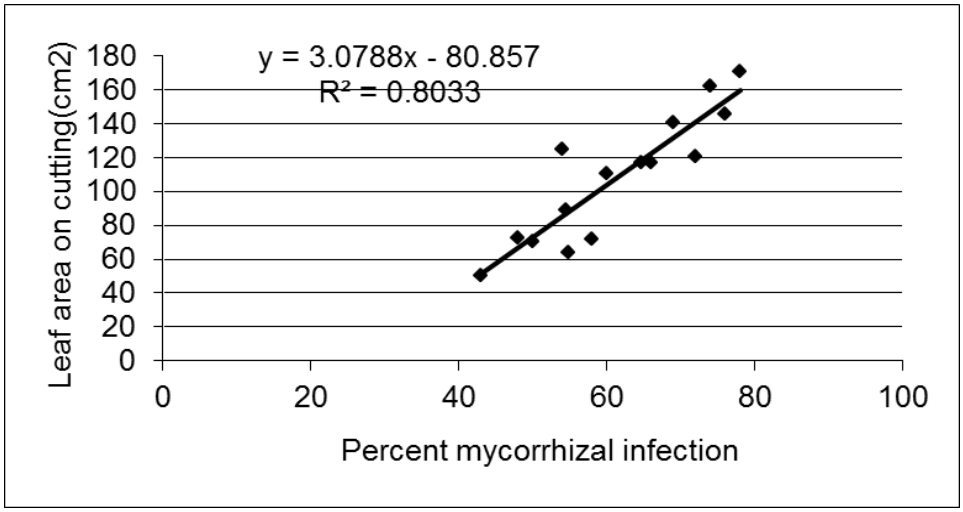

Fig. 5: Correlation between percent mycorrhizal infection and *Milletia puguensis* cutting leaf area

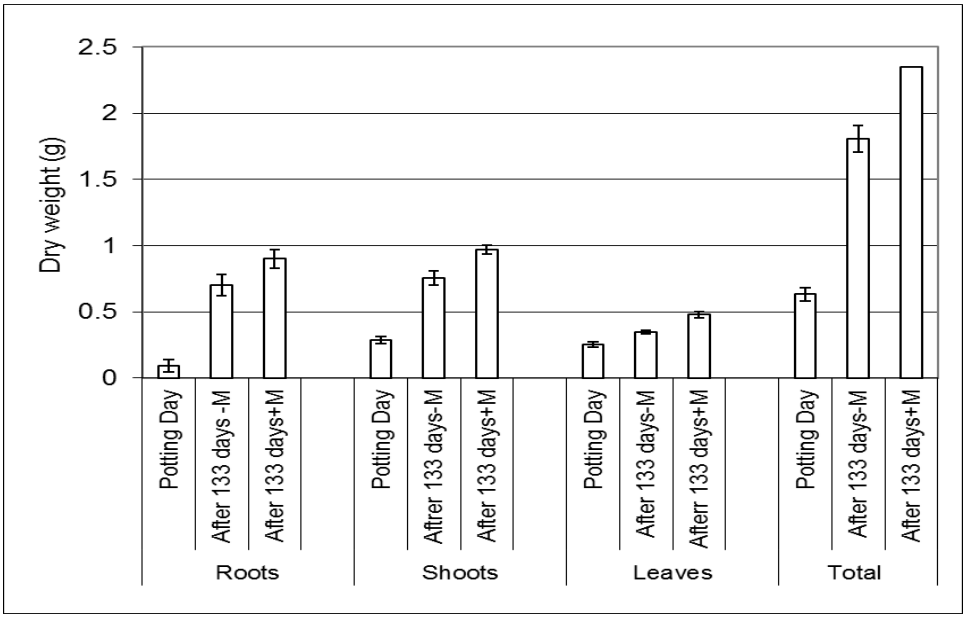

Fig. 6: Dry weights of cuttings grown with (+M) or without mycorrhizas (-M)

IV. DISCUSSION AND CONCLUSION

This work, reports for the first time on rooting of single-node leafy stem cuttings of *M. puguensis*, a plant endemic to Pugu forest in Tanzania. The plant *M. puguensis* is said to be in danger of extinction and needs urgent steps for its protection and conservation [11].

The plant *M. puguensis* has several socio-economic potentials. The plant has been reported to have antimalarial [21], [22] and anti-leishmanial compounds [23], [24]. In addition, a decoction of the roots of the plant is drunk to treat umbilical hernias [25].

M. puguensis is said to be in danger of extinction and needs urgent steps for its protection and conservation [11]. Conserving the plant through seeds has been observed to be problematic. Only a quarter of the flowers of this plant normally set mature fruits and for individual plants that manage to set fruits, the fruits normally contain a few seeds per pod[12] (Mulibo 1997). As such, the seed production abnormalities in *M. puguensis*, makes vegetative propagation the only viable method of propagating this plant for conservation purposes. This work reports for the first time, a method for vegetative propagation of *M. puguensis*, which is through rooting of its single-node leafy stem cuttings.

Mycorrhizal associations have been observed in this work to positively affect the growth of *M. puguensis*. Several plant species in the Family Fabaceae, have similarly been shown to have their growth positively affected by mycorrhizae. They include; *Amorpha crenulata* [26]; *Cullen australasicum, C. tenax, Lotus australis, L. pedunculatus* and *Medicago sativa* [27]; *Pterocarpus angolensis* [28]; *Medicago truncatula* [29] ; *Acacia etbaica* [30]; *Spartium junceum*, *Medicago arborea*, *Coronilla emerus* and *Retama monosperma* [31], also *Albizia saman* [32]. It can thus be concluded that, to ensure best survival and growth of vegetatively propagated *M. puguensis* plants, one has to inoculate the rooted plants with mycorrhizas before planting them in the field.

ACKNOWLEDGEMENTS

I would like to thank Sida/SAREC for supporting this work financially.

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