Evaluation of the effect of arbuscular mycorrhizal fungi infection in three aromatic plants.

Evaluación de la colonización por hongos micorrízico arbusculares en tres plantas aromáticas.

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ABSTRACT

The aim of this study was to evaluate the effect of the colonization of arbuscular mycorrhizal fungi in the production of secondary metabolites in *Petroselinum crispum*, *Salvia officinalis* and *Dysphania ambrosioides*. Three treatments were established: Control (C), commercial inoculum (CI) and native inoculum (NI). Every 15 days height, stem diameter and number of leaves were measured. The percentage of colonization was assessed by staining of roots, and identification of flavonoids through TLC thin layer chromatography, finally the concentration of total phenols was evaluated by spectrophotometry from the ethanol extracts of each plant. The results of development of biomass and the percentage of colonization show statistically significant difference for the three variables evaluated ($P \leq 0.05$) from each culture in the treatment of NI with respect to control, showing a greater effect on plants of *S. officinalis* (51% and 91%, respectively). Chromatography reveals the presence of flavonoids in the three plants; however this is more intense for the treatment of NI, showing increased production of total phenols in *S. officinalis*. Finally, the chemical characterization of the substrate shows a higher assimilation of nitrogen and phosphorus (0.11% and 0.35 mg / kg respectively) in *S. officinalis* associated with a native inoculum (NI).

Key words: arbuscular mycorrhizal fungus, *Petroselinum crispum*, *Salvia officinalis*, *Dysphania ambrosioides,* secondary metabolites.

RESUMEN

El objetivo de este estudio fue evaluar el efecto de la colonización de hongos micorrízicos arbusculares en la producción de metabolitos secundarios en *Petroselinum crispum, Salvia officinalis* y *Dysphania ambrosioides*. Se establecieron tres tratamientos: control (C), inóculo comercial (CI) e inóculo nativo (NI). Cada 15 días se midieron la altura, el diámetro del tallo y el número de hojas. El porcentaje de colonización se evaluó mediante tinción de raíces e identificación de flavonoides mediante cromatografía en capa fina de TLC, finalmente se evaluó la concentración de fenoles totales mediante espectrofotometría a partir de los extractos de etanol de cada planta. Los resultados del desarrollo de biomasa y el porcentaje de colonización muestran una diferencia estadísticamente significativa para las tres variables evaluadas ($P \leq 0.05$) de cada cultivo en el tratamiento de NI con respecto al control, mostrando un mayor efecto sobre las plantas de *S. officinalis* (51 % y 91%, respectivamente). La cromatografía revela la presencia de flavonoides en las tres plantas; sin embargo, esto es más intenso para el tratamiento de NI, mostrando un aumento en la producción de fenoles totales en *S. officinalis*. Finalmente, la caracterización química del sustrato muestra una mayor asimilación de nitrógeno y fósforo (0.11% y 0.35 mg / kg respectivamente) en *S. officinalis* asociado con un inóculo nativo (NI).

Palabras clave: hongo micorrízico arbuscular, *Petroselinum crispum, Salvia officinalis, Dysphania ambrosioides*, metabolitos secundarios.

INTRODUCTION

The Arbuscular Mycorrhizal Fungi (AMF) are obligate symbionts, which form mutual associations with most agricultural crops of interest, increasing the amount of nutrients absorbed by the plant, especially phosphorus (P), copper (Cu) and zinc (Zn), they are considered as biological resource because they generate environmental benefits by improving the physicochemical and biological soil conditions (Aguilera *et al.* 2008). Also they can induce the production of phenolic compounds and plant alkaloids, they play a positive role on them (Mera *et al.* 2009; Zeng *et al.* 2013). Currently, the AMF have been reported to contribute to the growth of the host plant and the synthesis of secondary metabolites in some aromatic plants (Zubek *et al.* 2009).

Aromatic plants are those plants that have active ingredients with a pharmacological action for health (Harborne 1998, Manach *et al.* 2005, Mera *et al.* 2009), the use of these plants for therapeutic purposes requires the urgent need for

scientific studies in order to discover the compounds contained in them which may have pharmaceutical application (Orhan *et al.* 2010, Zhang *et al.* 2012), within this broad group of plants is *Salvia officinalis* L., *Petroselinum crispum* M*.* and *Dysphania ambrosioides* L., commonly known as Salvia, peregil and epazote, respectively (Lu et a. 2001). These plants have been used to flavor food in the countries of South America, due to the presence of secondary metabolites such as flavonoids, coumarins, condensed tannins and saponins, these secondary metabolites provides a taste and smell to the food (Miliauskas *et al.* 2004, Delamare *et al.* 2007, Koochak *et al.* 2010), these substances are investigated by significant medical applications, such as, anti-inflammatory and digestive infections, antiradical, antioxidant, antimicrobial and antiviral (Seyyednejad *et al.* 2008, Hakkim *et al.* 2013).

Baricevic *et al.* (2001), Wong *et al.* (2006) and Bozin *et al.* (2007) with his research showed the antioxidant activity of methanol extract of *Salvia officinalis*, *Petroselinum crispum and Dysphania ambrosioides.* Lu *et al.* (2001) and Koochak *et al.* (2010) reports that these plants exhibit notable bactericidal and bacteriostatic activity against *Bacillus cereus*, *Bacillus megatherium*, *Bacillus subtilis*, *Aeromonas hydrophila*, *Aeromonas sobria* and *Klebsiella oxytoca*, inhibits growth due to the activity of active compounds known as secondary metabolites. Some studies have shown that the production of secondary metabolites in different parts of an aromatic plant can be improved or decreased depending on the associated AMF (Seyyednejad *et al.* 2008, Zeng *et al.* 2013). However, the degree of influence of different fungi AM may vary in the same plant or the same fungi in different aromatic plants. Therefore, the inoculum composed of different AMF may differ not only in their effectiveness in establishing the symbiosis and promote the growth of certain plants, but also in the production of higher contents of phenolic acids (Jurkiewicz *et al.* 2010). The hypothesis of this paper is that, the arbuscular mycorrhizal fungi associated with the aromatic plants allow the growth of biomass and produce high concentrations of secondary metabolites. Thus, the aim of this study was to evaluate the effect of the colonization of arbuscular mycorrhizal fungi in the production of secondary metabolites of aromatic plants of *Petroselinum crispum*, *Salvia officinalis* and *Dysphania ambrosioides*.

MATERIALS AND METHODS

The experiment was established in the Universidad Politécnica de Chiapas, located in the city of Tuxtla Gutiérrez, Chiapas, México. The geographic location was latitude 16° 45' 11 " north and longitude 93° 06' 56", corresponding to tropical

regions, with more than 1100 mm annual rainfall. During the experimental period the temperature of the greenhouse was maintained to 28°C, The air relative humidity was maintained at 60–65%. with the objective of evaluating the effect of the application of two AMF inoculum (native and commercial) on the development of aromatic plants of *Salvia officinalis*, *Petroselium crispum* and *Dysphania ambrosioides*, as well as the production of secondary metabolites.

Characterization of native inoculum: the native inoculum is obtained from an organic coffee plantation where the varieties of Arabic coffee and bourbon are sown; this is located in the municipality of Cintalapa, Chiapas, México. The geographic location was latitude 16° 40' 06" north and longitude 93° 38' 40", with more than 1018 mm annual rainfall. In the case of soil, he proceeded to take five samples of the plot at a depth of 30 cm. (Bennamoun *et al.* 2016). The characterization of the inoculum allows obtaining the number of spores of AMF g^{-1} rhizosphere soil. The spores were extracted and isolated according to Gerdemann and Nicholson (1963). This technique is used for sieving the coarse particles of the soil and retaining AMF spores and organic particles on sieves of different sizes. 50 g of soil was mixed with 500ml of water in the 1000 ml conical flask. The soil mixture was agitated vigorously to free the AMF spores from soil and allowed to settle for 5-10 minutes and the supernatant was decanted through sieves of 150 and 350 µm. Later, they were performed and mounted in lactoglycerol polyvinyl (PVLG) for identification under microscope (Schen *et al.* 1990, Méndez *et al.* 2011).

Establishment of plants and mycorrhizal inoculation: the plants *S. officinalis*, *P. crispum* and *D. ambrosioides* were propagated in germination trays with sand. One month after seeding were transplanted and inoculated for the three species, three different treatments, one consisting of 1g of commercial inoculum (CI), other by 10g native inoculum (NI) and the last, control group (C), without any inoculum. Ten replicates were used for each treatment. The substrate used consisted of a sterilized soil in autoclaved at 15 lb of pressure at 120 °C for 60 minutes (Karagiannidis *et al.* 2011a, 2011b). The application of mycorrhizal inoculants was performed at a dose of 1 g of IC and 10 g of NI, it was because the CI contains up to 70 spores g^{-1} of inoculum of *Glomus*, while the NI containing 7 spores g^{-1} of rhizosphere soil of *Glomus* and *Acaulospora*. The treatments were established in the greenhouse, during the experimental period the temperature of the greenhouse was maintained to 28°C, the air relative humidity was maintained at 60–65% and irrigation was performed with drinking water.

Plant growth responses and mycorrhizal colonization: during plant development, every 15 days were measured agronomic traits such as plant height measured by ruler from the floor base to the last leaf of the plant with digital vernier and number of leaves, in order to observe the plant growth effect related to fungal colonization. At 10 weeks, a destructive sampling was performed to determine the dry weight of foliage. For the percentage of mycorrhizal colonization, of roots they were rinsed with KOH and H_2O_2, and stained with trypan blue lactoglycerol (Phillips *et al.* 1970). The record of the frequency of root segments with mycorrhizal structures was observed with a compound microscope (40x) (Byomic Microscope 3, 5 inch LCD Deluxe), according to Giovannetti and Mosse (Giovannetti *et al.* 1980).

Plant material: the leaves of *S. officinalis*, *P. crispum* and *D. ambrosioides* were collected during the first 5 days of bloom. The leaves were air-dried and ground in a laboratory mill. The dried samples were stored at room temperature in closed containers in the dark until used.

Preparation of extracts: 20g of *S. officinalis*, *P. crispum* and *D. ambrosioides* leaves were added to 450 mL of methanol (Sigma- Aldrich) at 100%; this mixture was then sonicated using a Bandelin SONOREX ™ Digital Ultrasonic bath 10 P at room temperature during 2 h. Afterwards, the mixture obtained was filtered (filter paper Whatman No. 1) and centrifuged (Thermo scientific, SHKA® 200) at 3000 rpm during 10 minutes at room temperature. The supernatant was evaporated under vacuum in a rotary evaporator (Rotavapor R-215) at a temperature of 45°C and the residue was then suspended in 5 ml of methanol and stored at -20°C (Fowler *et al.* 2013).

Phytochemical analysis: the qualitative analysis was performed through a chemical analysis to detect the presence of major classes of secondary metabolites; it was determined using a silica gel thin-layer chromatography (TLC) reported by Harborne (Harborne 1992, Harborne 1998). Briefly, the chamber was saturated with mobile phase hexane (HYCEL): ethyl acetate (HYCEL): acetic acid (Avantor) (31: 14: 5). After, the stationary phase plates silica gel 60 F_{254} 10X20 cm aluminum base (Merck) were placed in the chamber. The standards of metabolite: developer reagent (flavonoids: quercetin: Reagent citrobórico) were used. Thus, the components were eluded during 20 minutes and were observed through a UV chamber, 245-365 nm (Chromato-Vue® C-75). Technique that allows the preliminary identification of a substance, based on differences in polarity of each of the elements present in an extract (Patra *et al.* 2013). The flavonoids, having in its structure different substituents have multiple polarities (Formica *et al.* 1995); the quantitative analysis was done using

a visible light spectrophotometer (DR5000- 03 HACH-USA) (Harborne 1992, Harborne 1998). Total phenols content was estimated as gallic acid equivalents (Singleton *et al.* 1999).

Chemical characterization of the substrate: in preparation of samples for chemical analysis proximal, the moisture of the treatments was assessed by drying at 105 °C for 24 h and Organic matter (OM) was determined by a muffle furnace at 550 °C (TMECC 2002, Taipale *et al.* 2016, Yağcı *et al.* 2016) Total nitrogen (N) was analysed by Kjeldahl method (AOAC 2000, Li *et al.* 2015). P contents were analyzed by colorimetric analysis in a spectrophotometer using the molybdate reagent ascorbic acid-ammonium (Shamim *et al.* 2015). Thus, the potential of hydrogen (pH) with the use of a potentiometer (HANNA HI 2211-01) was also determined. According to Konca *et al.* (2016).

Statistical analysis: Data are presented as mean (SD). For statistical analyses one way ANOVA was used at $p<0.05$ level of significance, with the Statgraphics PLUS program (1999).

RESULTS AND DISCUSSION

In native inoculum 7 spores of genus *Glomus* and *Acaulospora* g^{-1} of soil were found (Figure 1), which correspond to the species *Glomus clarum*, *Acaulospora excavata*, *A*. aff. *bireticulata*, *A. alpina* y *A. foveata.* Because the commercial inoculum has 70 spores g-1 attributable the genus *Glomus*, also identified in the native inoculum, an adjustment was made in the inoculum concentration to apply, so that 10g NI is applied, while for the treatments evaluated with commercial inoculum was added 1g.

Agronomic variables measured showed statistically significant differences ($p <0.05$) in the treatments evaluated. NI provided the highest values of biomass in the three crops (51% for *S. officinalis*, *P. crispum* 36% and 22% *D. ambrosioides*) (Table 1). The results were observed comparing treatments in terms biomass of each crop (Figure 2).

In the percentage of colonization in roots, structures such as vesicles, hyphae and arbuscules in NI and CI treatments were observed (Figure 3), compared to the control (uninoculated treatment). Statistically significant difference for the three treatments was observed in each crop. In Table 1 the percentages of colonization and presence of arbuscules for each culture and treatments are presented, in which can be observed that NI treatments increased colonization (85 to 91%) compared to CI

treatment (32 to 54%). The highest percentage of colonization was observed in roots of *Salvia officinalis*.

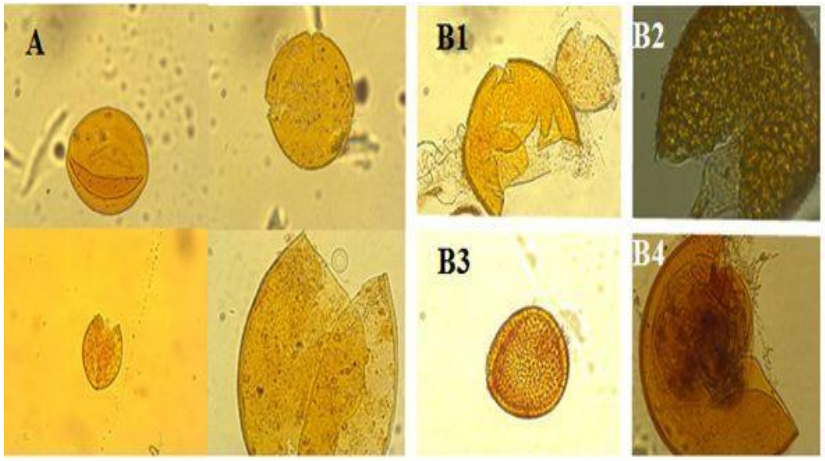

Figure 1 - Identification of spores belonging to the native inoculum (A- *Glomus clarum*; B1- *Acaulospora aff bireticulata;* B2- *Acaulospora excavata;* B3- *Acaulospora alpina;* B4- *Acaulospora foveata).* Spores mounted on a glass slide with a drop of PVLG + Melzer. Visualized with one objective of 40X.

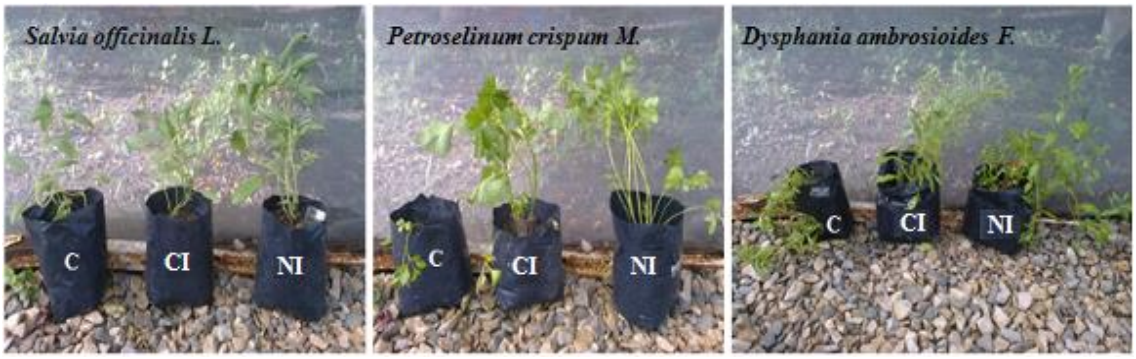

Figure 2 - Comparison of biomass among treatments (C= Control, CI= Commercial inoculum, NI= Native inoculum) of the three species evaluated (*Salvia officinalis, Petroselinum crispum* and *Dysphania ambrosioides*).

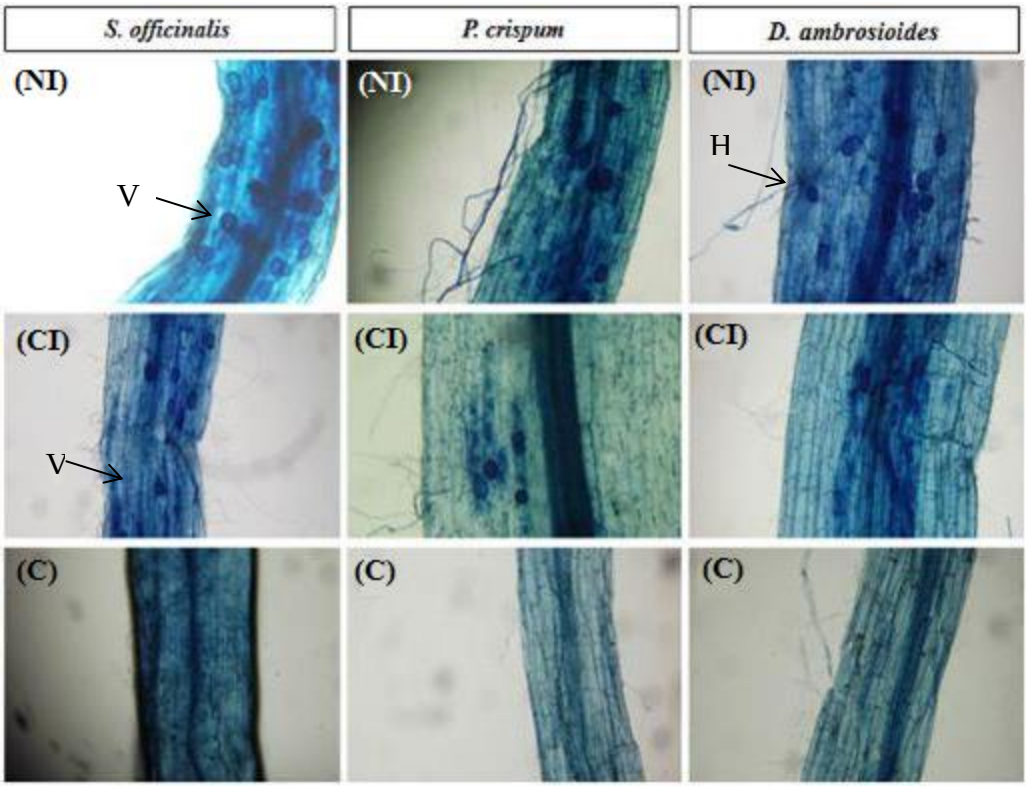

Figure 3 - Roots of *Salvia officinalis, Petroselinum crispum* and *Dysphania ambrosioides,* inoculated with the two treatments native inoculum (NI) and commercial inoculum (CI). In relation to control (C). Evaluated by the technique of clearing and staining of mycorrhizal roots, stained with trypan blue 0.05%. H: hyphae, V: vesicles.

Therefore, for qualitative identification and separation thereof, they used solvents of different polarities were used. In this work they were used medium polar solvents such as: hexane: ethyl acetate: acetic acid (31: 14: 5). The positive control used was naringenin; The Figure 4 shows the presence of this flavonoid indicated by arrow. When comparing the chromatograms of the positive control with the crude extract of the three plants evaluated orange spots are observed at a similar distance in relation to the positive control. The Figure 4 shows the chromatograms corresponding to crude extracts evaluated, the orange stains more intensely belong to NI, followed by treatment of CI compared to control.

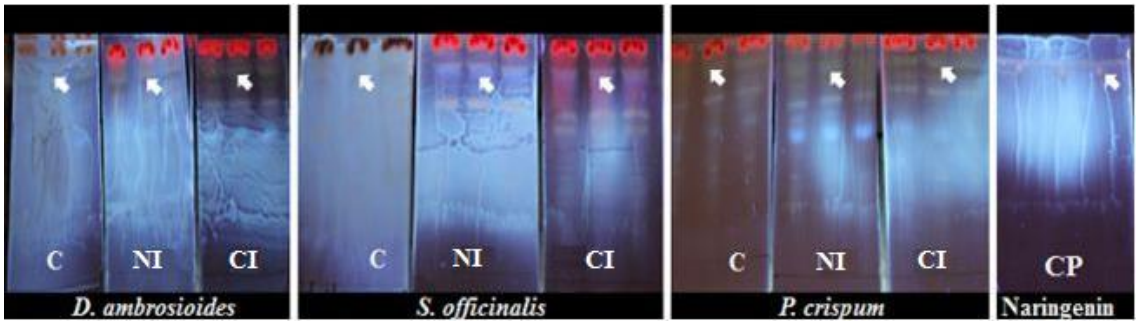

Figure 4 - Chromatograms of three plant species tested (*Salvia officinalis, Petroselinum crispum.* and *Dysphania ambrosioides)* by the influence of a commercial inoculum (CI) and a native inoculum (NI) with respect to control (C). Through the technique of thin layer chromatography on silica gel (TLC) analyzed in a UV chamber 245 at 365 nm (Chromato-Vue® C-75). The white arrows represent the presence of flavonoids with respect to the positive control (CP), naringenin.

Table 1. Quantification of agronomic factors and the percentage of root colonization of *Salvia officinalis, Petroselinum crispum* and *Dysphania ambrosioides* with native inoculum (NI) and commercial inoculum (CI).

Plant Species	Treatments	Height (cm)	Number of leaves	Colonization (%)	Presence/absence of arbuscules
Salvia officinalis	C	14.31 ± 0.13^{a*}	12.02 ± 0.15^{a}	0 ^{c}	A
	CI	11.80 ± 0.11^{b}	10.03 ± 0.12^{a}	54 ^{b}	P
	NI	25.03 ± 0.08^{c}	13.04 ± 0.01^{a}	91 ^{a}	P
Petroselinum crispum	C	15.39 ± 0.12^{ab}	08.05 ± 0.09^{b}	0 ^{c}	A
	CI	08.41 ± 0.13^{b}	04.02 ± 0.07^{c}	32 ^{b}	P
	NI	17.05 ± 0.07^{a}	13.01 ± 0.04^{a}	89 ^{a}	P
Dysphania ambrosioides	C	19.82 ± 0.13^{b}	01.85 ± 0.06^{c}	0 ^{c}	A
	CI	23.02 ± 0.00^{ab}	02.45 ± 0.08^{b}	46 ^{b}	P
	NI	31.01 ± 0.10^{a}	03.05 ± 0.07^{a}	85 ^{a}	P

C= Control, CI= Commercial inoculum, NI= Native inoculum, P= Presence, A= Absence

^{ab} Different literals denote a statistically significant difference (Tukey, p≤0.05)

* The values are average of ten replicates (n=10).

For quantitative analysis of secondary metabolites present in the methanol extracts, the total phenolic concentration was evaluated by the colorimetric method of Folin-Ciocalteu using gallic acid as standard. Comparing the control of each extract with treatment of NI, it shows that there is a statistically significant difference. However there is no significant difference when comparing treatments of NI with respect to CI for *D. ambrosioides* but there are significant statistical difference between NI treatments with respect to CI in *P. crispum* and *S. officinalis*. For treatments of CI with respect to control, there is no significant statistical difference in any of the evaluated extracts. Is worth mentioning that the plant with the highest concentration of total phenols was *S. officinalis*, the above can be seen in Table 2.

When evaluating the assimilation of nutrients especially N and P, it is observed that there is greater assimilation by treatments that include arbuscular mycorrhizal fungi (Table 3). However, N and P assimilation was more evident in native inoculums, as observed in *Salvia officinalis* with an assimilation of 50% reducing from 0.22% of total nitrogen to 0.11% and for phosphorus decreased from 0.68 to 0.35mg/Kg.

During experimental development, spores of the genus *Glomus* and *Acaulospora* were identified in the NI and CI, these spores were highly effective in root colonization as demonstrated in the evaluations, however the NI was more eficient so that 91% of colonization was observed for *S. officinalis*, 89% for P. crispum and 85% for *D. ambrosioides*, proving to be more efficient because the genera identified have been reported with high efficiency in different soil types (Kapoor *et al.* 2002). Due to the response of plants evaluated in symbiotic association with mycorrhizal. The positive role of the symbiosis established by the AMF in the growth of the plants was identified through assessment of the development of biomass; The NI showed the highest values in each treatment, this was because AMF represent an alternative to promote the growth of some plants (Szakiel *et al.* 2011). Previous work has shown that plants inoculated with AMF reflect an increased in phosphate, micronutrients and nitrogen in plants increased biomass (Karagiannidis *et al.* 2011). According Rajan *et al.* (2000), the hyphae extramatricales produced by AMF's activity as extensions of roots and increased root system surface, make more efficient uptake of water and nutrients diffusion limited and this effect more pronounced in the absorption of P in deficient soils that item, as shown in Table 3 with the phosphorus absorption values reflected in the decrease in phosphorus in the substrate of the three evaluated plants.

Table 2. Concentration of total phenols in relation to root colonization of *Salvia officinalis, Petroselinum crispum* and Dysphania *ambrosioides* with native inoculum (NI) and commercial inoculum (CI).

Plant Species	Treatments	Concentration (mg/g dry matter)
Salvia officinalis	NI	$9.1 \pm 0.11^{a*}$
	CI	7.8 ± 0.06^{b}
	C	6.7 ± 0.05^{c}
Petroselinum crispum	NI	3.0 ± 0.06^{a}
	CI	2.7 ± 0.11^{b}
	C	2.0 ± 0.13^{c}
Dysphania ambrosioides	NI	3.0 ± 0.09^{a}
	CI	2.9 ± 0.00^{a}
	C	2.7 ± 0.02^{b}

C= Control, CI= Commercial inoculum, NI= Native inoculum, P= Presence, A= Absence

[ab] Different literals denote a statistically significant difference (Tukey, $p \leq 0.05$)

[*] The values are average of ten replicates (n=10).

In the present investigation it has shown that the benefit AMF increase in the concentration of these secondary metabolites in plants, this also proven by Toussaint *et al.* (2007); Smith (2008); Zubek (2009).

Recognizing the effects of AMF on the biochemical pathways of the host plant could benefit the pharmaceutical industry; developing varieties of plants with improved growth and increased levels of active compounds (Zubek *et al.* 2009).

This investigation showed that the AMF benefits the increase in the concentration of these secondary metabolites in plants; it also tested by Toussaint *et al.* (2007); Smith (2008); Zubek (2009).

Table 3. Chemical characterization of the substrate used after 45 days of evaluation in *Salvia officinalis*, *Petroselinum crispum* and *Dysphania ambrosioides* for the native inoculum (NI) and commercial inoculum (CI).

C= Control, CI= Commercial inoculum, NI= Native inoculum, P= Presence, A= Absence

[ab] Different literals denote a statistically significant difference (Tukey, p≤0.05)

Plant Species	Treatments	Available Phosphorus (mg/Kg)	M.O (%)	pH	Total nitrogen (%)
	Initial control substrate	0.70 ± 0.11	5.89 ± 0.08	6.81 ± 0.05	0.23 ± 0.01
Salvia officinalis	C	0.68 ± 0.09[a]	5.89 ± 0.02[a]	6.51 ± 0.06[a]	0.22 ± 0.01[a]
	CI	0.49 ± 0.12[b*]	4.12 ± 0.12[b]	6.61 ± 0.07[a]	0.16 ± 0.12[a]
	NI	0.35 ± 0.09[c]	2.95 ± 0.13[c]	7.01 ± 0.05[a]	0.11 ± 0.02[b]
Petroselinum crispum	C	0.67 ± 0.06[a]	5.66 ± 0.07[a]	6.71 ± 0.04[a]	0.20 ± 0.15[a]
	CI	0.50 ± 0.02[b]	4.25 ± 0.05[b]	6.51 ± 0.00[a]	0.18 ± 0.03[a]
	NI	0.45 ± 0.13[c]	3.13 ± 0.06[c]	7.21 ± 0.01[a]	0.12 ± 0.02[b]
Dysphania ambrosioides	C	0.65 ± 0.07[a]	5.79 ± 0.00[a]	6.21 ± 0.13[a]	0.21 ± 0.14[a]
	CI	0.49 ± 0.06[b]	4.15 ± 0.09[b]	6.81 ± 0.06[a]	0.16 ± 0.01[a]
	NI	0.37 ± 0.08[c]	3.03 ± 0.13[c]	7.11 ± 0.03[a]	0.12 ± 0.11[b]

[*] The values are average of ten replicates (n=10).

So too, Toussaint *et al.* (2007) and Szakiel, Paczowski (2011) AMF show that not only are involved in the development and improvement of plant growth. In this way, they are closely related to the production and quality of active compounds present as secondary metabolites, clearly the results of this research confirm the foregoing, due to for the most outstanding total phenol concentrations evaluated in three plant species correspond to the treatments inoculated with AMF with respect to the control treatment. It has been shown that establish symbiosis arbuscular mycorrhizae can improve synthesis of some amino acids and contribute to the accumulation of specific metabolites by promoting the absorption of N (Toussaint *et al.* 2004, Smith 2008), it is currently known that tyrosine and phenylalanine are important precursors of phenolic compounds (Petersen and Simmonds 2003). So that, some research has shown that

AMF symbiotic activity promotes absorption of P, N, minerals and other nutrients that contribute to the accumulation of secondary metabolites of plants (Kapoor *et al.* 2002, Zeng *et al.* 2013).

In conclusion, arbuscular mycorrhizal fungi play an important role in establishing symbiosis with plants, same as demonstrated in the further development of biomass that is important in the case of aromatic and medicinal plants, because they offer a higher amount of secondary metabolites, also show that exercise has a significant effect on the production of secondary metabolites in relation to the concentration of total phenols. Therefore, future research should focus on the mechanism of the AMF which increase the content of the active ingredients of the inoculated plants, implemented the production of plants with high concentrations of secondary metabolites that can be targeted to a pharmacological evaluation for therapeutic purposes, for health due to their antioxidant potential. The application of native samples may represent an important alternative on crop yields and production of secondary metabolites, with respect to commercial inoculants.

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