# Vesicular Arbuscular Mycorrhizal Inoculation Influences Growth, Nutrient Absorption and Hyphae Colonization of Rough Lemon (*Citrus limon*) Seedlings

D. K. Chebet*[1], F. K. Wanzala[2]and W. Kariuki[3]

[1]Seeds, Crops and Horticulture Science Department, University of Eldoret,

[2]Horticulture Department, Jomo Kenyatta University of Agriculture and Technology, Kenya

[3]Scotts Christian University, Kenya

***Corresponding author's email address:** chebet@uoeld.ac.ke

**Abstract**

*Many researchers continue to illustrate the benefits of Vesicular Arbuscular Mycorrhiza fungi on crop output, particularly under unfavourable growth environment. In Sub–saharan Africa, mycorrhizae research on economically important fruit seedlings have attracted inadequate attention. This study was carried out to ascertain the effect of Vesicular Arbuscular mycorrhiza fungi on growth, nutrient absorption and hyphal colonization of rough lemon (Citrus limon) seedlings raised under low phosphorus levels in sand culture and also in low nutrient sand/soil planting media. The study found out that lemon seedling height, number of leaves, leaf area and stem thickness were increased by VAM inoculation in relation to non-inoculated seedlings raised under corresponding P levels in sandy media and also under low sand/soil media. The fresh and dry weights of roots, leaves and stems, and the leaf concentration of phosphorus, nitrogen and potassium were also increased by VAM inoculation. Inoculation also enhanced mycorrhizae hyphal colonization of roots and upraised the root absorptive surface area. This study demonstrates that VAM inoculation improves the competence of lemon seedlings to uptake and use scarce soil nutrients thereby enhancing seedling performance. Being a cheap and readily available technology, VAM incorporation is recommended in nursery propagation of seedlings to ensure a good start of clean and healthy fruit seedlings.*

**Keywords:** Rough lemons, Vesicular arbuscular fungi, phosphorus, potassium, colonization

## INTRODUCTION

Crop productivity in Sub Sahara Africa is hampered by a decline in soil biological, physical and chemical properties which include drought stress, low nutrient reserves, soil degradation, soil acidity, phosphorus fixation and lack of soil biodiversity. Other adverse effects include natural resource degradation and proliferation of harmful microbes and pests and disappearance of beneficial soil organisms (Cardoso and Kuyper, 2006). To address these challenges, practices such as indiscriminate use of pesticides and inorganic fertilizers and high yielding crop varieties were adopted in the 20$^{th}$ Century as part of the green revolution package (Dalgaard *et al.*, 2003). Although this technology increased overall global food supply, reduced starvation and improved nutrition, millions of rural inhabitants in third world countries continue to suffer from declining household food production and are not food secure (Stocking, 2003). This raises queries on the suitability and sustainability of these agricultural practices in tropical agriculture (Dalgaard *et al*., 2003).





The contribution of Vesicular Arbuscular Mycorrhiza fungi to crop productivity has been widely demonstrated, especially under unfavourable growth conditions. Inoculation has been demonstrated to facilitate absorption majorly of immobile nutrients like phosphorus and zinc but also nitrogen, potassium, calcium and magnesium to a though to a limited extend (Rutto et al., 2002a; Muok and Ishii 2006, Chebet et al., 2020a). Studies further show that VAM fungi enhance tolerance to water stress and ensure rapid restoration following drought stress (Fidelibus et al., 2001) and imparts resilience to flooding (Muok and Ishii 2006, Chebet et al., 2020b) and high soil salinity (Muok and Ishii 2006, Chebet, 2020). Mycorrhizal inoculation counteracts against harmful soil borne pathogens and pests (Elsen et al., 2003) and facilitates overall crop productivity after transplanting (Wamocho, 1998).

In Sub-Saharan Africa, many mycorrhizal researches have concentrated on field and vegetable crops while major tropical fruit crops have so far received little research considerations (Guissou, 2009). This is critical because soils in the tropics, particularly in fruit orchards and seedling nurseries have been reported to have very low mycorrhizal content (Wamocho, 1998; Querejeta *et al.*, 2003). As an illustration of this point, the VAM spores in 103 orchards in 25 areas in Kenya (representative of 13 types of soil and 4 climatic conditions) were demonstrated to be less than 200 spores per 25-gram soil sample (Wamocho, 1998). The colonization of roots by VAM hyphae were also scarce (<30%) in orchards in Kenya (Wamocho, 1998). Related research in fruit seedling nurseries in Ethiopia and Somalia showed that indigenous mycorrhizae associations were sparse, even in unpasteurized soils, causing poor quality seedlings to be transplanted (Michelson, 1992). This contrasts to findings in Japan where the number of VAM spores is over 1000 in 25-gram soil sample inspite of substantial use of inorganic fertilizers and chemicals (Ishii *et al*., 1992). Root hyphae colonization of citrus roots in Japan is also on average over 70% (Ishii *et al.*, 1992).

In view of the limited VAM research on tropical fruit seedlings, this study was undertaken to elucidate the influence of VAM fungi on overall growth, nutrient absorption and hyphal root colonization in phosphorus-deficient rough lemon (*Citrus limon*) seedlings.

## MATERIALS AND METHODS

### Treatments and Experimental Design

The experiment was conducted at JKUAT (1255 m asl, 1.03°S, 37.01°E). Lemon seeds were grown in polythene sleeves (20 cm diameter, 25 cm depth) and germinated in sterile sand. It consisted of two types of VAM inoculation (inoculated and non inoculated) and four phosphorus levels (0, 0.44, 0.88, and 1.68 ppm) duplicated six times per treatment. The plants were watered weekly with 300 ml of half-strength Hoagland's nutrient solution (Millner and Kitt, 1992) amended to the equivalent P concentrations (Table 1).

**Table 1: Half-strength Hoagland's nutrient solution used in the study the effect of VAM fungi on growth, nutrient uptake and root colonization of Lemon seedlings**

**The concentration of the Hoagland's nutrient solution used in the experiment**

Mineral element g/500 ml solution Final concentration [a](μM) $Ca(NO_3)_2$ $4H_2O$ 118.10 5000 $KNO_3$ 50.55 5000 $MgSO_4$ 124.24 2000 NaFeEDTA 1.84 100 $Na_2MoO_4{\cdot}2H_2O$ 0.24 0.4 $H_2BO_3$ 3.09 20 $NiSO_4{\cdot}6H_2O$ 0.26 0.4 $ZnSO_4{\cdot}7H_2O$ 1.44 1 $MgCl_2{\cdot}4H_2O$ 1.98 2 $CuSO_4{\cdot}5H_2O$ 0.62 1 $CoCl_2{\cdot}6H_2O$ 0.24 0.4

[a] *Modified from Millner and Kitt (1992)*

The second experiment was conducted in a low-nutrient soil and sand media (1:1 vol/vol) using a two-by-two factorial design with two types of VAM (VAM inoculated and non-inoculated) and two media conditions (sterilized and non-sterilized), duplicated six times per treatment. Twenty-five grams of VAM inoculum containing approximately 200 spores of a mixture of *Glomus caledonium, Glomus etunicatum, Gigaspora magarita,* and *Scutellospora sp.* was used for inoculation (Dudutech, 2013). To ensure homogeneity, the non-inoculated treatments received an equivalent amount of autoclaved VAM inocula.

**Plant Growth Measures**

A two-week inoculation waiting period ended with weekly measurements of seedling height, the number of leaves, and stem girth.

**Biomass and Nutrient Analysis Measurement**

After the experiment, leaves, stems, and roots were measured for both fresh and dry weights. Atomic absorption spectrophotometry method was used to elucidate the Ca, Mg, and K contents (Maqueda and Morillo, 1990). Leaves were then dried in an oven pulverized with a mortar and pestle. One gram was weighed out from each treatment, heated in a muffle furnace set at 550ºC for 5 hours. The ensuing ash was upraised by pouring Hydrochloric acid over it, and 20% water added to the final volume. After this, 200 μL was transferred to ten milliliters and evaluated for Ca, Mg, and K. Phosphorus content was elucidated calorimetrically at 730 nm using a UV-VIS spectrophotometer (Fogg and Wilkinson, 1958). To figure out nitrogen content, micro-Kjeldahl method was used (Ogg, 1960).

**Determination of VAM Hyphae Root Colonization**

This was accomplished through the use of the Grid Intersect Method (Giovannetti and Mosse, 1980). After the experiment was completed, the fine roots (1 0.2 cm) were removed and cleaned in 10% KOH, followed by staining in a 0.05 percent tryphan blue, glycerol, and lactic acid (1:1:1) solution. The frequency of mycorrhizal colonization of the roots was determined using a compound microscope, with recordings collected for a total of ten fields (10 grids). The percentage of mycorrhizal hyphae colonization was calculated by multiplying the prevalence of colonization as a percentage of the total number of grids by 100. (Wamocho, 1998).





**Statistical analysis**

The obtained data were analyzed using the Genstat software (2013). All treatment means were tested for LSD, and Duncan's multiple range test was used to segregate the means (Little and Hills, 1978).

## RESULTS

**Plant Height**

Seedlings subjected to varied phosphorus concentrations did not show any significant difference in height in the first 12 weeks from transplanting (Fig. 1). Mycorrhizal seedlings subjected to 0.44, 0.88ppm and 1.68 ppm P had the highest plant height on 16$^{th}$, 20$^{th}$ and 24$^{th}$ week after transplanting. From 28$^{th}$ to 32$^{nd}$ week, mycorrhizal seedlings subjected to 0.44 and 0.88 ppm P had the highest plant height (Fig. 1). Non mycorrhizal treatment that was not supplied with P had the lowest plant height (Fig. 1).

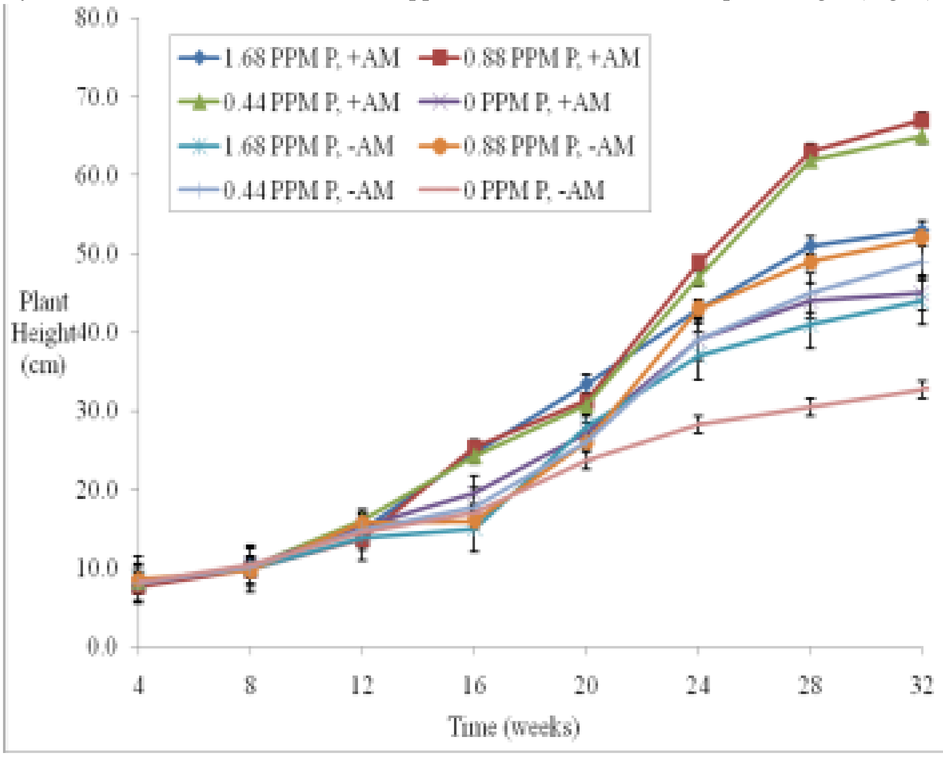


**Fig. 1: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Phosphorus levels on the plant height (cm) of Rough Lemon (*Citrus jambhiri*) Seedlings**

VAM seedlings grew to a greater height than non-inoculated controls in an experiment conducted in low-nutrient sterilized and unsterilized media (Fig. 2). There was no discernible difference in plant height across VAM treatments, regardless of whether the media were sterilized or not (Fig. 2). Plant height was considerably greater in non inoculated treatments grown in sterilized soil than in identical treatments grown in unsterilized soil (Fig. 2)



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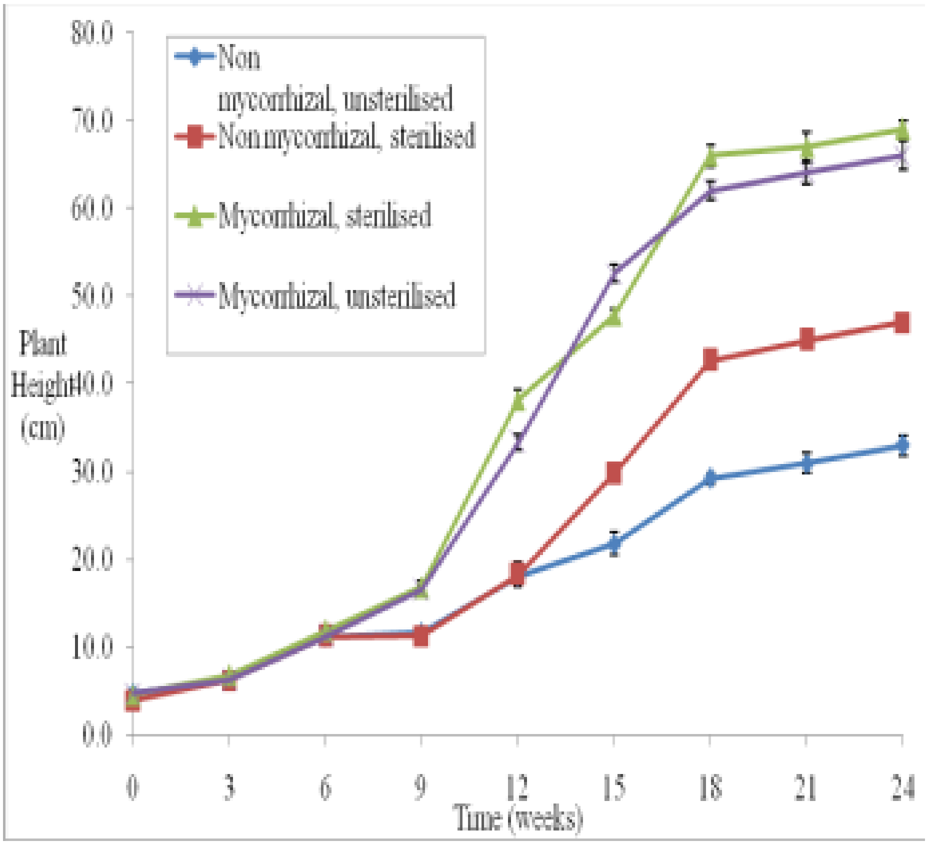


**Figure 2: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Soil Sterilization on plant height (cm) of Rough Lemon (*Citrus jambhiri*) Seedlings**



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**Table 2: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Phosphorus level on leaf number, stem thickness, fresh and dry weights and <u>leaf area of Rough Lemon (*Citrus jambhiri*) Seedlings</u>**

**Leaf**

**Treatment Leaf no. Stem Fresh weight (g) Dry weight (g) Girth Leaf Stem Root Leaf Stem Root**

**area (cm$^2$)**

0 PPM P, -AM 38.2d[z] 1.0a 4.5d 5.8a 10.5e 0.6d 1.3a 2.4d 230.2d 0.44 PPM P, -AM 42.7cd 1.0a 5.3c 5.9a 13.0cd 0.8cd 1.5a 3.9bc 281.4bcd 0.88 PPM P, -AM 42.6cd 1.0a 5.7c 5.9a 13.5bcd 1.1b 1.5a 3.5c 290.7[bcd] 1.68 PPM P, -AM 45.2bc 1.1a 5.6c 5.9a 12.7d 1.0bc 1.2a 3.6bc 258.6cd 0 PPM P, +AM 43.3bcd 1.0a 5.5c 5.8a 12.8d 0.8cd 1.4a 3.3c 275.1bcd 0.44 PPM P +AM 54.2a 1.1a 6.7ab 6.3a 15.3a 1.6a 1.6a 4.8a 320.8ab 0.88 PPM P +AM 58.6a 1.1a 7.0a 6.2a 15.2ab 1.4a 1.5a 4.7a 362.7a 1.6 PPM P +AM 48.6b 1.0a 6.5b 5.9a 14.5bc 1.1b 1.4a 4.3ab 300.0bc LSD ($p \leq 0.05$) 5.4 0.2 0.4 0.49 1.7 0.2 0.4 0.7 62.1 CV (%) 16.3 8.9 7.4 7.8 12.6 14.3 9.8 10.7 12.7 *[z]Column values followed by different letters are significantly different at p<0.05 n=6). +AM = inoculated with Vesicular Arbuscular Mycorrhiza Fungi, -AM = non-inoculated treatments*





**Leaf Number, Leaf Area, Stem Girth and Fresh and Dry Weights** Between all treatments, there was no significant variation in stem girth or stem fresh and dry weights (Table 2). VAM treatments containing 0.44, 0.88, and 1.68ppm P resulted in the maximum leaf and root fresh and dry weights, leaf number, and leaf area (Table 2). The uninoculated treatments (0 ppm P) had the fewest leaves, the lowest leaf and root fresh and dry weights, and the smallest leaf area (Table 2).

In low-nutrient sterilized and non-sterilized media, VAM treatments produced considerably more leaves, leaf and root fresh and dry weights, and leaf area than non inoculated treatments. Stem thickness and stem fresh weights did not differ significantly across treatments. Mycorrhizal plants grown in sterilized or unsterilized media did not exhibit significant differences in any of the parameters examined (Table 3). When non-inoculated plants were grown in sterilized soil, their leaf and root fresh weights were considerably greater than when non-inoculated plants were grown in unsterilized media (Table 3).

**Mycorrhizal Hyphal Colonization**

Colonization of Mycorrhizal Hyphae was seen in both inoculated and non-inoculated seedlings cultivated in unsterilized media, which had extremely low root colonization levels. Inoculated seedlings did not exhibit a significant change in hyphal colonization percentage. Seedlings that were not infected and were grown in sterilized media lacked hyphal colonization (Table 4).

**Table 3: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Soil Sterilization on the leaf number, stem girth, biomass and leaf area of Rough Lemon (*Citrus Jambhiri*) seedlings**

[z]Column values followed by different letters are significantly different at $p<0.05$ n=6). +AM = inoculated with Vesicular Arbuscular Mycorrhiza Fungi, -AM = non inoculated treatments, +ST = sterilized soil, -ST, unsterilized soil

**Table 4: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Soil Sterilization on mycorrhizal root colonization (%) of Rough Lemons (*Citrus jambhiri*)**

**Treatment % Root Colonization** Non Mycorrhizal, Unsterilized

[z]7.1 ± 4.5

Non Mycorrhizal, sterilized 0

Mycorrhizal, Unsterilized 51.1 ± 2.9

Mycorrhizal, sterilized 55.3 ± 2.4

[z]Means ±SE (N=6)

**Table 5: Influence of Vesicular Arbuscular Mycorrhiza Fungi and Soil Sterilization on the leaf nutrient levels (%) of Rough Lemon (*Citrus jambhiri*) Seedlings**

**N (%) P (%) K (%) Ca (%) Mg (%)** -AM-ST 2.0 ± 0.1[z] 0.2 ± 0.05 2.1 ± 0.2 2.8 ± 0.1 1.6 ± 0.1 -AM+ST 2.0 ± 0.1 0.3 ± 0.07 1.9 ± 0.2 3.1 ± 0.2 1.7 ± 0.2 +AM-ST 2.3 ± 0.1 0.4 ± 0.05 2.6 ± 0.1 3.0 ± 0.1 1.6 ± 0.1 +AM+ST 2.3 ± 0.2 0.4 ± 0.04 2.6 ± 0.1 3.1 ± 0.1 1.6 ± 0.2 [z]Means ±SE (N=6)





**Leaf Nutrient Content**

Inoculated seedlings had significantly larger percentages of N, P, and K than non inoculated seedlings (Table 5.0). There was no statistically significant difference in the Ca and Mg contents of any of the treatments (Table 5.0).

## DISCUSSION

The results of this study indicate that VAM fungi enhance lemon growth by increasing seedling height, leaf number and area, biomass accumulation (fresh and dry weights), and nutrient absorption. Chebet et al. (2020b) observed similar findings for papaya, Al Karaki (2013) for sour oranges, Suri and Choudhary (2013) for soybeans, and Tas (2013) for sweet corn. Kadhe and Rodrigues (2009) showed an increase in phosphorus absorption in papaya, observing that the leaf petiole of VAM plants contained 0.42–0.63% total phosphorus, compared to 0.35 percent P in non-mycorrhizal plants. Additionally, when linseed was inoculated with *Glommus mosseae* or *Glommus intraradices* and their intergration, a considerable rise in shoot P concentration was observed (Rydlová et al., 2011).

This study was conducted in both sand culture and sand and soil (1:1 vol/vol) media, both of which were deficient in nutrients. VAM fungi provide an effective conduit for phosphorus to be extracted from huge volumes of soil and transported to the cortical tissues of the host roots in nutrient-deficient soils (Smith and Smith, 2011). This is because the VAM hyphae have a relatively tiny size, which enables them to enter extremely thin soil pores, hence increasing the volume of soil investigated (Smith and Read, 2008; Schnepf et al., 2011). When compared to non-inoculated seedlings, VAM seedlings showed a larger root fresh weight, indicating a greater root mass. Similarly, VAM seedlings had a higher rate of mycorrhizal hyphal colonization than non inoculated seedlings. As a result, it is hypothesized that the increased mass of VAM treatments resulted in increased absorptive area for nutrients and water, resulting in improved mycorrhizal treatment performance.

In the experiment carried out in sand media under varied phosphorus levels, VAM inoculation in combination with moderate P caused the highest growth response. Initially, VAM plants subjected to high phosphorus level (1.68 ppm) had the highest increase in plant height. However, a gradual reduction in growth occurred such that by the end of the study, the rate of growth had reduced significantly. This shows that benefits of mycorrhization are lost when soils are rich in phosphorus. This was also observed in sunflower whereby mycorrhiza + 200 kg P/Ha treatment combination did not show significant difference in seed yield when compared to nonmycorrhizal + 200 kg P/Ha treatment (Vaseghmanesh *et al*., 2014).

Mycorrhizal inoculation was used to boost the leaf nitrogen content in this study. This was also observed in chickpeas (Yaseen et al., 2012), and may be a result of increased soil exploration caused by VAM treatment (Sundar et al., 2010). Additionally, this study revealed an increase in potassium uptake. Kadhe and Rodrigues (2009) observed a similar discovery in pawpaw, demonstrating that VAM plants had leaf petiole total K levels ranging from 2.68 to 4.39 percent, while non-mycorrhizal plants had total K levels of 2.26 percent. Chebet et al. (2020b) observed beneficial effects of VAM inoculation on K absorption in papaya, cowpea and sorghum (Bagayoko et al., 2000), and finger millet (Rao et al., 1983). While enhanced K intake can be linked to increased soil exploration and K supply to lemon roots, further benefits can be related to the fact that VAM fungus bind soil particles together and to the roots, enhancing nutrient uptake (Estrada-Luna et al., 2000).





Mycorrhizal inoculation played a greater role than media sterilization in determining plant performance. This was demonstrated by the absence of significant changes in all parameters between sterilized and unsterilized low nutrition sand:soil (1:1 vol) media. Whereas non-mycorrhizal seedlings fared badly in this study, seedlings grown in sterilized media outperformed those grown in non-sterilized media. This could occur as a result of eradicating all organisms by soil sterilization. This was useful because it eradicated dangerous microbes, which resulted in increased performance of non mycorrhizal seedlings grown on sterilized media. On the other hand, the absence of sterilization may be advantageous because beneficial microbes are not destroyed. A minor number of mycorrhizal root colonizations were identified in the unsterilized treatment. This was supposed to help the plants by inhibiting the growth of harmful microbes in the soil medium, as Elsen et al. observed (2003).

## CONCLUSION AND RECOMMENDATIONS

This study found out that lemon seedling growth in low nutrient media was enhanced by vesicular arbuscular mycorrhizal inoculation. The improved growth occurred via greater plant height, leaf number and stem girth, increased leaf area and fresh and dry weights of roots, leaves and stems and increased uptake of phosphorus, nitrogen and potassium. The study therefore recommends the adoption of VAM inoculation during nursery propagation of tropical fruit seedlings. This will enable transference of mycorrhizae fungi into orchards at transplanting. It is also recommended that VAM spores be incorporated into the planting hole at transplanting time or introduction into fruit orchards as a regular practise, to replace those that are lost via tillage practices, soil erosion and fungicidal sprays. This is a regular practise in Japan were orchards are regularly introduced via sprinkler irrigation (Ishii et al. 1992). To bridge the knowledge gap, the government should assist training of smallholder farmers, fruit seedling propagators, extension service personnel, and policymakers on the benefits of vesicular arbuscular mycorrhizal technology. The training should emphasize the isolation, identification, and selection of better VAM strains with increased crop diversification and survival characteristics during shipping, storage, and after soil application.

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