

EVALUATION OF METHODS TO ESTIMATE MYCORRHIZAL INOCULUM POTENTIAL IN FIELD SOILS

EVALUACIÓN DE MÉTODOS PARA ESTIMAR EL POTENCIAL MICORRÍZICO DE SUELOS DE CAMPO

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ABSTRACT

Arbuscular Mycorrhizal Fungi constitute a group of microorganisms that form symbiotic relationships with plant roots in most ecosystems. Mycorrhizal potential or inoculum potential (IP) are terms used in many studies as an indicator of propagule density and mycorrhizal activity in the soil. It allows the quality and infectivity of soil inoculum to be evaluated and is used as a biological indicator. A number of methods have been used traditionally to assess the mycorrhizal potential to colonize a plant. These methods include: Infection Unit (IU), Mean Infection Percentage (MIP), Most Probable Number (MPN) and Mycorrhizal Soil Infectivity (MSI), and others. This study reviews the state of the art of these methodologies and discusses pros and cons, and how the methodologies can be implemented and applied most effectively. We evaluated three techniques (IU, MIP and MPN) varying the field soil types as inoculum, plant hosts and proportions of substrate to inoculum (w/w). The statistical analysis detected that the treatment with the factors combining: MIP x Corn x Proportion 4:1 of Clay soil to inoculum was the most significant ($F=2.41$, $p=0.0915$). Hence, the data presented in this paper should allow researchers to select an effective method for assessing AM inoculum potential.

Key words: Arbuscular Mycorrhizal Fungi, Inoculum Potential, Soil Infectivity.

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RESUMEN

Los Hongos Micorrízicos Arbusculares son un grupo de microorganismos que forman relaciones simbióticas con las raíces de las plantas en la mayoría de los ecosistemas. El Potencial Micorrízico o Potencial de Inóculo (PI) son términos que se utilizan en muchos estudios como indicador de la densidad de propágulos y la actividad de micorrizas en el suelo, lo que permite evaluar la calidad e infectividad del inóculo del suelo y también usarlo como un indicador biológico. Tradicionalmente se han utilizado varios métodos para evaluar el potencial del suelo para colonizar una planta, tales como: Unidad de Infección (UI), Porcentaje de Infección Medio (PIM), Número Más Probable (NMP), Infectividad Micorrízica del Suelo (MSI), y otros. Este estudio revisa el estado del arte de estas metodologías, analiza pros y contras, y cómo la metodología puede ser implementada y aplicada con mayor eficacia para medir la infección micorrízica. Se evaluaron tres técnicas (IU, PIM y NMP) variando tipos de suelos de campo, plantas trampa y proporciones de sustrato-inóculo (p/p). El análisis estadístico demostró que el tratamiento con la combinación de factores: PIM x Maíz x Proporción 4:1 de suelo arcilloso fue el más óptimo ($F=2.41$, $p=0.0915$). La información presentada en este estudio asistirá a los investigadores a seleccionar un método efectivo para medir el potencial micorrízico de los suelos.

Palabras clave: Hongos Micorrízicos Arbusculares, Potencial de inóculo, Infectividad Suelo.

1 INTRODUCTION

Arbuscular Mycorrhizal Fungi (AMF) establishes symbiotic associations with over 80% of plants. The association promotes the plant's photosynthetic rate, protection from pathogens, tolerance to environmental stress, water balance, and nitrogen fixation in the soil by associative symbiotic bacteria (Andrade, 2010). All of these beneficial effects sustain soil health; and thus there is an increase in studies assessing the mycorrhizal potential in soils.

There are a variety of microbial parameters that can be used as diagnostic indicators of soil quality. Measurement of the status and activities of specific microbial communities, such as arbuscular mycorrhizal fungi and nitrifying bacteria, contributing to soil processes has the potential to provide particularly rapid and sensitive means of characterizing changes to soil quality (Bending *et al.*, 2004). Mycorrhizal potential or inoculum potential (IP) are terms used in many studies as an indicator of propagule density and mycorrhizal activity in the soil. The IP is defined as the ability of a fungal inoculum in the soil to infect the root system of plants under standard conditions (Dimond & Horsfall, 1960; Reyna *et al.*, 2006) meaning that this parameter allows the quality and infectivity of the inoculum to be evaluated (Hall, 1976; Smith & Smith, 1981). Another definition of IP could be the number of viable fungal propagules and structures with their original infectivity (Liu & Luo, 1994). Therefore, IP can be used as a biological indicator (Chang *et al.*, 2001; Vasconcellos *et al.*, 2013).

Most of the studies report different methods used to determine the ability of soils to produce mycorrhizal colonization. The Most Probable Number (MPN) of infective propagules, Infectious Unit (IU), Mean Infection Percentage (MIP), measurements of the number of spores, mycorrhizal colonization percentage of roots, and external mycelia (Sieverding, 1991; Morton *et al.*, 1993) are also used as indicators of potential soil infection. These techniques use several variables such as host plant, inoculation time and proportion of substrate-inoculum. This complicates the replication of these methods for measuring soil mycorrhizal potential in different field soils. Furthermore, there are unclear criteria of selection of the methodology used to measure the mycorrhizal potential, except by the criteria of being the method most reported in literature, as in the case of MPN (Porter, 1979; Moorman & Reeves, 1979; Powell, 1980; Franson & Bethlenfalvai, 1989). Therefore, a review and comparative study is needed in order to test the methodologies currently available.

This study aims to assess the MPN, MIP and IU techniques to measure the mycorrhizal potential of field soils, varying parameters such as soil type, type of plant host and the proportion of substrate (background soil which is sterilized medium to which the inoculum is added) to inoculum (with two types of natural soil), through monitoring the mycorrhizal potential at 14, 21 and 60 days, in order to determine the most efficient technique and propose an available method for research in this field.

2 MATERIALS AND METHODS

2.1. Overview of approach to analysis

To test the mycorrhizal inoculum potential methods, a systematic review of the literature available that performs the test was carried out, and the methods described were experimentally tested in a greenhouse assay.

2.2. Literature search and dataset construction

The NCBI, Google Scholar, and Web of knowledge databases (1958-2015) were searched using the key words *Arbuscular Mycorrhizal Fungi*, *Most Probable Number*, *Mean Infection Percentage*, *Infection Unit*, *Soil Inoculum Potential*, *Vesicular Arbuscular Mycorrhiza* and *Soil Infectivity* on 12 June 2015. An available "study" was defined as any measure of soil inoculum potential and soil infectivity using any of the three methods (MPN, MIP, IU). Studies were not included using other methods to measure the mycorrhizal inoculum potential. When data were not clearly described the authors of the papers were contacted. Papers were excluded, if no reply was received after one and half week. From each study, data were collected based on the type of container, plant host, soil type, test time, the ratio of inoculum to sterile soil used, potential and mycorrhizal colonization percentage, all with and without mycorrhizal inoculation. In addition, we recorded measures of mycorrhizal colonization in percentage (%), and inoculum potential in MPN/ g of soil (MPN), Percentage % (MIP), and IU/g of root (IU). When the data of mycorrhizal colonization percentage was not given, we contacted authors seeking this information.

2.3. Testing the methods

We obtained two samples of soil from guava plantations. The first one collected from the Tumbaco Experimental Farm of the National Institute of Agricultural Research (INIAP) located in Tumbaco, belonging to the Pichincha Province (UTM latitude: 9976119.33, UTM Length: 788105.50, Ellipsoidal height: 2420 m). The other soil sample was collected in Pedro Vicente Maldonado (PVM), northwest of the Pichincha Province, (UTM latitude: 717137.48, UTM Length: 9080.08, Ellipsoidal height: 620 m).

2.4. Characterization of soil samples

The physical-chemical analyses were carried out in the Soil Department of the National Institute of Agricultural Research (INIAP). Parameters such as moisture, pH, organic matter, and total elements (N, P, S, K, Ca, Mg, Zn, Cu, Fe and Mn) of the soil samples were analyzed.

2.5. Spore extraction

AM spores were obtained following the method of Sieverding (1991) and Oehl *et al.* (2005). Spores were isolated from soil particles using a dissecting microscope, where major taxonomic characteristics were observed such as spore size, color, shape, suspending hyphae and spore saccule. Subsequently, spores were fixed on slides with polyvinyl-lacto-glycerol (PVLG) and it was mixed with Melzer reagent to observe major taxonomic characteristics such as the presence and type of ornamentations on the wall of the spore, reaction to Melzer, number of germinal walls, and wall thickness, using a light microscope (Schenck and Pérez, 1990; Blaszkowski, 2012).

2.6. Characterization of spores

The characterization of the spores in each type of soil inoculum was made in the first instance by observing the color and shape of them. Subsequently, a microscopic determination of subcellular structures such as spore walls, germinal walls and the form of mycorrhizae was carried out. To establish the taxonomy of spores, morphological characteristics were contrasted with images and information available on the website of International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) of the West Virginia University.

2.7. Implementation of techniques to measure the mycorrhizal potential

Soil was prepared using a 1:1 (w/w) mixture of gravel and soil (low nutrient), which was sterilized twice using steam at 180°C for 50 minutes with a rest between steaming of 12-24 hrs. The second sterilization was done in order to eliminate all the microorganisms remaining after the first sterilization. After drying, the mixture was stored in plastic bags in a dry place.

The proportion of inoculum in pots was determined by weight field soil, it was weighed and mixed with sterile soil at ratios of 4:1 and 9:1 substrate:inoculum, respectively. Mixtures of substrate-inoculum were deposited in two types of containers, either: black plastic bags of 10.16 cm diameter by 15.24 cm height (for MIP and IU), and plastic cups of 250 mL (for MPN). Corn and oat plants were germinated four days before starting the experiment in, wet filter paper in sterile containers and planted in to experimental treatments.

The plants were kept in the greenhouse under controlled conditions of temperature (17-22 °C) and relative humidity (70-74 %), irrigated every two days.

To assess the techniques for measuring the mycorrhizal potential, a full factorial randomized block design was used. The analyzed factors were plant host (2), ratio of sterile to live soil (2), measures mycorrhizal potential (3), and soil type (2), a total of 24 treatments (Table 1). Each treatment was replicated twelve times, resulting in a total of 288 pots. To test the likelihood of cross contamination, sterile controls were established for each assay.

Table 1. Treatments used in the mycorrhizal potential experiment.

Analyzed factors	Characteristics
Proportion	Sterilized soil-inoculum 4:1 and 9:1
Plant Host	Corn (<i>Zea mays</i>), Oat (<i>Avena sativa</i>)
Soil type (inoculum)	Tumbaco Soil, Pedro Vicente Maldonado Soil
Mycorrhizal potential	MPN, IU, MIP

2.8. Evaluation of the mycorrhizal potential

To evaluate the mycorrhizal potential, plants were not irrigated for 3 days in order to produce water stress, ensuring the symbiosis between roots and fungus. After 14, 21 and 60 days of establishment, the roots of the host plants were harvested, these roots were rinsed with water and stored in sterile plastic bags at 17°C labeled for their subsequent staining and analysis according to the method described by Phillips & Hayman (1970), Gemma & Koske (1988), and McGonigle *et al.* (1990).

Infection Units: After 14 days of trial, 96 harvested roots were weighed and stained with Trypan Blue to assess the number of infection units and the mycorrhizal percentage colonization, using the methodology described by Franson, & Bethlenfalvay (1989). The evaluation lasted 4 days, and in addition hyphae, external mycelium, coils, vesicles and spores were scored.

Mean Infection Percentage: After 21 days of trial, 96 harvested roots were stained with Trypan Blue to assess the mean infection percentage, using the methodology described by Moorman & Reeves (1979). The evaluation lasted 5 days, and in addition hyphae, external mycelium, coils, vesicles and spores were scored.

Most Probable Number: Roots were harvested after 60 days then weighed and stained to assess the potential of AMF colonization and the mycorrhizal percentage colonization, following the methodology proposed by Porter (1979) and Sieverding (1991). The evaluation lasted 5 days, and in addition hyphae, external mycelium, coils, vesicles and spores were scored.

2.9. Statistical analysis

An ANOVA was used to analyze the data and estimate differences among means. In addition, Tukey's test (α 0.05%) was carried out to find means that are significantly different from each other. All data were processed with the statistical program Infostat® (2014).

3 RESULTS

3.1. Literature Systematic Review

The MPN technique demonstrates that all the propagules have the ability to infect the host plant (Liu & Luo, 1994). However, it has several limitations. It fails to detect dormant and viable spores or the fungal specificity to host plants (Adelman & Morton, 1986). In addition, results are likely to be influenced by edaphic variables like the temperature in which the plant were grown and length of time the assay run (Wilson & Trinick, 1982; Adelman & Morton, 1986); as well as manipulations of the inoculum, and type of inoculum (Wilson & Trinick, 1982).

The MIP technique has the advantage over the MPN by requiring fewer assays to obtain a result (Giovannetti and Mosse 1980). However, like MPN, it is sensitive to the environment and other variables associated to host plant and soil. Franson & Bethlenfalvay (1989) used irregular dilutions instead of a consistent dilution series. They stated few researchers have used the IU method, thus it still an open question. A summary of the strengths and weakness of these techniques are shown in Table 2.

According to the INVAM website (2013) more research is necessary to optimize harvest times to avoid the complications produced from secondary colonization, especially when the concentration of live soil in the assays are high. (Table 2).

Table 2 Strengths and weaknesses of currently used methods to estimate mycorrhizal infection potential.

Method	Strengths	Weaknesses	Comments
MPN	<ul style="list-style-type: none"> -Provides a single result that could be easily compared directly with other methods -Lower dilution is used, and reduces confidence intervals, increasing the accuracy of the test (INVAM, 2013) 	<ul style="list-style-type: none"> -More trial time (4-8 weeks) -Many dilutions used in the assay -Not detect all the fungi structures -Does not consider environmental effects -High confidence intervals, with a low dilution factor (INVAM, 2013) -Primary and secondary fungal infection can occur -Few studies of standardization 	<ul style="list-style-type: none"> -MPN is the method most used to calculate inoculum potential, however this method estimates the propagule numbers from a statistical table.
MIP	<ul style="list-style-type: none"> -Less trial time (21-30 days) -Measures primary mycorrhizal colonization, and secondary colonization does not occur (Giovannetti & Mosse, 1980) -Easy measurement of mycorrhizal potential -Less dilutions used in the assay 	<ul style="list-style-type: none"> -Does not consider environmental effects -Colonization percentage is not accurate in lower dilutions -Few studies of standardization 	<ul style="list-style-type: none"> -MIP is a simple method to use as an option to MPN.
IU	<ul style="list-style-type: none"> -Less trial time (14 days) -A relationship 1:1 between number of infective propagules and the number of infection units (INVAM, 2013) -Measures primary mycorrhizal colonization and secondary colonization does not occur (Franson & Bethlenfalvay, 1989) 	<ul style="list-style-type: none"> -Does not consider the environmental effects -Few studies of standardization -Use of irregular dilutions -Difficult to identify the type of infection of many fungal species by the short duration of the trial 	<ul style="list-style-type: none"> -IU is a simple method to use as additional option of MPN or MIP -Few researchers used this method

Electronic searching identified 527 publications, of which a subset of 50 satisfied the inclusion criteria (c. 9.48%) of the systematic review (figure 1). Articles consisted of MPN, MIP and IU techniques which measured the inoculum potential of soils. The variables most cited in these techniques are described in Table 3.

Table 3 Strengths and weaknesses of currently used methods to estimate mycorrhizal infection potential.

Method	Variables				
	Type of container	Host plant	Test time	Soil type	Sterile soil to inoculum ratio
IU	plastic tubes of 25-100 mL	onion, grasses, lettuce	14-30 days	sandy loam and silty	1:1
MIP	plastic tubes of 100-150 mL	corn, sorghum, grasses	30-60 days	sandy and sandy loam	1:1
MPN	plastic tubes of 400-500 mL	grasses, corn, onion	60-120 days	sandy loam and silty	1:1 and 1:2

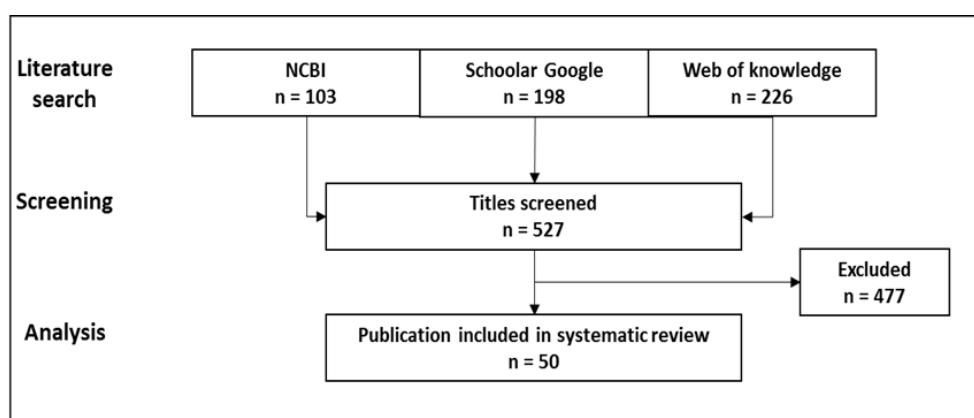


Figure 1 Flowchart diagram of the complete study selection process.

3.2. Experiment

3.2.1. Characterization of soil samples

Physical-chemical analysis of soil sampled from the Tumbaco Experimental Farm of the National Institute of Agricultural Research (INIAP), showed that the textural class was sandy-loam, the pH was slightly acidic, high level of organic matter (7.60%), and high levels of nitrogen, phosphorus and potassium. The textural class of the soil sampled from Pedro Vicente Maldonado was Clay and showed that the pH was slightly acidic, low level of organic matter (4.17%) with low levels of nitrogen, phosphorus (Table 4).

Table 4 Physical-chemical analysis of soil samples from the Tumbaco Experimental Farm and Pedro Vicente Maldonado.

Soil Sample	N	P	S	K	Ca	Mg	Zn	Cu	Fe	Mn	pH	Soil texture (%)		
	(ppm)			(meq/100 mL soil)			(ppm)					Sand	Silt	Clay
Tumbaco	61	114	25	1.7	60.8	4.9	16.1	11.5	232	15.9	6.3	45	37	18
Pedro Vicente Maldonado	1.5	0.03	-	-	-	-	-	-	-	-	6.4	40	40	60

3.2.2. Characterization of spores

AM fungal spores extracted from the sandy loam soil of Tumbaco Experimental Farm were identified as: *Diversispora trimurales* (Koske & Halvorson, 1989), *Acaulospora delicate* (Walker *et al.*, 1986), *Diversispora spurca* (Pfeiffer *et al.*, 1996), *Claroideoglopus etunicatum* (Walker *et al.*, 1986), *Acaulospora colombiana* (Spain, 1992) and *Acaulospora lacunose* (Morton, 1986).

AM fungal spores of the following species were found in the clay soil of Pedro Vicente Maldonado: *Rhizoglopus intradices* (Biermann & Linderman, 1983), *Acaulospora laevis* (INVAM, 2013), *Dentiscutata heterogama* (Koske & Walker, 1985) and *Acaulospora colombiana* (Spain, 1992).

3.2.3. Evaluation of the mycorrhizal potential

The mycorrhizal potential was measured in the twenty four treatments, and the results of each technique were expressed in colonization percentage and inoculum potential in the units of each method (Table 5, Table 6 and Table 7), and the results are described below.

3.2.3.1. Infection Unit (IU)

IU values (IU/g root fresh weight) and colonization percentage obtained after 14 days of the establishment of the experiment are shown in Table 5 and figure 2, where the combination of study factors (IU x Plant host x Soil type x Proportion substrate-inoculum) were detailed.

Table 5 Mean values of field root colonization and infective propagules in IU test.

Treatment N°	Plant host	Soil type	Ratio (w/w) substrate to inoculum	Colonization %	*IU/g root fresh weight
1	Corn	Clay	4:1	54.17	5.00
2			9:1	35.42	4.00
-		Control	-	0.00	0.00
3		Sandy loam	4:1	54.17	6.00
4			9:1	41.67	6.00
-		Control	-	0.00	0.00
5	Oat	Clay	4:1	50.00	20.00
6			9:1	35.00	22.00
-		Control	-	0.00	0.00
7		Sandy loam	4:1	45.83	20.00
8			9:1	24.17	9.00
-		Control	-	0.00	0.00

*IU was calculated by directly counting infection units per g fresh weight of host plant root using the methodology described by Franson & Bethlenfalvay (1989).

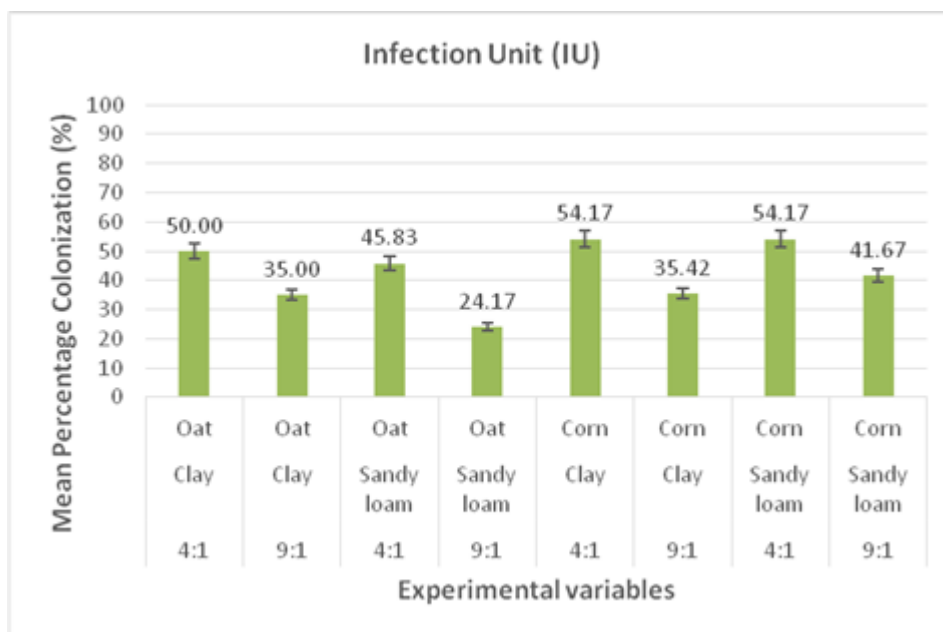


Figure 2 Percentage colonization mean values and standard errors of treatments in two types of soils with 4:1 and 9:1 ratio of sterile to inoculum, grown with either oat or corn plants, employing the IU method to estimate the mycorrhizal potential.

Trials in which corn was used as plant host and a proportion 4: 1 of clay soil or sandy loam soil obtained the highest mycorrhizal potential, with values of 5.00 UI/g root fresh weight (54.17% of colonization) and 6.00 UI/g root fresh weight (54,17% colonization), respectively (Table 5 and figure 2). In addition, it was found that there is no significant difference between the method and the type of soil ($p=0.2233$, $F=1.51$), therefore similar results were obtained in both treatments.

3.2.3.2. Mean Infection Percentage (MIP)

MIP values and colonization percentage obtained after 21 days of the establishment of the experiment are summarized in Table 6 and figure 3, where the combination of study factors (MIP x Plant host x Soil type x Proportion substrate-inoculum) were detailed.

Table 6 Mean values of field root colonization and infective propagules in MIP test.

Treatment N°	Plant host	Soil type	Proportion substrate-inoculum	*MIP %
1	Corn	Clay	4:1	81.67
2			9:1	67.50
-		Control	-	0.00
3		Sandy loam	4:1	78.75
4	9:1		62.50	
-	Control	-	0.00	
5	Oat	Clay	4:1	64.17
6			9:1	56.25
-		Control	-	0.00
7		Sandy loam	4:1	40.42
8	9:1		62.08	
-	Control	-	0.00	

*MIP was calculated using the methodology described by Moorman & Reeves (1979).

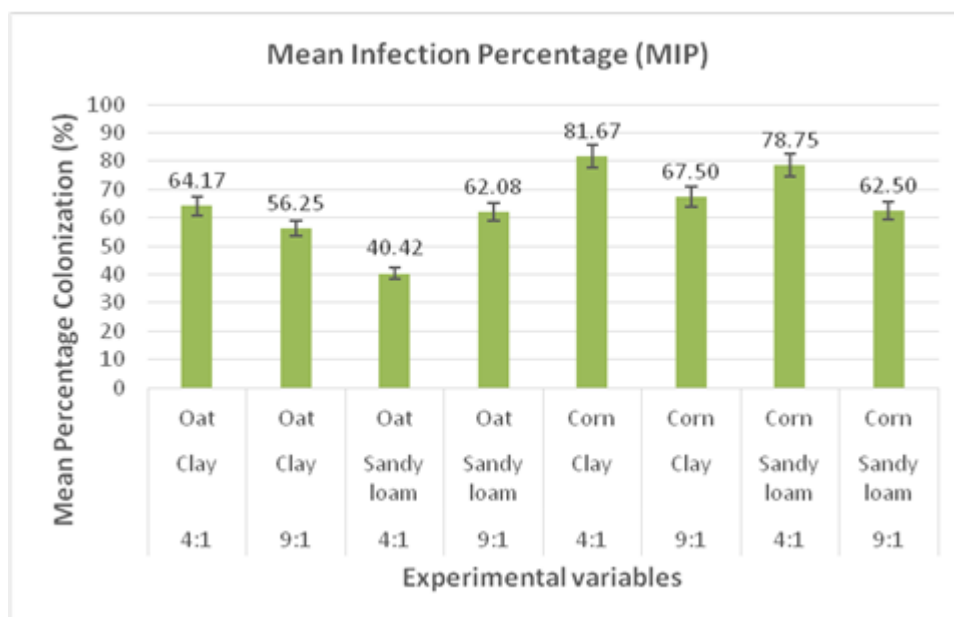


Figure 3 Percentage colonization mean values and standard errors of treatments in two types of soils with 4:1 and 9:1 ratio of sterile to inoculum, grown with either oat or corn plants, employing the MIP method to estimate the mycorrhizal potential.

The treatments in which corn and clay soil were used in a proportion of 4: 1 of substrate-inoculum showed the highest level of mycorrhizal potential, showing a

value of mycorrhizal potential (81.67%) (Table 6 and figure 3). The second highest value of mycorrhizal potential (78.75%) was obtained only when we used sandy loam soil (Table 6 and figure 3); however, no significant difference was found in the interaction between the MIP and the type of soil ($p=0.2233$, $F=1.51$).

3.2.3.3. Most Probable Number (MPN)

Mean values of MPN (MPN/g of soil) and colonization percentage obtained after 60 days of the establishment of the experiment are shown in Table 7 and figure 4, where the combination of study factors (MPN x Plant host x Soil type x Proportion substrate-inoculum) were detailed.

Table 7 Mean values of field root colonization and infective propagules in MPN test.

Treatment N°	Plant	Soil type	Proportion of aliquots showing infection at indicated dilution		Colonization %	MPN/g of soil	95% Confidence interval
			4:1	9:1			
1 and 2*	Corn	Clay	7/12	6/12	49.58	46.60	26.60-81.80
3 and 4*		Sandy loam	3/12	6/12	41.46	26.00	13.50-50.20
5 and 6*	Oat	Clay	3/12	6/12	41.25	26.00	13.50-50.20
7 and 8*		Sandy loam	5/12	10/12	54.17	52.40	31.10-88.30

*MPN calculation requires the use of at least two dilutions, for that reason two treatments with 4:1 and 9:1 proportions (dilutions) were used to calculate this number. The MPN was calculated following the methodology proposed by Porter (1979) and Sieverding (1991).

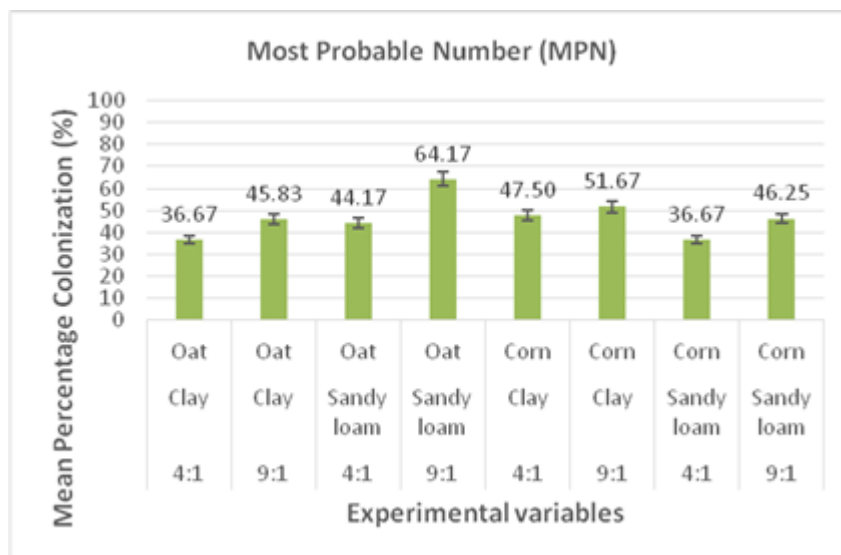


Figure 4 Percentage colonization mean values and standard errors of treatments in two types of soils with 4:1 and 9:1 ratio of sterile to inoculum, grown with either oat or corn plants, employing the MPN method to estimate the mycorrhizal potential.

The best results of mycorrhizal potential were found in trials where oat was used as a host plant and sandy loam soil at a proportion of 9:1 of substrate-inoculum, with values of 52.4 MPN/g of soil, and 64.17% of colonization (Table 7 and figure 4). Nonetheless, when corn was used as host plant, the best values were found using clay soil at a proportion of 9:1 of substrate-inoculum, with values of 46.6 MPN/g of soil and 51.67% of colonization (Table 7 and figure 4).

3.2.4. Statistical analysis

Overall the ANOVA and also Tukey test (α 0.05) revealed not significant difference between the MPN (46.61%) and IU (42.55%) methods ($F=40.61$, $p<0.0001$), however they differ significantly from the MIP (64.17%). A significant difference was detected in the percentage of mycorrhizal colonization between oat (47.40%) and corn (54.83%) ($F=12.74$, $p=0.0004$). There was not significant difference in using a clay soil (52.15%) or a sandy loam soil (50.07%) ($F=1.00$, $p=0.3179$), same was observed for the proportion of inoculum-substrate 4:1 (52.85%) and 9:1 (49.38%) ($F=2.78$, $p=0.0965$).

Analyzing the interactions between the method and the plant host; MIP x Corn (72.60%), MIP x Oat (55.73%) and IU x Oat (38.75%) were significantly different ($F=6.99$, $p=0.0011$). MIP x Clay soil (67.40%) differs significantly of any of the other methods combined with two types of soil ($F=1.51$, $p=0.2233$). In addition, the interactions IU x Proportion 9:1 (67.40%) and MIP x Proportion 4:1 (67.40%) were significantly different ($F=14.79$, $p<0.0001$). When Plant host x Soil was

compared, there is no significant difference between Oat x Sandy loam (46.81%) and Oat x Clay soil (47.99%); however they differ significantly with Corn x Clay soil (56.32%) ($F=0.19$, $p=0.6649$). Concerning to Plant-Proportion, Corn-Proportion 4:1 (58.82%) differs significantly from the other treatments ($F=4.70$, $p=0.0311$), and there is no significant difference in the relationship Soil-Proportion ($F=3.01$, $p=0.0840$).

In the interaction Method x Plant Host x Soil Type, there was only significant difference between the treatments IU x Oat x Sandy Loam Soil (35.00%) and MIP x Corn x Clay Soil (74.58%) ($F=5.49$, $p=0.0046$). Whereas in Method x Plant Host x Proportion, there was only significant difference between the treatments UI x Oat x Proportion 9:1 (29.58%) and MIP x Corn x Proportion 4:1 (80.21%) ($F=2.98$, $p=0.0526$). For the interaction Method x Soil x Proportion, it was found that IU x Sandy loam soil x Proportion 9:1 (32.92%) and MIP x Clay soil x Proportion 4:1 (72.92%) were significantly different ($F=0.95$, $p=0.3887$). In terms of Plants x Soil x Proportion, there was only significant difference between Oat x Sandy loam soil x Proportion 4: 1 (43.47%) and Corn x Clay Soil x Proportion 4: 1 (61.11%) ($F=0.94$, $p=0.3342$).

Finally, the four way interaction (Method x Plant x Soil x Proportion) of this study, MIP x Corn x Clay soil-Proportion 4:1 (81.67%) had the highest percentage of mycorrhizal colonization ($F=2.41$, $p=0.0915$) (Table 8).

Table 8 Tukey test (α 0.05) of 24 treatments with the factors combining: Method x Host Plant x Soil Type x Proportion substrate to inoculum (DMS=26.30265, Error: 311.9867 gl: 253).

Treatment	Method	Plant host	Type soil	Proportion	Mean
1	IU	Oat	Sandy loam	9:1	24.17 ^a
2	IU	Oat	Clay	9:1	35.00 ^{ab}
3	IU	Corn	Clay	9:1	35.42 ^{ab}
4	MPN	Oat	Clay	4:1	36.67 ^{abc}
5	MPN	Corn	Sandy loam	4:1	36.67 ^{abc}
6	MIP	Oat	Sandy loam	4:1	40.42 ^{abcd}
7	IU	Corn	Sandy loam	9:1	41.67 ^{abcde}
8	MPN	Oat	Sandy loam	4:1	44.17 ^{abcde}
9	IU	Oat	Sandy loam	4:1	45.83 ^{abcde}
10	MPN	Oat	Clay	9:1	45.83 ^{abcde}
11	MPN	Corn	Sandy loam	9:1	46.25 ^{abcde}
12	MPN	Corn	Clay	4:1	47.50 ^{abcde}
13	IU	Oat	Clay	4:1	50.00 ^{abcde}
14	MPN	Corn	Clay	9:1	51.67 ^{bcde}
15	IU	Corn	Sandy loam	4:1	54.17 ^{bcdef}
16	IU	Corn	Clay	4:1	54.17 ^{bcdef}
17	MIP	Oat	Clay	9:1	56.25 ^{bcdefg}
18	MIP	Oat	Sandy loam	9:1	62.08 ^{cdefg}
19	MIP	Corn	Sandy loam	9:1	62.50 ^{cdefg}
20	MIP	Oat	Clay	4:1	64.17 ^{defg}
21	MPN	Oat	Sandy loam	9:1	64.17 ^{defg}
22	MIP	Corn	Clay	9:1	67.50 ^{efg}
23	MIP	Corn	Sandy loam	4:1	78.75 ^{fg}
24	MIP	Corn	Clay	4:1	81.67 ^g

Means with common letter are not significantly different ($p \leq 0.05$)

4 DISCUSSION

4.1. Infection Unit (IU)

Infection Unit method was applied to make sure inoculum potential in clay and sandy loam soils by Franson & Bethlenfalvay (1989) and Cantrell & Linderman (2001), respectively. These studies used different host plants (grasses), in spite of this variation they obtained similar IU results between the two soils similar to this study there was not significant difference in terms of host plant ($p=0.0046$, $F=5.49$), hence oat obtained a mycorrhizal potential value very close to corn. Furthermore, there was a significant difference ($p=0.0526$, $F=2.98$) when the proportion of substrate-inoculum was varied, because the proportion 9: 1 reduced the results of mycorrhizal potential (Table 4 and figure 1). Franson & Bethlenfalvay (1989), authors of this method, used a series of irregular dilutions rather than a constant dilution for reasons not specified.

IU testing has a short duration of 14 days and only measure primary infections. The short duration can produce uncertainty in the results of mycorrhizal potential, because of the probability of contact of propagules with the root can be affected by the types of propagules present in inoculum, mixing, host species, etc (Morton, 2013). This would explain the variation in our results. Furthermore, this method has been the most poorly reported in the scientific literature. Consequently, there are not sufficient data.

4.2. Mean Infection Percentage (MIP)

The results from MIP test can be explained in terms of the diversity found in mycorrhizal inoculum. Sieverding & Toro (1985) stated that *A. colombiana* meets high specificity in soils with acid pH, which facilitates the cation exchange, allows the symbiosis and sporulation. However, León (2006) found that there are biotypes of *A. colombiana*, which prefer sandy soils and others prefer clay soils. This assay shows greater presence of this morphotype in both inoculum, and thus, it would assume that there is not a difference whether clay or sandy loam soils are used.

There are no studies about the use of the proportion of 9: 1 of substrate to inoculum. Nevertheless, Koide & Mooney (1987) and Morton (2013) recommend using a 1:10 proportion of substrate to inoculum for applying the MIP method in tests performed with tilled field, test inoculum, pot culture and commercial inoculants. This contrasts with the results of this study, because the proportion of 4:1 of substrate to inoculum resulted in a higher mycorrhizal potential. Moorman & Reeves (1979) stated that a bioassay is more accurate at 30 days after planting, since at the time the infection is mainly by propagules and not by the growth of the fungus in the roots; however, the relative proportions of mycorrhizal inoculum may need to be modified for a more precise potential infection bioassay.

To analyze the interaction between plant host and MIP, it is known that about 90% of all species of angiosperms belong to families that establish mycorrhizal relationships (Trappe, 1987), as is the case of corn and oat. Highest levels of root colonization by AMF detected in corn under organic management are supported by several previous studies (Ryan *et al.*, 1994; Galván *et al.*, 2009;

Verbruggen *et al.*, 2010). Besides, Moorman & Reeves (1979) and Morton (2013) mentioned that corn was the perfect host for bioassays because it is highly mycotrophic, produces abundant roots in three weeks, and fungal structures are easily observed. This is fully correlated with this assay.

On the other hand, when oat was used as plant host, there was a decrease in the potential mycorrhizal (64.17%) (Table 5 and figure 2). This may be due to environmental factors to which spores of mycorrhizal types inoculated were exposed to affecting their life cycle and germination capacity, settlement and development of the external mycelium (Read, 1999).

Additionally, this study found significant difference between the Plant Host and Method ($p=0.0011$, $F=6.99$), suggesting some degree of affinity of the fungus by the host plant. These results are confirmed by some studies that have shown that there may be some specificity relationship to the host by the physiology of the plant, different radical formation and chemical signals that may occur between the fungus and the plant (Manske & Vlek, 1994; Sieverding, 1991; Sánchez *et al.*, 2009).

4.3. Most Probable Number (MPN)

The results of this study are partially similar to those cited by Bagyaraj & Stürmer (2008), who reported that the host plant recommended in MPN test are the c4-grasses like corn, sorghum, millet, etc. Moreover, Porter (1979) said that the host plant used to measure the mycorrhizal potential must be highly mycotrophic

Additionally Gnekow & Marschner (1989) mentioned that sterilized soils ignores negative effects of soil organisms e.g. pathogens, and thus the soil sterilization may result in improved plant growth, especially when these are used with weakly mycotrophic species like cereals (Plenchette *et al.*, 1983). On the other hand, the inoculum potential value may increase because is directly proportional to the length and weight of the root (Liu & Luo, 1994). Additionally, any study does not specify which plant is better to use in the MPN method. Nevertheless Wilson & Trinick (1982) said that a plant with rapid root growth is required to provide a high root density, because this may be an important factor to enhance the MPN test. Thus, it may be assume that oat has a rapid root growth and consequently it would be important to carry out another study to check their growth rate.

In order to discuss the proportion of substrate-inoculum in the measurement of mycorrhizal potential, it is important to mention that Porter (1979) and Wilson & Trinick (1982) said that the use of a minor amount of soil with inoculum in comparison with the total volume of sterilized soil, decreases any errors produced by the effect of inoculum dilution on the nutritional status, growth, or infection of plant roots. Besides the soil nutrient status, especially the phosphorus level, must be appropriate, because it affects the number of infection points formed (Jasper *et al.*, 1979; Onguene & Kuyper, 2005). This is consistent with this study because the best results of mycorrhizal potential and colonization percentage were in the dilutions with a proportion of 9:1 of substrate-inoculum; due to the fact that the soil samples had high levels of phosphorus.

In general, agricultural field and fallow soils have high mycorrhizal inoculum potential (Onguene, 2000; Onguene & Kuyper, 2005). In this study, the best result for MPN method was obtained using the sandy loam soil from an agricultural field. According to Kalinhoff *et al.* (2009), the high values of infectivity are obtained from sandy loam soil where corn and fallows crops are grown, unlike the sandy clay loam soil of forest. Therefore, these data are consistent with the results obtained in our study because oat was planted in sandy loam soil.

There are other methods to study the fungi and its characteristics as a proxy of inoculum potential, among which are the selection of spores, DNA or RNA extraction from individual spores, amplification of gene regions, and clone and sequence of amplicons (INVAM, 2013). However the methods more cited evaluate AMF infection (MPN, MIP, IU and MSI) (Carter & Gregorich, 2007). MSI is similar to MPN method and is considered more precise; however, it requires the examination of hundreds and thousands of root system samples (Plenchette *et al.*, 1983).

Based on data and the statistical analysis obtained in this assay, the standardization of the techniques that measure the mycorrhizal potential must be done modifying variables such as the host plant, soil type and proportion of inoculum substrate. These variables allow parameterize studies which are focused on the detection of AMF potential of soils (Liu & Luo, 1994; Cantrell & Linderman, 2001).

The results of this study, on the three techniques used in this assay (MPN, MIP, IU), support conclusions of others authors (Hayman & Stovold, 1979; Schenck & Kinloch, 1980; Adelman & Morton, 1986) that the measurement of percentage infection in root samples provides a relative estimate of mycorrhizal potential.

The statistical analysis detected that the treatment with the following interaction MIP x Corn x Clay soil x Proportion 4:1, was the most significant (α 0.05, $p > 0.05$, $F = 2.41$). Therefore, it could be recommended as technique to test mycorrhizal inoculum potential. MIP has advantage in comparison to MPN and IU methods, because it requires less soil sample to obtain significant results and takes less time to perform. However it is important to mention that is equally sensitive to the different factors (host, soil and proportion), and the environment (Moorman & Reeves, 1979; Giovannetti & Mosse, 1980).

5 CONCLUSIONS

There are a variety of related studies to measure the mycorrhizal potential, but those described in this experiment don't make a comparative analysis among them and their experimental variables.

The experimental variables used in the techniques that measure the mycorrhizal potential, vary in all documents described in this study.

The results of this study clearly show that the measurements of mycorrhizal potential in soils is affected by the selection of different factors such as host plant, soil type and substrate-inoculum proportion. Hence, the data presented in this paper should allow researchers to select an appropriate method of assessing AMF infection that is most suitable for their use.

Something to keep in mind is that the standardization does not take into consideration environmental conditions which can influence the fungal response. For this reason, it is essential to consider any disparities existing between experimental and environmental conditions.

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