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Native Mycorrhizae for Improving Seedling Growth in Avocado Nursery (*Persea americana* Mill.)

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Abstract

Objectives: This research aims to evaluate the efficiency of the use of native mycorrhizae on seedling growth of avocado to produce high quality avocado plants. **Methods**: Root and soil sampling was done in 14 avocado production sites from Interandean valleys of Ecuador. Native mycorrhizae inoculum and two control treatments (commercial product and absolute control without inoculation) were evaluated in both seed and seedlings. A randomized complete block design was applied for the trails. Analysis of variance was run to determine statistical differences and the Tukey test at 5% was used to determine ranges of significance. **Findings**: Soils showing largest number of spores and highest percentage of mycorrhizal colonization were collected in Tumbabiro (Imbabura) and San José de Minas (Pichincha). Compared to the absolute control, an increase in total phosphorus and dry matter content of 84% and 100%, respectively, was observed in trap plants. Using inoculum from Tumbabiro and San José de Minas to inoculate avocado seeds and seedlings, dry matter content increased by 44% while the percentage of total phosphorus increased by 42% compared to the controls. Although acceptable results were obtained with the commercial product about the percentage of phosphorus and dry matter, poor percentages of root colonization were obtained; whereas the native inoculum from Tumbabiro and San José de Minas produced better results improving avocado seedling growth in nursery. **Application/Improvements**: This research allows us to infer that native mycorrhizal strains are effective as inoculum to enhance the development of avocado seedlings.

Keywords: Arbuscular Mycorrhizal Fungi, Colonization, Inoculation, Phosphorous

1. Introduction

The center of origin of almost all recognized members of the genus *Persea* occurs primarily in the region located from the central part of Mexico through Guatemala to Central America[1]. The avocado (*Persea americana* Mill.) belongs to the Lauraceae family and there are actually three botanical varieties and cultivated subspecies. These types are Mexican (cultivar 'drymifolia'), Guatemalan (cultivar 'guatemalensis') and from the West Indies (cultivar 'american')[2].

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In the Andean region, the avocado is a traditional fruit in the diet of Ecuadorians. Its delicate flavor makes it the perfect complement to dishes and it is commonly consumed fresh. In Ecuador, avocado consumption has an annual increase of 1.4%. The production of this fruit is no longer only for local consumption and it has become a product with high export potential[3] because of its nutritional characteristics and high yields per unit area. The main avocado producing areas are Carchi, Imbabura, Pichincha, Tungurahua, Azuay and Loja[4].

The avocado, like other fruit species, needs a period of growth in a nursery prior to transplantation in commercial orchards. In this stage where the use of mycorrhizal-arbuscular fungi has a high potential as they play an important role in the growth and nutrition of higher plants such as most fruit species[5,6].

The benefits of inoculation with the fungi that form arbuscular mycorrhizae can be seen in improved seedling survival, increase of plant growth in shorter time, reduce of nursery time, saving in fertilizer costs, and increased production and product quality[7]. Modification of the root system by symbiotic association with these fungi helps to improve the absorption and transport of water and nutrients from the soil to the root by increasing the volume of soil explored which is reflected in better plant growth[8]. These fungi are obligate symbionts. They cannot complete their life cycle in the absence of a host plant and need to be associated with the root in order to obtain carbon from photosynthesis. In addition, they form an extension of the plant roots beyond the nutrient zone in the soil[9].

This research aims to evaluate the efficiency of using of native mycorrhizae in the development of avocado seedlings of the 'National' cultivar (from the Mexican strain) because currently in Ecuador there are no records of seedling growth inoculated with these fungi. The use of mycorrhizal fungi for the production of avocado plants in the nursery stage should be considered a regular practice because of their benefits to plant growth. These microorganisms deserve special interest for increasing root system efficiency in the absorption of nutrients, especially phosphorus, and promoting absorption of trace elements[10,11]. Furthermore, the use of these fungi will reduce pesticide application because mycorrhizae create physical barriers against pathogenic organisms in the soil, becoming an environmentally friendly alternative that will help to avoid soil contamination and will promote an approach to new technologies geared towards a trend towards sustainable management that can be integrated into the process of integrated fruit production[8].

2. Materials and Methods

The research was conducted in the nursery and laboratory of the Tumbaco Experimental Farm belonging to the National Institute of Agricultural Research, located in the province of Pichincha at an altitude of 2,348 meters above sea level, latitude 00°13'00" South and longitude 78°24'00" West, with an average temperature of 23°C and with 41% relative humidity.

3.1 Analysis of Mycorrhizal Content in Soil from Avocado Orchards

Collection of Biological Material

Roots and soil samples were collected in avocado production areas where there was a low use of agrochemicals for crop development and that were grown mainly by organic management. The sites were selected in the Andean valleys of Ecuadoras shown in Table 1.

Soil Sampling:

The quantification of spores was done around the crown of established avocado plants (selected at random), at a depth of 20 cm. The sample was composed of ten subsamples of 100 g each. They were transported using a cooler to the laboratory, which were spread on paper and dried at room temperature under shade for 15 days. After this time the quantification of spores was performed.

Table 1. Soils sampled in producing areas of avocado crop in Ecuador

Soil	Province	County	Parish	Site	Altitude (msnm)	Latitude	Longitude
s1	Imbabura	Urcuquí	Tumbabiro	El Bohio	2,120	00°28'3"N	78°11'0"W
s2	Azuay	Cuenca	Paute	Guallaguzho	2,120	02°46'39"S	79°45'32"W
s3	Pichincha	Quito	San José de Minas	La Playa	1,980	0°09'58"N	78°23'10"W
s4	Tungurahua	Patate	Los Andes	Lote 1	2,038	01°14'18"S	78°30'04"W
s5	Tungurahua	Patate	Los Andes	Lote 2	2,038	01°14'18"S	78°30'04"W
s6	Tungurahua	Patate	Los Andes	Lote 3	2,038	01°14'18"S	78°30'04"W
s7	Tungurahua	Patate	Los Andes	Lote 4	2,038	01°14'18"S	78°30'04"W
s8	Tungurahua	Patate	Los Andes	Lote 5	2,038	01°14'18"S	78°30'04"W
s9	Pichincha	Quito	Atahualpa	San José de Atahualpa	2,042	0°82'04"N	78°22'79"W
s10	Pichincha	Quito	Perucho	Cementerio	2,761	0°66'46"N	78°22'65"W
s11	Pichincha	Quito	Chavezpamba	Bellavista	1,129	0°73'73"N	78°24'10"W
s12	Azuay	Cuenca	Gualaceo	Nauiq - Nayuntur	2,330	03°19'47"S	79°03'28"W
s13	Imbabura	Cotacachi	San Francisco	Piavachupa	2,360	0°19'56"N	78°14'57"W
s14	Pichincha	Quito	Puéllaro	Munango	2,060	0°37'10"N	78°15'39"W

Root sampling:

Using a shovel dug to a depth of 40 cm around the crown of trees to obtain mainly tertiaries roots (healthy, young and slightly lignified). Samples were transported using a cooler to the laboratory.

Estimated variables:

The variables evaluated the population of mycorrhizal spores in soil through quantification of spores by the method of sieving and centrifugation[12]. Rate of mycorrhizal colonization in roots was measured by the clarification and root staining method, following the technique of Phillips and Hayman[13].

Treatments and Analysis data:

The treatments consisted of soils sampled in the avocado producing areas described in Table 1. The experimental unit consisted of a sample of 1 kg of soil made up of 10 subsamples of 100 g each from the sampled sites. A Complete Block Design (CBD) was applied to set the assay. Analysis of variance (ANOVA) was run to determine statistical differences and the Tukey test (at 5%) was used to determine ranges of significance. For statistical data analysis the R software version 3.3.1 was used. Soils corresponded to samples collected in Tumbabiro, Paute, San José de Minas and Los Andes-Patate lote 2 that showed the best results were selected for further research in the next phase.

2.2 Propagation on Trap Plants (*Sorghum vulgare*)

Soil sampling for physical-chemical analysis:

For physics-chemical analysis a sample of 1 kg of the selected soils was used. In the plantation, 10 sub-samples (100 g) were taken at 20 cm depth, taking a zig-zag route to obtain a final mixed sample (1 kg). The sample was labeled and sent to the Soil and Water Laboratory in the Santa Catalina Research Site for the respective processing. For the moisture and density analysis, a specific cylindrical bore was introduced at 20 cm depth to obtain the sample which was dried in an oven at 110°C.

Treatments:

Soils that achieved the best average spore quantification and root colonization constituted the inoculum that was multiplied in trap plants, treatments being as follows: s1 = San José de Minas, s2 = Tumbabiro), s3 = Paute, s4 = Los Andes-Patate (lote 2) and s5 = control (sterile sand). The experimental unit consisted of a pot of 2 kg capacity, where 10 seeds of *S. vulgare* were sown.

Experimental design and statistical analysis:

A Randomized Complete Block Design (RCBD) was used, with five replications. ANOVA and Tukey tests at 5% were utilized to determine differences between treatments. The statistical analysis was done in R version 3.3.1.

Estimated variables:

The variables evaluated were total phosphorus concentration in plant tissue which was measured 90 days after sowing; the total concentration phosphorus in plant tissue was determined by bromatological analysis. Dry matter produced 90 days after sowing; the fresh weight (g) of the whole plant was measured; then samples were placed in an oven for 24 hours at 110°C to determine the dry weight and establish their percentage ratio. Population of mycorrhizal spores in the soil was measure using 20 g of soil; 90 days after sowing the trap plants spore quantification was performed by sieving and centrifugation method described by Herrera[12]. Mycorrhizal colonization rate in roots was evaluated 90 days after sowing, using the clarification and staining root method following the technique of Phillips and Hayman[13].

Specific management of the experiment in trap plants:

For disinfection, *S. vulgare* seeds were placed in alcohol (70%) for three minutes, bathed with a chlorine solution (1.5%) for two minutes and rinsed five times with sterile distilled water. 500 g of sterile sand, 1 kg of sampled soil mixed with rice husks and finally a layer (100 g) of the same soil was placed in each pot. Then 10 seeds were sown at a depth of 0.5 mm. After 90 days, three random

plants were taken for assessing root colonization. Another three plants were used for determining the percentage of dry matter. The remaining four plants were maintained to continue generating the inoculum that was used in the next stage. Fertilization was performed using ammonium nitrate (2 g/l) once a month. Irrigation was carried out three times per week (500 ml of water per pot).

2.3 Evaluation of the Inoculum in Avocado Seeds and Seedlings

This phase was divided into two trials where the inoculum was evaluated in avocado seeds and seedlings. The research was conducted in the nursery of the Tumbaco Experimental Farm, whose location and environmental characteristics correspond to that described above in the section on the multiplication of inoculum in the trap plants.

Treatments:
These were constituted by four inoculums, a commercial control and an absolute control: i1 = San José de Minas, i2 = Tumbabiro, i3 = Paute, i4 = Patate, i5 = commercial control (*Glomus* sp.), i6 = absolute control (without inoculation). The experimental unit consisted of an avocado seed or seedling, sown or transplanted into a pot of 4 kg capacity containing substrate with 100 g of inoculum.

Variables evaluated:
Dry matter percentage: was measured at 120 days after seeds and seedlings were inoculated, the fresh weight of the whole plant (in the remaining five plants) was assessed[13]; samples were placed in an oven for 24 hours at 110°C for measuring dry weight and calculating dry matter. Total phosphorus: in the plant tissue was recorded after 120 days by bromatological analysis. The total phosphorus was estimated by foliar analysis using the molybdenum-vanadate colorimetric method; while the molybdenum blue method was applied for phosphorus determination in the soil[14].

Experimental design and statistical analysis:
A RCBD was used, with 10 replications. ANOVA and Tukey tests at 5% were utilized to determine differences between treatments. Statistical analysis was done in R version 3.3.1.

Specific management of seeds and seedlings:
Avocado seeds were placed in alcohol (70%) for three minutes, then soaked with a solution of chlorine (1.5%) for two minutes and rinsed five times consecutively with sterile distilled water. They were subsequently immersed in a solution of gibberellic acid at a concentration of 1,250 ppm for 24 hours for pre-germination. Ten days before using the inoculum, the trap plants (*S. vulgare*) were cut and the pot was watered. The inoculum was prepared by mixing the roots of the trap plants, which had been cut into pieces of about 1 cm, with potting soil, removing the sand layer. Three kilograms of sterile substrate composed of black soil, rice husks and pomina (proportion 2:1:1) was placed in pots of 4 kg capacity. Then 100 g of the inoculum was placed in each pot. The avocado seed was sown and covered with a layer of 100 g of sterile substrate. In the case of seedlings, these were previously germinated and after 90 days were transplanted into the pots. Fertilization was performed using ammonium nitrate (2 g/l) once a month. Irrigation was carried out three times per week (500 ml of water per pot).

3. Results and Discussion

3.1 Analysis of Mycorrhizal Content in Soil

According to Table 2, the soils with the highest number of spores of mycorrhizal fungi and a high percentage of colonization corresponded to samples collected in Tumbabiro, Paute, San José de Minas and Los Andes (Lote 2 - Patate), being the genus *Glomus* the most frequent in these soils. Table 3 shows the physical characteristics of the soils that showed the best results in number of spores and percentage of colonization. The best results were observed in the soil sampled in Tumbabiro. This soil has a sandy loam texture allowing good drainage, providing an adequate environment for the presence of these microorganisms[15]. Moreover, mycorrhizal fungi act in the soil structure by providing more aggregation and stability, and preventing erosion processes[16].

Table 2. Population of mycorrhizal spores and percentage of colonization in soils sampled from avocado producing areas in Ecuador

Soil	Origin	Number of spores /100g soil		Colonization Percentage (%)	
s1	Tumbabiro	2,485	ab	18.79	a
s2	Paute	2,723	a	15.28	ab
s3	San José de Minas	2,025	bcd	18.15	a
s4	Los Andes Lote 1- Patate	2,205	abc	6.35	de
s5	Los Andes Lote 2- Patate	2,281	abc	16.20	ab
s6	Los Andes Lote 3- Patate	1,215	efg	8.09	cde
s7	Los Andes Lote 4- Patate	1,610	de	9.60	cd
s8	Los Andes Lote 5-Patate	970	fgh	6.30	de
s9	San José de Atahualpa	1,345	ef	12.06	bc
s10	Perucho	770	gh	7.15	de
s11	Chavezpamba	1,440	ef	8.00	cde
s12	Gualaceo	1,775	cde	9.80	cd
s13	Cotacachi	1,400	ef	4.75	e
s14	Munango	485	h	4.16	e

Table 3. Physical characteristics of the soils used for the multiplication of the inoculum using the trap plants

Soil	Origin	Texture	% humidity	Density	pH	Organic matter (%)	% colonization	Spores in 100g
s3	SJ. Minas	sandy loam	30.90	1.12	6.30	5.00	18.15	2,485
s1	Tumbabiro	sandy loam	37.10	1.33	7.80	3.30	18.79	2,025
s2	Paute	clay loam	46.20	1.30	6.90	5.60	15.28	2,723
s5	Patate	loam	56.50	1.36	7.40	7.40	16.20	2,281

3.2 Multiplication on Trap Plants (*Sorghum vulgare)*

Dry matter percentage: When analyzing this variable, it can be seen that mycorrhizal treatments showed the best results, which is consistent with another reasearch[17] where found that mycorrhizal fungi contribute to higher dry matter. Plants developed in the soil from San José de Minas showed the highest percentage of dry matter content with 45.5% of biomass; whereas the control without inoculation had just 21.22%. *S. vulgare* it has been observed that mycorrhizal plants increase their growth, their amount of photosynthetic pigments and stomata conductance in comparison to non-mycorrhizal plants[18]. Apparently the flow of carbohydrate is regulated by the host plant and depends on the species of fungus.

Total phosphorus:
The highest percentage of phosphorus was observed in plants grown in the soils of San José de Minas and Tumbabiro with an average of 0.24% in both cases; whereas the control (without inoculation) showed the lowest percentage (0.13%) in Table 4. Mycorrhizal fungi contribute to increased phosphorus uptake by the plant[17,19]. Some authors[11,18] reported that mycorrhizal fungi contribute to greater absorption of N, P, K, Zn and Cu by the plant.

Population of Mycorrhizal spores in the soil: The greatest spore population was observed in soil from Tumbabiro (3,835 spores 100 g^{-1} soil); while the control showed only 68 spores 100 g^{-1} of soil. Tumbabiro soil has a sandy texture which favors the formation and function of mycorrhizal fungi[20]. The opposite occurs in compacted soil where fertility and distribution of plant roots, and therefore the hyphae of arbuscular mycorrhizae in the rhizosphere, are reduced either by direct effect on the communities of fungi or indirectly through their effects on the host plant.

Table 4. Variables evaluated during multiplication of inoculum trap plants

Origin	Variables		
	Dry matter (%)	Phosphorus Total (%)	Number of Spores/100g of soil
Tumbabiro	40.16 b	0.24 a	3,835 a
Paute	31.82 c	0.22 a	3,002 b
San José de Minas	45.50 a	0.24 a	3,016 b
Los Andes - Lote 2 Patate	26.50 d	0.20 a	2,834 b
Control (sterile sand)	21.22 e	0.13 b	67.60 c

Percentage of root colonization: It has been considered that soil mycorrhizae potential is influenced by different factors such as host plant, soil type, and the proportion between the substrate and the inoculum[21]. The highest percentage of root colonization was observed in the soil of Tumbabiro, with an average of 30.34%; while the lowest percentage (4.56%) was obtained by the absolute control (Table 4). These results show that the type of spores in the soil was effective in the trap plants because the infection rate exceeds 20%, which is a positive result as is mentioned by Herrera[12]. In addition, higher percentage of root colonization is expressed in more growth of the aerial part of the plant (stem and leaves) and that greater root development does not always occur[22]. The low colonization observed in the control, despite the use of sterilized soil, can be explained by the persistence of spores in the soil as populations of mycorrhizal fungi are composed of spores of different ages and in different states of dormancy or quiescence[23].

3.3 Evaluation of the Inoculum in Avocado Seeds and Seedlings

Percentage of root colonization:

In terms of seeds, the highest percentages of colonization (Table 5) were obtained for the treatments that used the inoculum from San José de Minas (14.08%) and Tumbabiro (12.38%). The absolute control showed the lowest percentage of colonization (2.70%). This value, was explained is accounted for the persistence of spores in the soil[23]. In terms of seedlings, the highest percentage of colonization is shown in Table 5. It was observed

Table 5. Variables evaluated at 120 days after inoculation by mycorrhizae in seeds and seedlings of avocado

Origin	Variables			
	Phenological state	Dry matter (%)	Total Phosphorus (%)	Colonization (%)
Tumbabiro	Seed	21.56 a	0.14% a	12.38ab
	Seedling	22.70 ab	0.12% a	9.65b
Paute	Seed	19.48 ab	0.11% bc	6.30c
	Seedling	22.12 b	0.10% ab	5.51d
San José de Minas	Seed	22.14 a	0.13% ab	14.08a
	Seedling	23.28 a	0.12% a	13.53a
Los Andes - Lote 2 Patate	Seed	18.96 ab	0.10% c	6.24c
	Seedling	22.08 b	0.09% ab	6.20c
Commercial control	Seed	21.28 ab	0.12% bc	11.24b
	Seedling	22.34 ab	0.11% a	7.57bc
Absolute control	Seed	16.56 b	0.08% d	2.70d
	Seedling	21.18 c	0.08% b	2.33e

in the treatment with inoculum from San José de Minas (13.53%), while the absolute control showed the lowest percentage (2.33%)[24].

The inoculation with these fungi can be performed in seedbeds or during the transplantation process[25]. However, problems can occur when the inoculation is made in seedbeds as the seeds may germinate slowly or may produce certain substances that inhibit the intra-radical process of colonization. On the other hand, if it is considered that inoculation with these fungi might not produce the expected results in most tree fruit species because the seedling in its early stages depends on the content of reserves held in its cotyledon, the results obtained in this research show that there was variation in the percentage of colonization in the roots of avocado seedlings depending on the soil that was used as the inoc-

ulum, confirming an interaction between the plant and the fungus. It is adequate to inoculate seedlings once they present the first pair of green leaves (indicator of root presence), to encourage direct contact between the root and the inoculants, ensuring rapid establishment and expression of the symbiosis[5].

Percentage of dry matter:

In terms of seeds, the best result was observed in the treatment which used inoculum from San José de Minas, with an average of 22.14%. It has been reported that a higher percentage of root colonization (as is the case with San José de Minas soil) is expressed in greater growth of the plant (stem and leaves)[22]. The absolute control showed the lowest percentage, with 16.56%.

In terms of seedlings, inoculum from the soil of San José de Minas and Tumbabiro obtained the highest percentages of dry matter compared to the other treatments. This increase is due to the colonizing action of mycorrhizae fungi. These two treatments also achieved the greatest percentages of root colonization. The interaction of rhizosphere microorganisms with mycorrhizal fungi produces a symbiotic association that allows greater colonization and, therefore dry matter production because absorption and nutrient availability is greater for the plant[24]. Mycorrhizal inoculum had no significant effect on fruit dry matter[25]. In addition, carbohydrates synthesized in the leaves allow the development of mycorrhizal fungi[9]. These carbohydrates are produced more in seedlings than seeds because seedlings start with a certain amount of leaves and are capable of photosynthesis, thus benefiting the fungus with carbohydrates.

Total phosphorus in plant tissue:

In seed inoculation (Table 5), the best results were obtained by the inoculum from Tumbabiro (14%) and San José de Minas (13%). It is worth mentioning that the commercial control (with 12%) occupied the second rank of significance. On the other hand, the absolute control showed the lowest percentage (8%).

In seedlings, the highest proportion of total absorbed phosphorus was observed in the same soils as those mentioned above for seeds (Table 5) and the absolute control showed the lowest value. It can be inferred that these results are due to the action of mycorrhizae fungi that colonized the roots, which performed their function through external hyphae that are heavily branched and increase the number of absorption sites[26]. Furthermore, there is greater absorption of this mineral by the plant because these fungi correct soil pH through organic agents and solubilizers, making available the phosphorus retained in the soil[27,28] shown in Table 6. Phosphorus concentration in tissue is related to the structure that colonizes the root[29]. Thus, when the root is colonized by arbuscules the amount of phosphorus in tissue decreases because fungi also absorb phosphorus for growth; whereas when the root is colonized by vesicles and mycelium, phosphorus becomes available to the plant shown in Figure 1.

Table 6. Phosphorus percentage retained before and after inoculation in the soil used for planting seeds and seedlings of avocado

Origin	Phenological state	Initial Phosphorus Retained (%)	Final Phosphorous Retained (%)
Tumbabiro	Seed	94.50	76.60
	Seedling	94.50	75.70
Paute	Seed	94.50	71.50
	Seedling	94.50	73.20

Table 6 Continued

San José de Minas	Seed	94.50	74.30
	Seedling	94.50	72.90
Los Andes lote 2 Patate	Seed	94.50	67.50
	Seedling	94.50	72.50
Commercial Control	Seed	94.50	72.00
	Seedling	94.50	67.80
Absolute Control	Seed	94.50	90.07
	Seedling	94.50	91.30

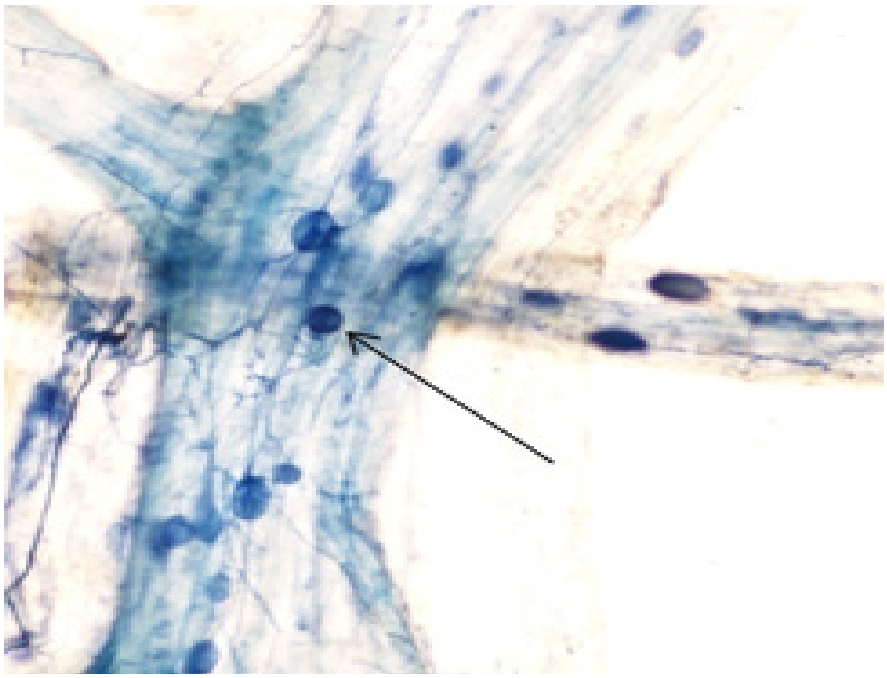

Figure 1. Vesicles observed in secondary and tertiary roots of avocado seedlings. The arrow indicates the vesicle inside the root.

4. Conclusions

The soil sampled in Tumbabiro and San José de Minas showed the best results both in quantity of spores and in the percentage of colonization in roots. In trap plants, the soils mentioned are increased by 84% and 100% the total amount of phosphorus and the percentage of dry matter respectively in comparison with the control. Plants produced from the avocado seeds and seedlings inoculated with the soils from Tumbabiro and San José de Minas showed a 44% increase in the percentage of dry matter compared to the control without inoculation; while the percentage of total phosphorus increased 42%. It is worth mentioning that the commercial control showed an acceptable performance; however, it was always behind the best treatments already mentioned, and it also did not display a good percentage of root colonization. This research allows us to infer that native mycorrhizal strains are effective in the development of avocado seedlings.

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