RESPONSE OF *CAPSICUM CHINENSE* TO MYCORRHIZAE INOCULATION AND LOCAL MYCORRHIZAL DIVERSITY ASSOCIATED WITH THE CROP IN PUERTO RICO

by

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ABSTRACT

Capsicum chinense is widely cultivated in the Caribbean region. Most types are pungent, such as 'Scotch Bonnet' and 'Habanero'. In Puerto Rico, non-pungent types called "ají dulce" are consumed as part of the local cuisine. Currently, in commercial agriculture, the use of chemical fertilizers dominates the local market, while biological ones are overlooked. The purpose of this research was to characterize and identify the endomycorrhizae associated with locally grown *C. chinense* in the western area of Puerto Rico. We collected “ají dulce” roots and surrounding soil from plants growing in experimental plots at UPRM. Plants were grown under six treatments: (1) commercial Promix® BX, (2) Promix® Mycorrhizae (*Glomus intraradices*), (3) Promix® mixed with 25% soil, (4) Promix® BX plus inorganic (N-P-K) fertilizer, (5) Promix® Mycorrhizae (*Glomus intraradices*) plus inorganic fertilizer and (6) Promix® mixed with 25% soil plus inorganic fertilizer. Aji dulce planted in Promix® Mycorrhizae without fertilizer had greater plant height, and a larger number of branches, leaves and fruits, compared to the other substrates. But when fertilizer was added, there was little or no positive response of plants in Promix Mycorrhizae. To determine the percentage mycorrhizae infection, slides were done utilizing Trypan blue-stained root cuts. Differences in percentage infection were found among treatments with Promix® + Soil exhibiting the highest percentage infection. DNA extractions and PCR reactions using specific primers for mycorrizhae were performed. Cloning of PCR products for sequencing were done. Based on GenBank database searches, we established that the genera *Scutellospora, Diversispora, Acaulospora, Racocetra, Funneliformis, Glomus, and Paraglomus* are present in local soils.

RESUMEN

Capsicum chinense es ampliamente cultivado en la región del Caribe. La mayoría de sus tipos son picantes como el 'Scotch Bonnet' y el 'Habanero'. En Puerto Rico, los tipos no picantes son llamados "ají dulce" y son consumidos como parte de la cocina local. Actualmente, en la agricultura comercial, el uso de los fertilizates químicos es lo que domina el mercado local, mientras que los biológicos son pasados por alto. El propósito de esta investigación es caracterizar e identificar las endomicorrizas asociadas a *C. chinense* crecido localmente en el área oeste de Puerto Rico. Se recolectaron raíces de “ají dulce” y suelo adyacente a las plantas creciendo en predios experimentales de UPRM. Estas plantas estuvieron expuestas a seis tratamientos usando (1) Promix® BX para uso general comercial, (2) Promix® Mycorrhizae (*Glomus intraradices*), (3) Promix® con 25% del suelo del invernadero de UPRM, (4) Promix® BX para uso general comercial con fertilizante, (5) Promix® Mycorrhizae (*Glomus intraradices*) con fertilizante y (6) Promix® con 25% del suelo del invernadero de UPRM con fertilizante. Aji dulce plantado en Promix® Mycorrhizae sin fertilizante tuvo mayor altura de la planta, cantidad de ramas, hojas y frutos comparado con los otros substratos. Pero cuando se añadia fertilizante había poca o ninguna respuesta positiva de las plantas con Promix® Mycorrhizae. Además, para calcular el porciento de infección, se hicieron laminillas utilizando cortes de raíces teñidas con Trypan blue. Por consiguiente, diferencias en el porcentaje de infección fueron encontradas entre los tratamientos donde Promix® + suelo con fertilizante obtuvo el porciento de infección más alto. Se realizaron extracciones de DNA y reacciones de PCR utilizando cebadores específicos para micorrizas. Se clonaron los productos de PCR y se secuenciaron. Usando búsquedas en

GenBank el análisis sugiere la presencia en el suelo de UPRM de los siguientes géneros de micorrizas: *Scutellospora, Diversispora, Acaulospora, Racocetra, Funneliformis, Glomus,* y *Paraglomus.*

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DEDICATION

To my mom Olga, who has always loved me unconditionally and encouraged me in all my endeavors, who also showed me understanding; to never give up on my goals and supported my career whichever way she could. Also, she has been proud of me since day one.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Glossary

AMF- Arbuscular Mycorrhizal Fungi

PGPR- Plant Growth-promoting rhizobacteria

1 General Overview

1.1 INTRODUCTION

1.1.1 *Capsicum chinense* Jacquin

Peppers, such as bell peppers, banana peppers, sweet chili peppers, chilies, and jalapeños, taxonomically belong to the genus *Capsicum* (Heiser and Smith, 1953). Among the genus *Capsicum* there are five domestic species: *C. annuum, C. baccatum, C. frutescens, C. chinense* and *C. pubescens*. According to the Census of Agriculture in Puerto Rico (2012), 508 farms are partially or completely dedicated to the production of peppers. In 2012, Puerto Rico produced 5,500 tons of peppers (*Capsicum* spp.) (FAOSTAT 2014). During the same year, USA expended $409 million in imports of green and dry peppers. According to the Agriculture Department of Puerto Rico (2015), in the tax year 2013-2014, 1,601,900 pounds of *Capsicum chinense* were produced on the island.

Capsicum chinense Jacquin is widely cultivated in the Caribbean region (Lim 2013). Most types are pungent, such as 'Scotch Bonnet' and 'Habanero'. In Puerto Rico, non-pungent types are preferred and referred to as "ají dulce" or sweet chili pepper. In terms of area cultivated, sweet chili pepper is a minor crop in Puerto Rico. However, it is an essential ingredient in the local cuisine. Previous studies (Constantino et al. 2008) have established that Mexico has the greatest genetic diversity of chilies and peppers (*Capsicum* spp.), and this country occupies second place in terms of world production. Also, Mesoamerica, which includes central Mexico to the northwestern of Costa Rica, is considered one of the most relevant regions where crops were domesticated in the Americas (Aguilar-Meléndez 2006, Hughes et al 2007, and Pickersgill 2007). Another fact is that archaeological evidence from Mexico shows that humans had been using wild chiles as a food source approximately since ~8000 B. C., not only as condiments, but also as components of their sophisticated diet (Aguilar-Meléndez 2006, Smith 1967). However, yields are generally very low with a mean of 13.7 t ha^{-1} (Constantino et al. 2008). Despite this, *Capsicum* spp. represents one of the main ten horticultural exports of Mexico (FAOSTAT 2012). Peppers are known for being a good source of vitamins A, C, E and for their antioxidant properties (Antonious et al. 2009). Besides their use in cuisine, they are recognized and used as part of ornamental and medicinal practices.

Currently in the field of agriculture are commonly use chemical fertilizers as a stimulant for growth in crops. In Puerto Rico the use of biological products as fertilizers is unusual because the agriculturist is used to the results that they obtained by chemical fertilizaers. There are a few works in Puerto Rico related to mycorrhizae but none of them including *Capsicum.*

1.1.2 Mycorrhizae

Even though *C. chinense* is widely grown on the island, no previous report describes the diversity and abundance of fungi associated with the plant. **Mycorrhizae** are fungi that have a symbiotic relationship with the roots of vascular plants (Castillo et al. 2009). This symbiosis is present in 95% of terrestrial plant species in almost all natural habitats (Trappe 1987, Vacacela Quizhpe 2009). Three types of mycorrhizae exist based on the morphological structures they form in the plant roots (Vacacela Quizhpe 2009): [1] **Ectomycorrhizae**, well-known for dense mantle and Hartig net presence, [2] **Ectendomycorrhizae**, which includes the arbutoid and monotropoid types, and [3] **Endomycorrhizae**, which are characterized by intracellular colonization of the root cells by the fungi. The latter is divided into ericoid, orchidioid and arbuscular types (Read 1999). All glomeromycotean fungi, except one genus, are known to form arbuscular mycorrhizae (Redecker and Raab, 2006).

1.2 LITERATURE REVIEW

1.2.1 Endomycorrhizae

Some characteristics of **Arbuscular Mycorrhizal Fungi** (AMF) are that the mantle is not formed in this group and fungi colonization of the root can be inter and intracellular producing structures in the root cells known as vesicles and arbuscles. The purpose of the **vesicles** is food storage. **Arbuscles** increase contact area in the cell through hyphae ramification increasing nutrient exchange with the plant. Members of AMF are part of the Phylum Glomeromycota, which includes three classes: Archaeosporomycetes, Glomeromycetes, and Paraglomeromycetes, five orders: Archaeosporales, Diversisporales, Gigasporales, Glomerales and Paraglomerales, 14 families, 29 genera and approximately 230 species (Oehl et al. 2011). Among this phylum, many species form **glomoid spores,** which are spores alike in mode of formation (i.e. blastic development), and a spore wall structure like *Glomus*, which was the first genus studied in Glomeromycota (Blaszkowski and Chwat 2013; Schüßler et al. 2001). Previously, diversity and identification was based on spore morphology, spore formation, and spore wall structure (Gerdemann and Trappe 1974, Walker and Sanders 1986, Morton and Benny 1990, Schenck and Pérez 1990). Unfortunatedly, these few morphological characters lack significance to differentiate species in the Glomeromycota (Oehl et al. 2011). Commonly, glomerospores are morphologically similar with extensive diameter range, 80–500 μm (Simon et al. 1993). On the other hand, study of AMF diversity in mycorrhizal roots is scarce because of the difficulty of distinguishing AM fungal species based on hyphal morphology in roots (Shafiqua and Stephan 2013). DNA-markers have recently been developed to identify AM fungi (Redecker et al. 2003, Reddy et al. 2005).

1.2.2 Endomycorrhizae relationship with plants

AMF in natural and semi natural ecosystems are important because they improve plant productivity and diversity, as well as increase plant resistance against biotic and abiotic stresses (Smith and Read 2008). Also, they are important in ecological agriculture for the benefits provided to most cultivars and environment conservation by acting as biofertilizers, bioprotectors and biocontrol agents (Azcón- Aguilar et al. 2002). AMF colonized roots use extraradical mycelium for exploring a greater volume of soil, and translocating nutrients from soil to the plants more efficiently (Linderman 1992, Souchie et al. 2006). The extensive extraradical AMF mycelium improves capture of relatively immobile nutrients in soil such as Phosphorus (P) (Souchie et al. 2006), Copper (Cu) and Zinc (Zn), and also improves water absorption (Augé 2004). In exchange for these nutrients, endomycorrhizae can capture hexoses from the apoplast of the root cortical cells (Douds et al. 2005). Phosphorus is known to be one of the most essential elements for plant growth and development after nitrogen (N) (Tanwar et al. 2013). AMF greatly contribute to phosphorus uptake (Schnepf et al. 2009, Beltrano et al. 2013), in addition to other ions previously mentioned. Not surprisingly, it has been shown that the colonization potential of AM fungi decreases with increasing P concentration in the soil (Fusconi et al. 2005, Tanwar et al. 2013). Other studies, have found that AMF can also help plants alleviate detrimental effects of salinity on growth by modulating cell membrane stability at high P and salt soil concentration (Beltrano et al. 2013). The primary advantage of mycorrhizal hyphae in P uptake is the ability to extend deeper in the soil beyond the P depletion zone of the roots (Ravnskov and Jakobsen 1995). It has been reported that plant dependency on mycorrhizal fungi correlates with the level of soil fertility and receptivity of soil to inoculants (Covacevich and Echeverria 2008).

In order to take advantage of the mycorrhizae relationship, it is imperative to establish compatibility between host and AMF by selecting an appropriate fungal strain for a specific plant cultivar (Kaya et al. 2009, Tanwar et al. 2013). In addition, combinantion of beneficial microorganisms in soils have shown increase in crop resistance to diseases, abiotic stress and yield production. Consortium use of AM fungi, along with some rhizobacteria to enhance quantity and quality of plant production in agriculture, is a relatively recent technology (Tanwar et al. 2013). Among several free-living microorganisms, plant growth-promoting rhizobacteria (PGPR) have received special attention due to their beneficial effect on plant growth promotion by enhancing mineral nutrition (Gamalero et al. 2004) and synthesis of phytohormones (Gamalero et al. 2008). Nitrogen fixation, the solubilization of phosphorous in the rhizosphere and the production of phytohormones enhance plant growth directly. Hence, application of biofertilizers, including seed coating, is highly beneficial for various crops (Terry et al. 2002), reducing production costs by using lower volumes of inoculants (Taylor and Harman 1990). In the case of crops that require transplanting, such as the habanero chilli, one inoculation technique consists of dipping the roots of seedlings in a mixture of solid biofertilizer and water immediately before transplanting (Bashan 1998).

Recent studies have suggested that AMF-inoculated peppers benefit from association with PGPR by increasing drought resistance (Davies et al. 2002), reducing pathogenic infection and enhancing fruit production (Salami 2002, Garmendia et al. 2004), increasing P intake and chlorophyll content, exhibiting greater leaf development, and increasing carbohydrate content (Demir 2004). In addition, increased saline soil resistance has been shown in peppers (Turkmen et al. 2005, Kaya et al. 2009, Beltrano et al. 2013), and chilies (Selvakumar and Thamizhiniyan 2011). Differences in mycorrhizal responsiveness between different crops and among different genotypes within the same crop have also been observed (Declerck et al. 1995, Parke and

Kaeppler 2000, Linderman and Davis 2004). Finally, it is important to mention that mycorrhizal colonization significantly decreases with fertilizer application (Tanwar et al. 2013) warranting the search for alternatives to chemical fertilizers.

1.2.3 Previous Endomycorrhizae studies in Puerto Rico

In Puerto Rico, only a few studies have been done with mycorrhizae, for example in pines (Briscoe 1959), in *Coffea arabica* (Lebrón et al. 2012), in orchids (Otero et al. 2002, 2004; Bayman et al. 2016) in *Vanilla* (Porras-Alfaro and Bayman 2007) and in tropical forest (Bachelot et al. 2017). Briscoe (1959) worked on pine roots of introduced plants in Puerto Rico determining the inoculation effects, but did not identify mycorrhizae species. In the case of *Coffea arabica,* it was reported that the morphotypes present were typically *Glomus* and *Sclerocystis,* although no molecular tools were used to confirm these genera (Lebrón et al. 2012). Bayman et al. (2016) determined that ectomycorrhizae diversity associated with the orchid *Oeceoclades maculata* belonged mostly to members of the Basidiomycota. Otero et al. (2002) identified four fungal lineages related to the basidiomycete *Ceratobasidium* spp. from temperate regions in the tropical orchids: *Campylocentrum fasciola* (Lindl.) Cogn., *Campylocentrum filiforme* (Sw.) Cogniaux, *Erythrodes plantaginea* (L.) Fawcett & Randle, *Ionopsis satyrioides* (Sw.) Reichenbach f., *Ionopsis utricularioides* (Sw.) Lindl., *Oeceoclades maculata* (Lindl.) Lindl., *Oncidium altissimum* (Jacq.) Sw.; *Psychilis monensis* Sauleda, and *Tolumnia variegata* (Sw.) Braem. For the orchid *Tolumnia variegate* mycorrhizal isolates fell into four different clades of *Ceratobasidium,* while most of those from *I. utricularioides* were restricted to a single clade (Otero et al. 2004). At the same time, a study made in yet another orchid, *Vanilla,* related basidiomycetes in the genera *Ceratobasidium, Thanatephorus* and *Tulasnella* were found (Porras-Alfaro and Bayman 2007). Most recently, the study done by Bachelot et al. (2017)

sequenced soil for AMF diversity at the tropical rainforest in Puerto Rico (El Yunque National Forest) but they did not mention which species were found. The authors found that AMF diversity in soil counteracted negative effects from foliar damage on seedling mortality especially in rare tree species. In adittion, the effects of foliar damage and soil AMF diversity might foster tree species coexistence at El Yunque (Bachelot et al. 2017).

Although different species of mycorrizhae were found in each of the organisms studied in Puerto Rico, very limited data about glomeromycotan fungi are available for the island. A recent study (Urbina et al. 2016) on soil fungi of Puerto Rico reported only 1.6% of Glomeromycota present in the data. Soils in the island seem to be dominated by Ascomycota (79.6%) and Basidiomycota (17.5%) fungi. Nonetheless, mycorrhizae are present in association with different plants and soil types across the environment. In addition, no local information regarding the effect of endomycorrhizae on crops was found.

In order to better document the relationships between *C. chinense* and their root-associated fungi, this study gathers information about their diversity and then determine their importance in promoting growth in this crop. The purpose of this work was identifying endomycorrhizae diversity in local soil in western Puerto Rico that could be use as a biofertilizer in crops as *Capsicum chinense*.

1.3 Goals

1.3.1 Hypotheses

1. A great diversity of genera of endomycorrhizae will be found in soils of westerm Puerto Rico.
2. Endomycorrhizae used as biofertilizers in *C. chinense* plants will produce a better yield and growth than chemical fertilizers.

1.3.2 Objectives

1. Characterize and identify endomycorrhizae diversity in *C. chinense* grown in local soils.

2. Determine effects of substrates with commercial mycorrhizae vs. naturally occurring mycorrhizae from local soils on growth of *C. chinense*.

3. Study the effect on plant growth of adding inorganic fertilizer to substrates containing mycorrhizae.

2 MATERIALS AND METHODS

2.1 Confirmation of presence of endomycorrhizae in a field planting of **Capsicum chinense** *(Sweet chili pepper)*

2.1.1 Sample collection

Six mature *C. chinense* plant samples were collected from behind the Piñero Building at the University of Puerto Rico at Mayaguez (UPRM). These samples were collected using a small shovel (approximately 25 cm) to take soil and the complete plant, including the roots. Each plant was placed in a 1-gallon Ziploc® bag with wet paper towels inside to maintain moisture. Samples were placed in a cooler and transported immediately to the laboratory for further processing.

2.1.2 Clearing and staining roots to detect mycorrhizae

Roots from each pot were removed from the plant and cut into 5 to 6 cm samples. Following Brundrett et al. (1996), KOH 10% w/v was used to clear roots at 60° C for 1 hour. Cleared roots were rinsed with distilled water four times, then place in a 2% HCl solution and kept at room temperature for 20 minutes. The HCl solution was removed and roots rinsed four times with distilled water. Staining with Trypan Blue (0.4%) in glycerol was performed at room temperature for approximately 4 days (Bevege 1968, Phillips & Hayman 1970, Kormanik & McGraw 1982). After staining, roots were kept at room temperature (18°C) until processing for microscopic analysis. Thin cuts with scalpel were done after destaining the roots in distilled water to improve contrast.

2.1.3 Isolation and identification of AM fungi associates from roots and soil

A modification of a protocol from Gerdemann and Nicolson (1963) was used. Soil samples were weighed, then sieved using USA Standard Test Sieves (Fisher Scientific) with pores of 0.00237998 (P1), 5.334e-5 (P2) and 2.54e-5 (P3) meters. The soil was mixed with distilled water and sieved through P1 and the solution was collected. Afterwards, the soil was moved with a trowel to the other sieves to repeat the procedure. Samples were kept in small bottles (50 mL) and refrigerated until morphological characterization. The isolated spores were identified using keys of Schenck and Perez (1990).

2.2 Effects of mycorrhizae on **C. chinense** *growth and production*

2.2.1 Experiment 1

2.2.1.1 Experimental Design 1

Experiment 1 was conducted from July to December 2014. Sweet chili pepper (cultivar Carnaval) was seeded in plastic trays with 72 plug sheets cells [1.44" (Round diameter) X 2.38"] Deep filled with one of two planting mixes (treatments): (1) Pro-mix® BX General Purpose (P) and (2) Pro-mix® Mycorrhizae (with *Glomus intraradices* (PM) (all from Premier Tech Horticulture, Quakertown, Pennsylvania). At 30 days, seedlings were transplanted to 2 gallon plastic pots filled with the corresponding planting substrate. A total of 24 pots were used for each treatment. Pots were randomly arranged in the outdoor terrace of the Biology building at UPRM.

2.2.1.2 Data collection and analysis

Plant development was assessed by measuring plant height (cm), number of branches and number of leaves and number of fruits after six months of growth. Plant height was measured from soil surface to the growing tip of the plant. Treatments means were compared using t-test at the 0.05 level of probability.

2.2.2 Experiment 2

2.2.2.1 Experimental design 2

Experiment 2 was conducted from February to July 2015. Sweet chili pepper (cultivar Carnaval) was direct-seeded in 1-gallon plastic pots filled with a 3 x 2 treatment combination of substrates and fertilizer (Table 1) arranged in a completely randomized design with 10 replicates on the outdoor terrace of the Biology building at UPRM.

Table 1. Description of substrate-fertilizer treatment combinations in Experiment 2.

Treatment code	Substrate[1]	Inorganic N-P-K fertilizer[2]
Promix	Pro-mix BX®	Without
Promix+F	Pro-mix BX®	With
Promix+Myco	Pro-mix BX® + Mycorrhizae[3]	Without
Promix+Myco+F	Pro-mix BX® + Mycorrhizae[3]	With
Promix+Soil	Pro-mix BX® mixed with soil[4]	Without
Promix+Soil+F	Pro-mix BX® mixed with soil[4]	With

[1]All Pro-mix products are from Premier Tech Horticulture (Quakertown, Pennsylvania)
[2]Nurish Soluble Fertilizer 20-20-20 ME (Pan American Fertilizer, Guánica, Puerto Rico) at a rate of 20 grams/1 gallon (approximately, 300 mL per plant).
[3]This commercial product contains *Glomus intraradices*
[4]Soil, previously identified as containing mycorrhizae, was collected from behind the Piñero building on the UPRM campus and mixed with Pro-Mix BX® in the proportion of one part soil to three parts Pro-Mix BX®

Plants were watered 4 days per week or as necessary. After the first month, plants in pots were thinned to one seedling per pot. Whitefly (*Bemisia tabaci*) is a common pest of peppers (de

Lima and Campos 2008) among other insects such as aphids and mites; they were controlled using Spectracide® Malathion Insect Spray Concentrate. Plants were observed for 6 months.

2.2.3 Harvest and analysis

Plant development was assessed by measuring plant height (cm), number of branches and number of leaves on a weekly basis, beginning in the 2nd month. Plant height was measured from soil surface to the growing tip of the plant. The date of anthesis of the first flowers was recorded for each plant. Fruits were harvested, counted and weighed as they reached maturity. At approximately 6 months of age, plants were harvested by removing the whole plant completely from the pot. Plants were kept with their shoots, roots and soil in a labeled storage bad in a -20°C freezer.

2.2.3 Statistical analysis

The experimental data were analyzed as 3 x 2 factorial analysis of variance (ANOVA) using Infostat software (Di Rienzo et al. 2014). Treatment means were compared using Fisher's Least Significant Difference (F-LSD) at $\alpha = 0.05$.

2.2.4 Clearing and staining roots to detect mycorrhizae

This procedure was done as previously mentioned in our work.

2.2.5 Percent root colonization

After clearing and staining with 0.4% Trypan Blue as described above, percentage of mycorrhizal root colonization was assessed in each plant of Experiment 2. Total numbers of AMF vesicles (structures) were counted using a gridline modified slide under a light microscope (Nikon Eclipse E-200 with camera DS-Di2 and display Nikon Digital Sight DS-L3 [Nikon Corporation]). Percentage infection was determined as: [total number of mycorrhizal vesicles (structures) / total number of roots covering gridlines] x 100 (Utobo et al. 2011). When possible, all structures of AMF, i.e. arbuscules, vesicles, etc., were used to determine total colonization in root cortical cells. For counts used to determine percentage, only observed vesicles were taken into consideration. Several root cut samples were used for the determination of the infection percent.

2.3 Molecular characterization of local mycorrhizae

2.3.1 Total DNA extraction from soil and root samples

The rhizosphere (soil and roots) from pots from Experiment 2 that contained Pro-mix BX® + Soil was weighed and separated into 2 g samples. The soil and root segments were crushed in a mortar and pestle until a homogeneous solution was obtained (Lochan et al., 2011). DNA extraction was performed following instructions in the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc). Subsamples of 0.25 g were transferred to separate 2.0 mL PowerBead tubes containing 750 µL buffer solutions, both supplied by the kit. The tube and its contents were mixed with a vortex mixer. A total of 60 µL of kit solution C1 was added to the PowerBead tube and mixed with a vortex mixer for 10 min. The solution was then centrifuged at 10,000 x g for 30 sec at room temperature and the supernatant was transferred to a sterile 2 mL collection tube. A total of 250 µL of kit solution C2 was added, mixed for 5 sec and incubated at 4°C for 5 min. The tubes were centrifuged at room temperature at 10,000 x g for 1 min. Avoiding the pellet, approximately 600 µL of supernatant were transferred to a sterile 2 mL collection tube, 200 µL of kit solution C3 were added. The solution was mixed for 30 sec and incubated at 4°C for 5 min. Once again, the tubes were centrifuged at room temperature at 10,000 x g for 1 min, and avoiding the pellet, approximately 750 µL of supernatant were transferred to a sterile 2mL collection tube to which 1,200 µL of kit solution C4 were added. The solution was mixed for 5 sec. Approximately 675 µL were loaded in spin filters and centrifuged at room temperature at 10,000 x g for 1 min, after which the flow-through was discarded and 675 µL of the supernatant were added again to the spin filters. The tubes were again centrifuged at room temperature at 10,000 x g for 1 min; the remaining supernatant was added onto the spin filter and centrifuged at room temperature at 10,000 x g for 1 min. Afterwards, 500 µL of kit solution C5 were added, centrifuged at room temperature at 10,000 x g for 30 sec and the flow-through was discarded. The tube was again centrifuged at room temperature at 10,000 x g for 1 min, the spin filters were placed in 2 mL collection tubes and 50 µL of TE/10 were added at the center of the filter membrane. The tubes were centrifuged at room temperature at 10,000 x g for 30 sec and the spin filters were discarded. Lastly, these tubes were stored at -20°C until further use.

2.3.2 DNA extraction confirmation with electrophoresis

Electrophoresis of total DNA was performed in 1% agarose gels 1X TAE with ethidium bromide. The agarose gel was run at 90V and 70 mA for 45 min, then observed under UV light using S/N 101707-001 UV Transilluminator (UVP) and photographed with Samsung ISOCELL S5K2L1, Sony Exmor RS IMX260 and Olympus camera C-5060.

2.3.3 PCR amplification

DNA was amplified in a sterile PCR microtube with a final reaction volume of 25 µL containing 10 pmol of each primer (Table 1), GoTaq® Green/Colorless Master Mix (Promega), and genomic DNA. Conditions for PCR amplification were first a hot start at 60°C, an initial denaturation of 3 min at 95°C followed by five cycles of 30 s at 95°C, 30 s at 52°C, and 1.5 min at 72°C. Lastly, 25 to 30 cycles with annealing at 51°C were performed, followed by a final 5 min extension at 72°C and holding at 4°C (Redecker 1997, Shafiqua and Stephan 2013). PCR product was visualized by electrophoresis on 1.0% agarose gel with 1X TAE. Each sample for the pit was prepared with 2 µL of PCR product and 3 µL of loading buffer/dye.

2.3.4 Nested PCR amplification

A two-step nested PCR amplification was performed following Redecker (2000) (Table 2). DNA was amplified in a sterile PCR microtube with a final reaction volume of 100 µL containing 10 pmol of each primer, GoTaq® Green/Colorless Master Mix, and genomic DNA. Conditions for PCR1 amplification was as follows: hot start of 60°C, an initial 3 min denaturation at 95°C, followed by 5 denaturation cycles at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1.5 min.

Table 2: Primers used for PCR of arbuscular mycorrhizal fungi collected in Mayaguez, Puerto Rico.

Primer	Sequence	Reference
GLOM5.8R	TCC GTT GTT GAA AGT GAT C	Redecker 2000
GIGA5.8R	ACT GAC CCT CAA GCA KGT G	Redecker et al. 1997
ARCH1311	TGC TAA ATA GCC AGG CTG Y	
ACAU1660	TGA GAC TCT CGG ATC GG	
LETC1670	GAT CGG CGA TCG GTG AGT	
GLOM1310	AGC TAG GYC TAA CAT TGT TA	
ITS1	TCC GTA GGT GAA CCT GCG G	White et al. 1990
ITS4	TCC TCC GCT TAT TGA TAT GC	
NS31 modified	GGT TTC CCR TRA GGY GCC G	Simon et al. 1992
AM1 modified	GTT TCC CGT AAG GCG CCG AA	
VANS1	GTC TAG TAT AAT CGT TAT ACA GG	
AM1	GTT TCC CGT AAG GCG CCG AA	
AML1	ATC AAC TTT CGA TGG TAG GAT AGA	
AML2	GAA CCC AAA CAC TTT GGT TTC C	
AM1	GTT TCC CGT AAG GCG CCG AA	Schreiner and Mihara 2009
AMF-specific NS3	GCA AGT CTG GTG CCA GCA GCC	Shafiqua and Stephan 2013

The previously mentioned steps were followed by 30 cycles with the annealing at 54°C and a final 5 min extension at 72°C and holding at 4°C (Redecker et al. 1997, Redecker 2000). Conditions for PCR2 amplification were as follows: hot start of 61°C, an initial 3 min denaturation at 95°C, followed by 5 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 1.5 min, followed by 30 cycles with annealing at 64°C and a final 5 min extension at 72°C and holding at 4°C (Redecker et al. 1997, Redecker 2000). PCR2 products were visualized by electrophoresis on 1.0% agarose gel and 1X TAE. Each sample was prepared with 2 µL of PCR product and 3 µL of loading buffer/dye or using only 2 µL of PCR product when GoTaq® Green Master Mix (Promega) was used. Redecker (2000) determined specific ribosomal primer annealing sites and sequences that correspond to a target group of species or family.

Table 3: Primer set order used for two-step (PCR1 and PCR2), nested PCR for the identification of Arbuscular Mycorrhizal Fungi.

Forward	**Reverse**
PCR1	
NS5	ITS4
ARCH1311	ITS4
GLOM1310	
LETC1670	ITS4
ACAU1660	
PCR2	
ITS1	GLOM5.8R
ITS1	GIGA5.8R
ARCH1311	ITS4
ARCH1311	GLOM5.8R
ARCH1311	GIGA5.8R
GLOM1310	ITS4
GLOM1310	GLOM5.8R
GLOM1310	GIGA5.8R
LETC1670	ITS4
LETC1670	GLOM5.8R
LETC1670	GIGA5.8R
ACAU1660	ITS4

ACAU1660	GLOM5.8R
ACAU1660	GIGA5.8R

2.3.5 Cloning and sequencing

Cloning of PCR products from local soil samples was done with the pGEM®-T Easy Vector Quick Cloning Kit (Promega, Madison, WI) following the manufacturer's instructions. The transformation analysis was done using LB (Becton, Dickinson and company)/ampicillin (Amresco) /IPTG (Promega) /X-Gal (Promega) plates prepared in the lab. Plates were incubated overnight at 37°C and white colonies were selected for colony plating in cuadriculated LB/ampicillin plates. From these white colonies, the colony PCR technique were performed and amplified in a sterile PCR microtube with a final reaction volume of 50 µL containing 10 pmol of each primer, GoTaq® Green/Colorless Master Mix (Promega), and one colony per tube. Approximately 80 colonies per sample were amplified to confirm their respectively PCR fragment. These products were visualized by electrophoresis on 1.0% agarose gel. Each sample was used with 2 µL of PCR product as per the instructions for the GoTaq® Green Master Mix.

For the cases where more than one band of PCR were observed per colony, PCR bands were cut directly from the electrophoresis 2.0 % agarose gel and pieces were cleaned with the Wizard® SV Gel and PCR Clean-Up System (Promega). These products were visualized by electrophoresis on 1.0% agarose gel. Each sample was prepared with 2 µL of cleaned sample product and 3 µL of loading Buffer/Dye.

2.3.6 DNA sequencing

PCR products and transformants were sent to the Molecular Cloning Laboratories (MCLab, South San Francisco, California. https://www.mclab.com/DNA-Sequencing-

Services.html) for sequencing. The sequences obtained were analyzed and edited using Sequencher 4.3 (Gene Codes Corporation, Ann Arbor, Michigan).

2.3.7 Data analyses

Sequences were analyzed against the database of the National Center for Biotechnology Information (NCBI) using BLAST® (Basic Local Alignment Search Tool) in GenBank to determinate the percentage of similarity with species and strains already available in this database. The alignment and phylogenetic analysis (Maximum-Likelihood) were performed using the program Mega 7 (Tamura et al. 2011).

3 RESULTS

3.1 Confirmation of presence of endomycorrhizae in a field planting of **Capsicum chinense** *(sweet chili pepper)*

Through the techniques used we were able to confirm the presence of endomycorrhizae in *Capsicum chinense* soil and roots. The genera found in this experiment were *Glomus* and *Acaulospora*.

3.2 Effects of mycorrhizae on C. chinense *growth and production*

3.2.1 Experiment 1

For Experiment 1, plant development for each pot was assessed by measuring plant height (cm), number of branches and number of leaves at the end of the 122 days of this experiment. Since the Pro-mix® treatment did not produce any fruit, number of fruit was not statistically analyzed in this experiment.

C. chinense plants in Pro-mix® BX Mycorhizae showed considerably more development than those planted in Pro-mix® BX (Figure 1 and 2). Plant height was approximatedly four times greater when grown in the substrate containing mycorrhizae, and plants grown with mycorrhizae had approximately 8 times more leaves and branches, and these differences were significant ($p<0.05$). Only plants grown in Pro-mix® BX Mycorhizae produced fruits (Figure 1D).

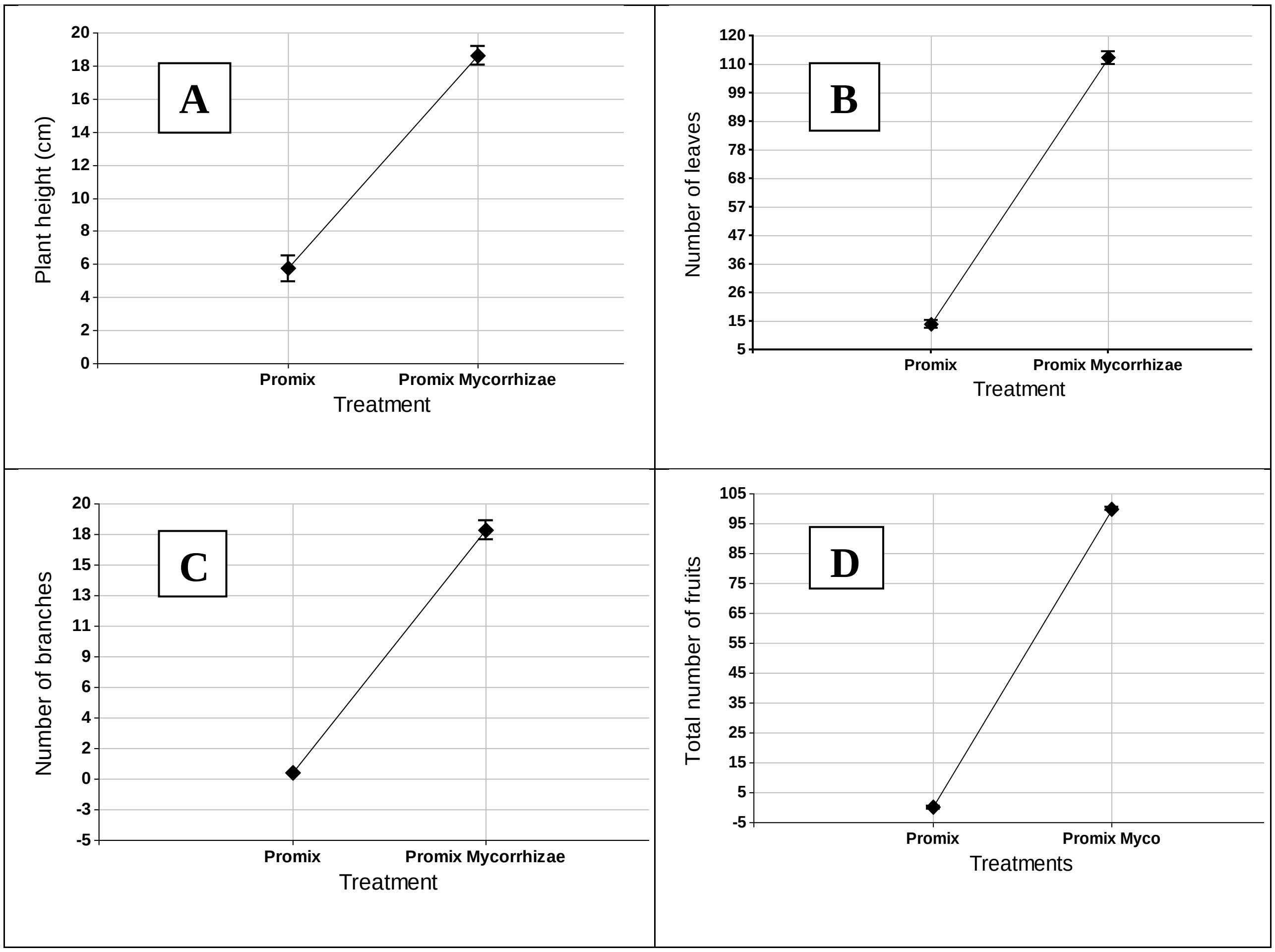


Figure 1: Experiment 1: (A) Plant height, (B) number of leaves, (C) number of branches, and (D) total number of fruit in *Capsicum chinense* four months after planting in either Pro-mix® BX (Promix) or Pro-mix® BX Mycorrhizae (Promix Mycorrhizae) . Bars represent ± Fisher's Least Significant Difference (LSD) at the 0.05 level of probability. Data from D was not statistically analyzed since no fruits were produced in the Promix treatment.

Figure 2: Experiment 1: Four month old *Capsicum chinense* grown in commercial substrates (left) Pro-mix® BX or (two plants on right) Pro-mix® BX Mycorrhizae. They were selected randomly for picture purpose.

3.2.2 Experiment 2

For plant height and number of leaves, few differences among treatments were observed during the first 68 days of plant growth (Figures 3 and 4). Counts on number of branches began 91 days after planting (Figure 5). The first fruits were harvested at 103 days after planting (Figure 6).

There were differences between treatments, and the relative differences among the planting substrate x fertilizer treatment combinations were not always the same. Therefore, in order to better study the interaction of treatments x time, analyses of variance were carried out on data taken every three weeks, beginning at 61 days after planting for plant height and number of leaves.

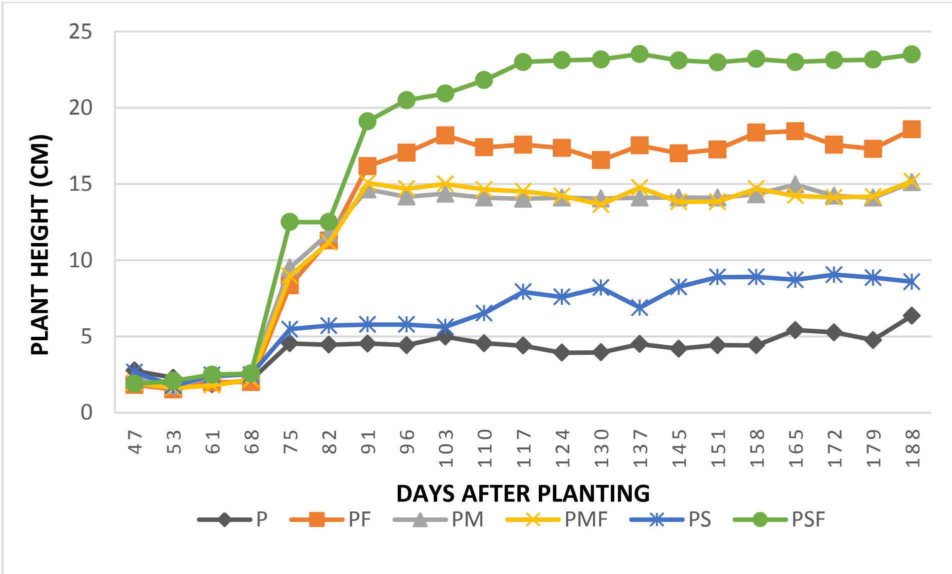


Figure 3. Plant height of *Capsicum chinense* during the period from 47 to 188 days after planting in three substrates (Pro-mix®-BX [P], Pro-mix®-BX Mycorrhizae [PM] and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] [PS]) with and without added fertilizer (F).

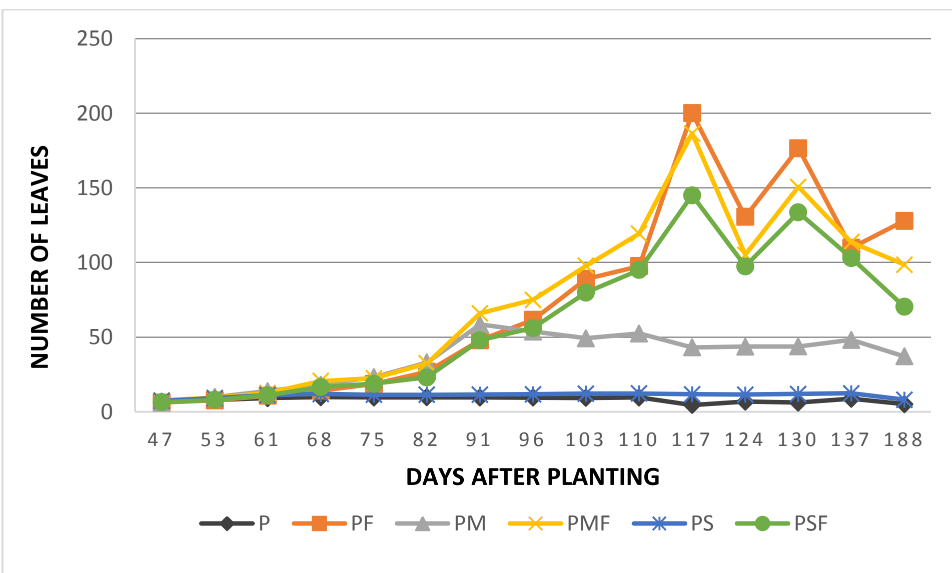


Figure 4. Number of leaves of *Capsicum chinense* during the period from 47 to 188 days after planting in three substrates (Pro-mix®-BX [P], Pro-mix®-BX Mycorrhizae [PM] and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] [PS]) with and without added fertilizer (F).

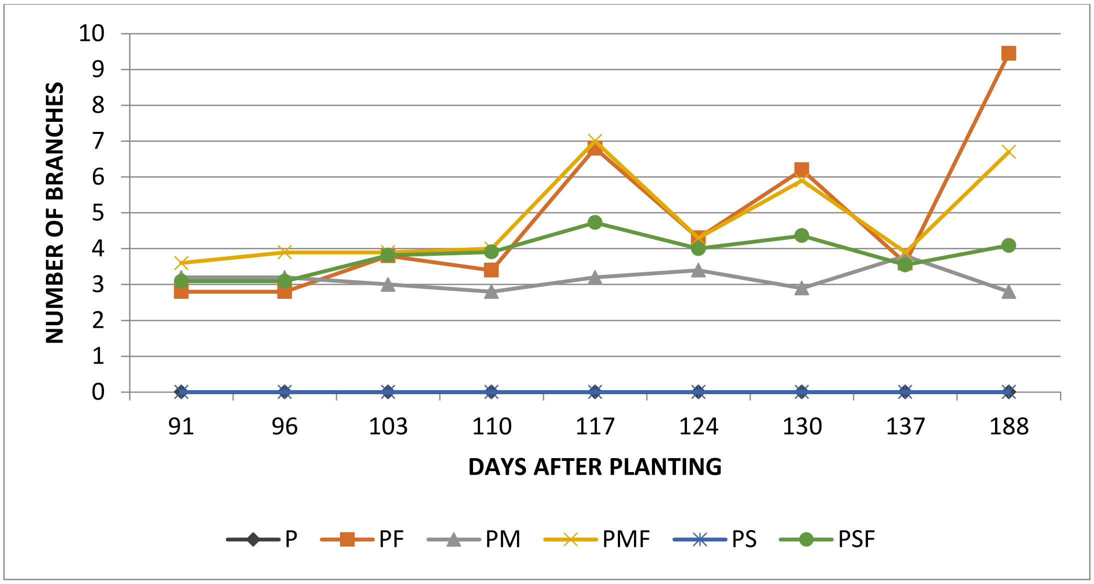


Figure 5. Number of branches of *Capsicum chinense* during the period from 91 to 188 days after planting in three substrates (Pro-mix®-BX [P], Pro-mix®-BX Mycorrhizae [PM] and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] [PS]) with and without added fertilizer (F).

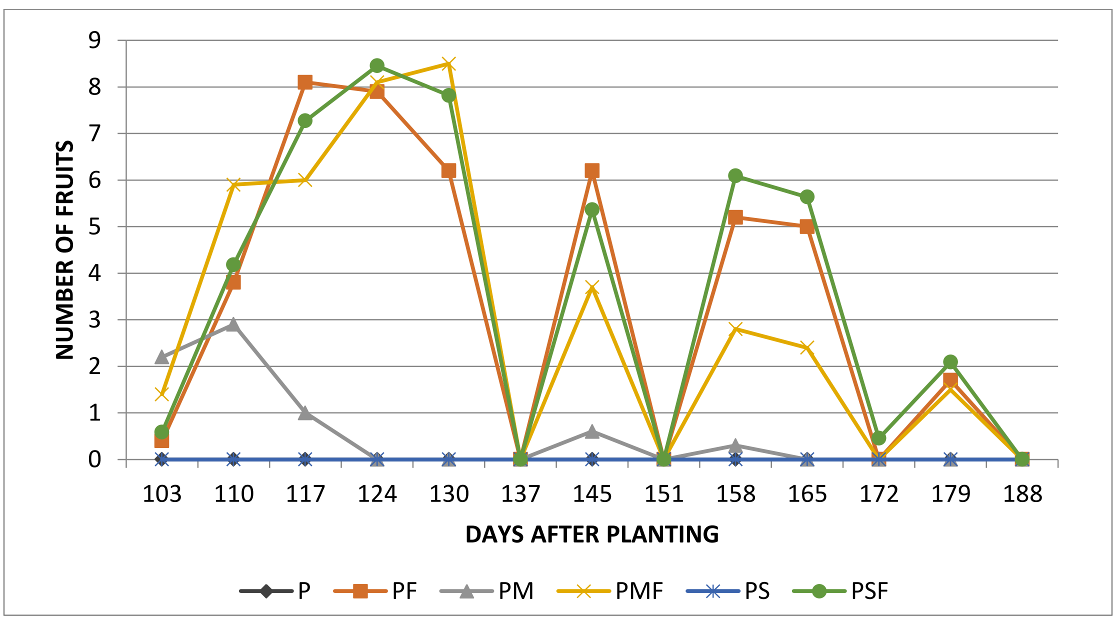


Figure 6. Number of fruit in *Capsicum chinense* during the period from 103 to 188 days after planting in three substrates (Pro-mix®-BX [P], Pro-mix®-BX Mycorrhizae [PM] and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] [PS]) with and without added fertilizer (F).

At 103 days, data on number of branches were also included in the analysis. Fruit production was not analyzed by date. Instead the total production (total weight, number of fruit and average fruit weight) was analyzed at the end of the study.

At 61 days after planting, there was no planting substrate x fertilizer interaction, or effect of fertilizer on plant height (Table 4). There were significant differences among substrates, with Promix+Soil producing plants significantly taller than either Promix or Promix+Myco (Figure 7). For number of leaves, the substrate x fertilizer interaction was significant (Table 4). In the absence of fertilizer, there were significant differences in number of leaves between substrates, and plants grown in Promix+Myco had the largest number of leaves (Figure 8). Adding fertilizer increased leaf production in *C. chinense* planted in Promix, but not in the other substrates.

Table 4. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height and number of leaves in *Capsicum chinense* 61 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height	Number of leaves
Planting substrate (S)	2	0.0030	<0.0001
Fertilizer (F)	1	0.3704	0.8577
S x F	2	0.1058	0.0143
Error	56		
LSD for S x F[1]		0.46463	1.34230
C.V.		24.4%	13.4%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

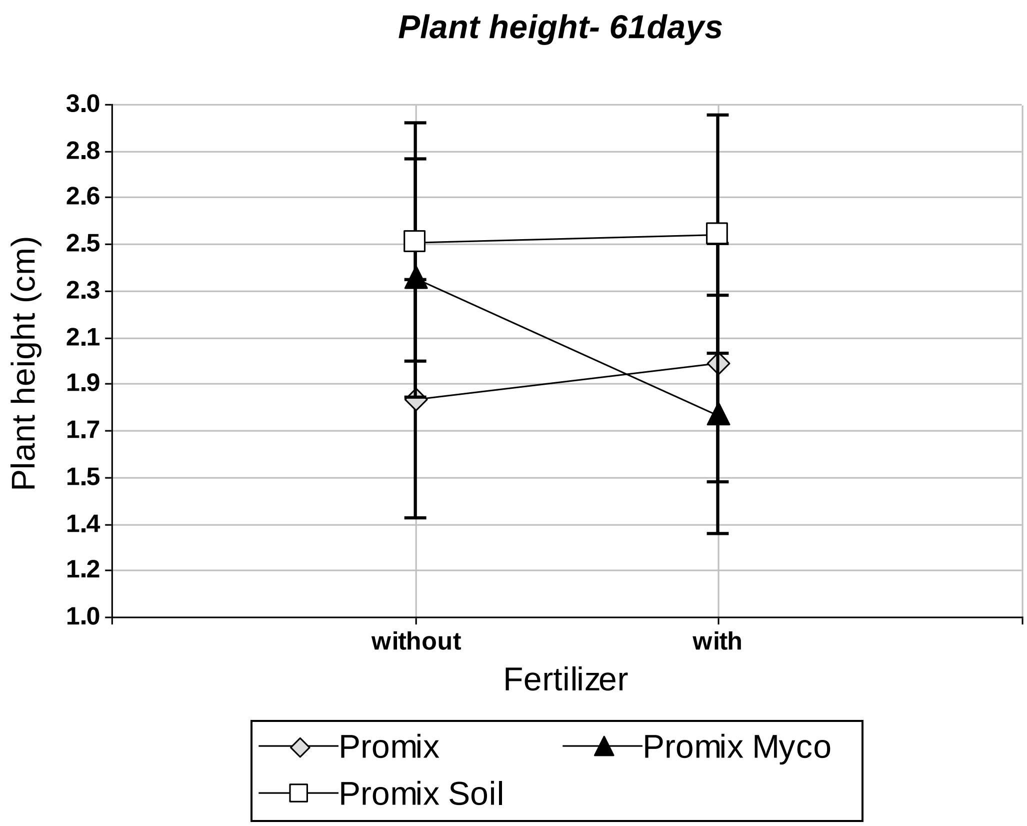


Figure 7. Mean plant height of *Capsicum chinense* 61 days after planting in Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil), with and without fertilizer. Bars represent ± the Least Significant Difference at the 0.05 probability level.

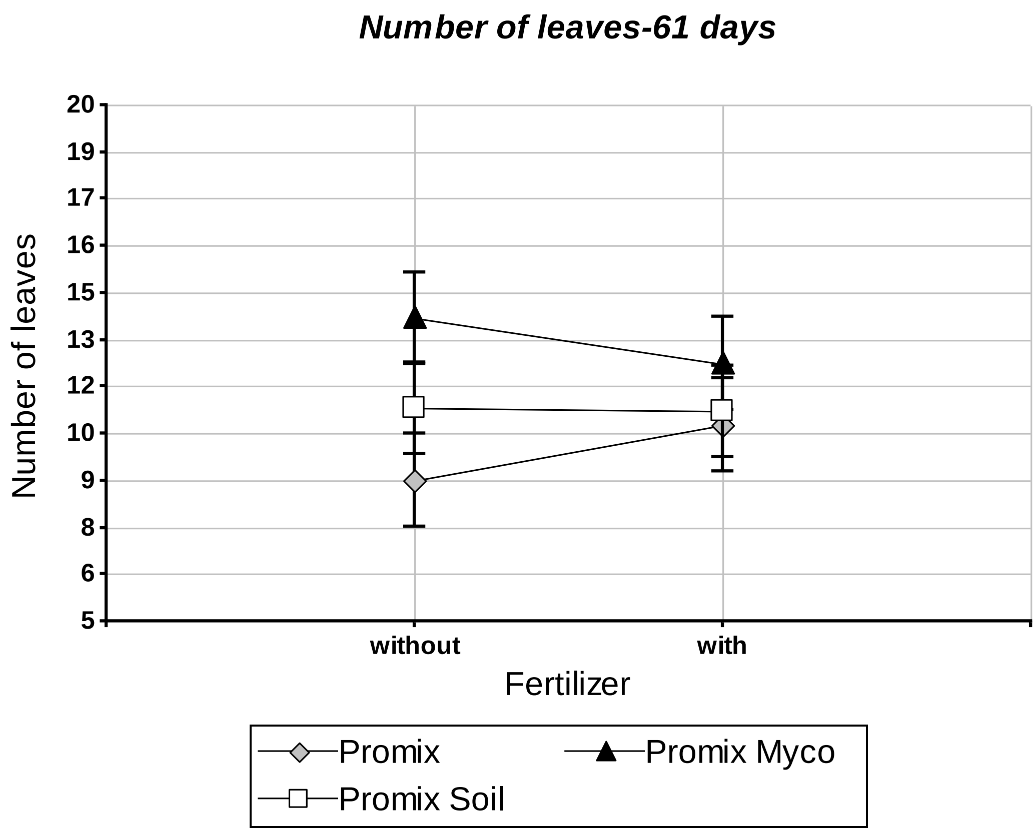


Figure 8. Mean of number of leaves of *Capsicum chinense* 61 days after planting in Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil) with and without fertilizer. Bars represent ± the Least Significant Difference at the 0.05 probability level.

At 82 days after planting there was a strong substrate x fertilizer interaction for both plant height and number of leaves (Table 5). For both variables, there were large differences between substrates when no fertilizer was added, and much smaller differences among substrates when fertilizer was added (Figures 9 and 10). Without fertilizer, both Promix and Promix+Soil produced much shorter plants and fewer leaves compared to Promix+Myco. When fertilizer was used, there was a significant increase in plant height and number of leaves when fertilizer was used with Promix or Promix+Soil, but there was no similar response in plants grown in

Promix+Myco. However, with fertilizer, Promix+Myco continued to have significantly more leaves than the other two substrates.

Table 5. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height and number of leaves in *Capsicum chinense* 82 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height	Number of leaves
Planting substrate (S)	2	<0.0001	<0.0001
Fertilizer (F)	1	<0.0001	<0.0001
S x F	2	<0.0001	<0.0001
Error	56		
LSD for S x F[1]		1.81193	3.94697
C.V.		21.7%	19.9%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

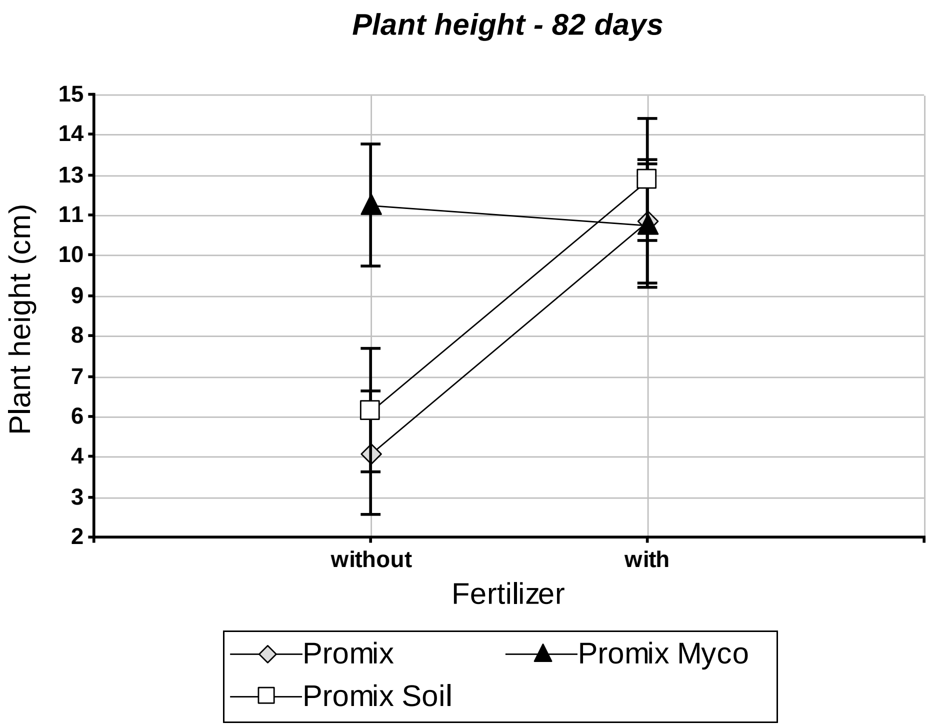


Figure 9. Mean plant height for 82 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

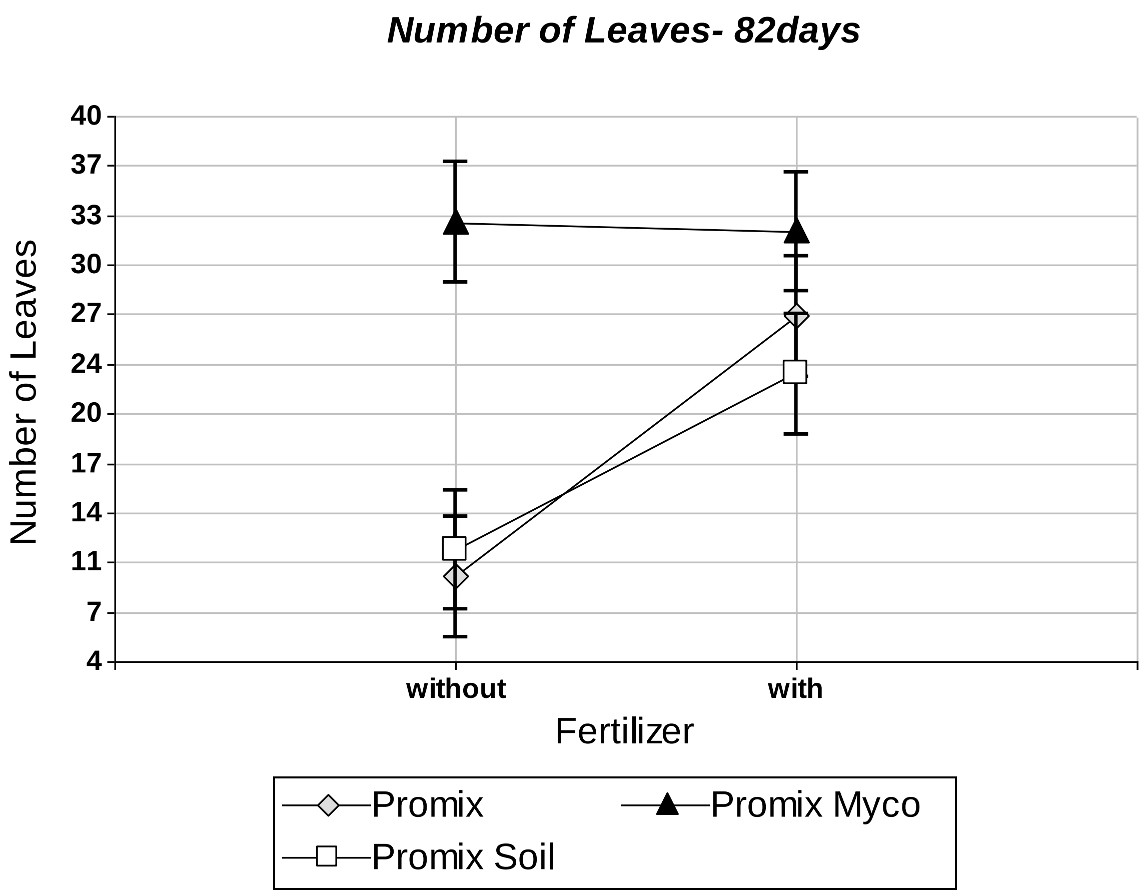


Figure 10. Mean of number of leaves for 82 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

At 103 days after planting, there were significant substrate x fertilizer interactions for plant height and number of branches, but not for number of leaves (Table 6). For plant height, plants in Promix and Promix+Soil responded strongly to the application of fertilizer, while plants in Promix+Myco did not (Figure 11). Without fertilizer, plants in Promix+Myco were significantly taller than plants grown in the other two substrates. When fertilizer was added, plants in Promix and Promix+Soil were taller than plants in Promix+Myco. For number of leaves, there was a clear, positive response of plants grown with fertilizer, no matter which substrate was used (Figure 12). Without and with fertlilzer, Promix+Myco produced plants with the greater number of leaves. Without fertilizer, branching was greatest in Promix+Myco, but these differences disappeared when fertilizer was applied (Figure 13).

Table 6. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height, number of leaves and number of branches in *Capsicum chinense* 103 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height	Number of leaves	Number of branches
Planting substrate (S)	2	0.0002	<0.0001	0.0014
Fertilizer (F)	1	<0.0001	<0.0001	<0.0001
S x F	2	<0.0001	0.0922	0.0027
Error	56			
LSD for S x F[1]		1.92297	15.66555	1.30285
C.V.		16.5%	32.5%	61.5%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

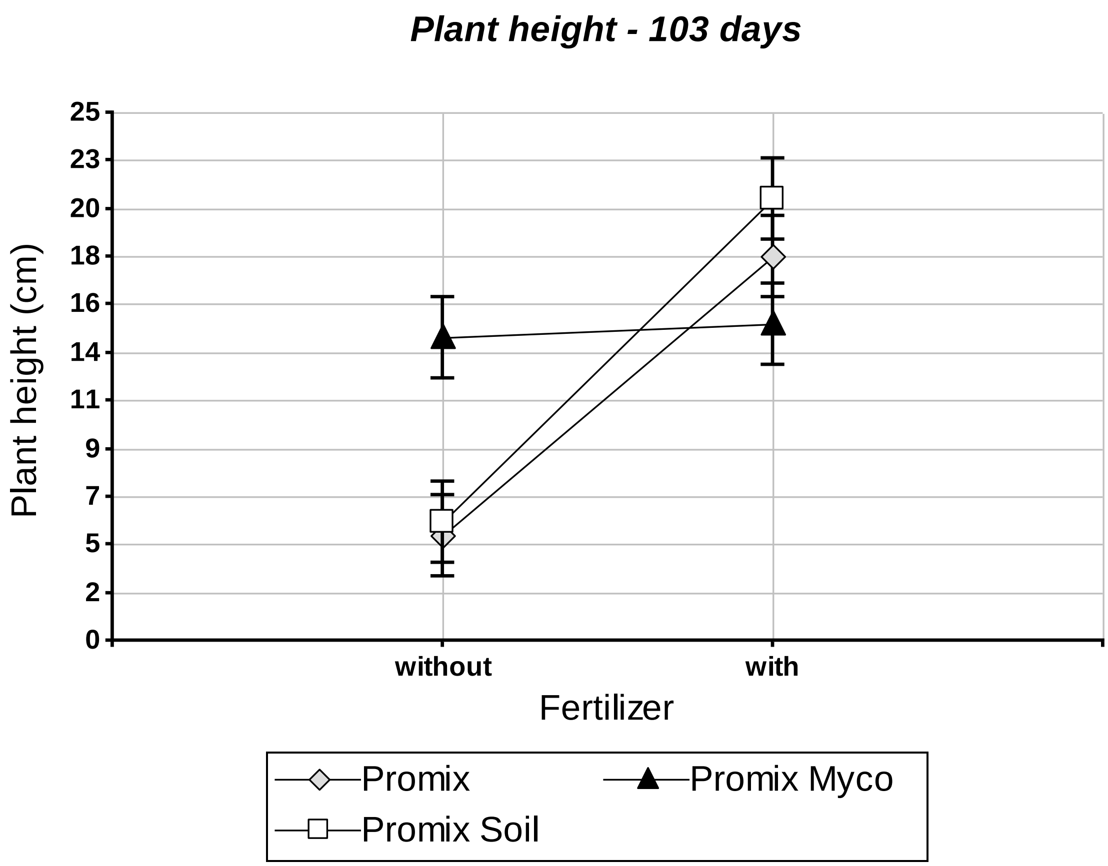


Figure 11. Mean plant height for 103 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

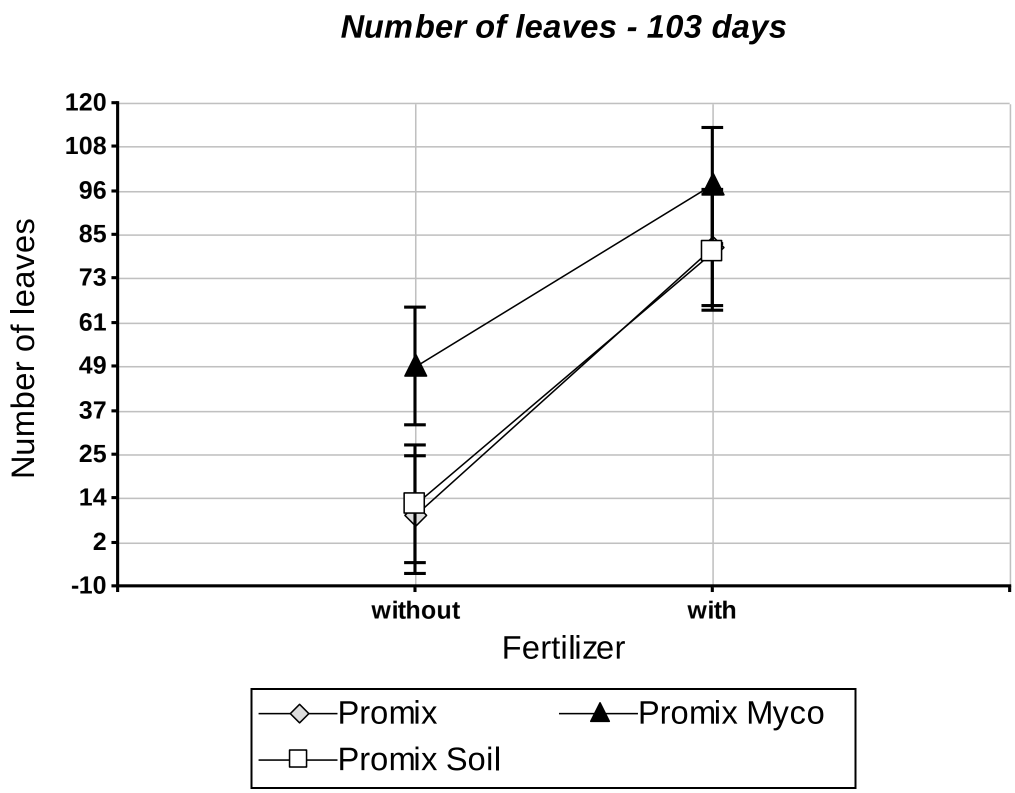


Figure 12. Mean of number of leaves for 103 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

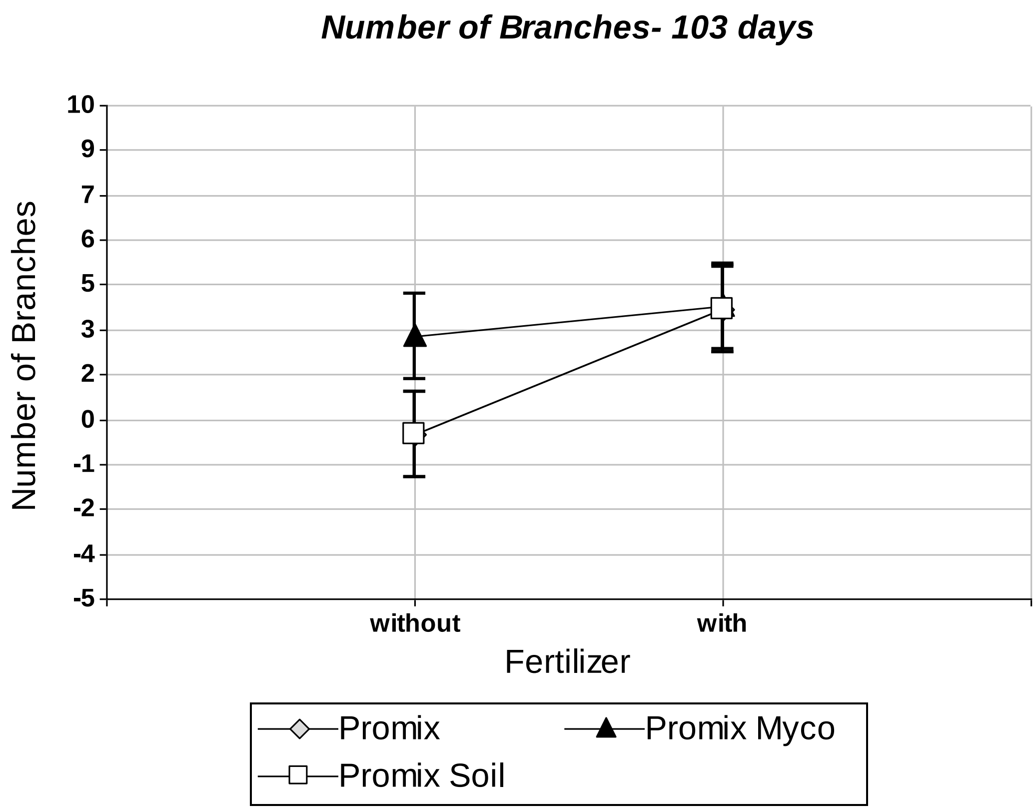


Figure 13. Mean of number of branches for 103 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

At 124 days after planting, a significant substate x fertilizer interaction was again observed in plant height, number of leaves and number of branches (Table 7). Without fertilizer, Plants in Promix- Myco were more developed than those in either of the other two substrates (Figures 14, 15 and 16). For Promix-Myco with fertilizer, there was no increase in plant height and number of branches. In contrast, the addition of fertilizer to Promix or Promix+Soil resulted in a significant increase in growth. The general appearance of plants at 124 days after planting is seen in Figure 17.

Table 7. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height, number of leaves and number of branches in *Capsicum chinense* 124 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height	Number of leaves	Number of branches
Planting substrate (S)	2	<0.0001	0.0012	0.0004
Fertilizer (F)	1	<0.0001	<0.0001	<0.0001
S x F	2	<0.0001	<0.0001	0.0013
Error	56[1]			
LSD for S x F[1]		2.39018	15.14283	1.36771
C.V.		20.2%	26.2%	58.6%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

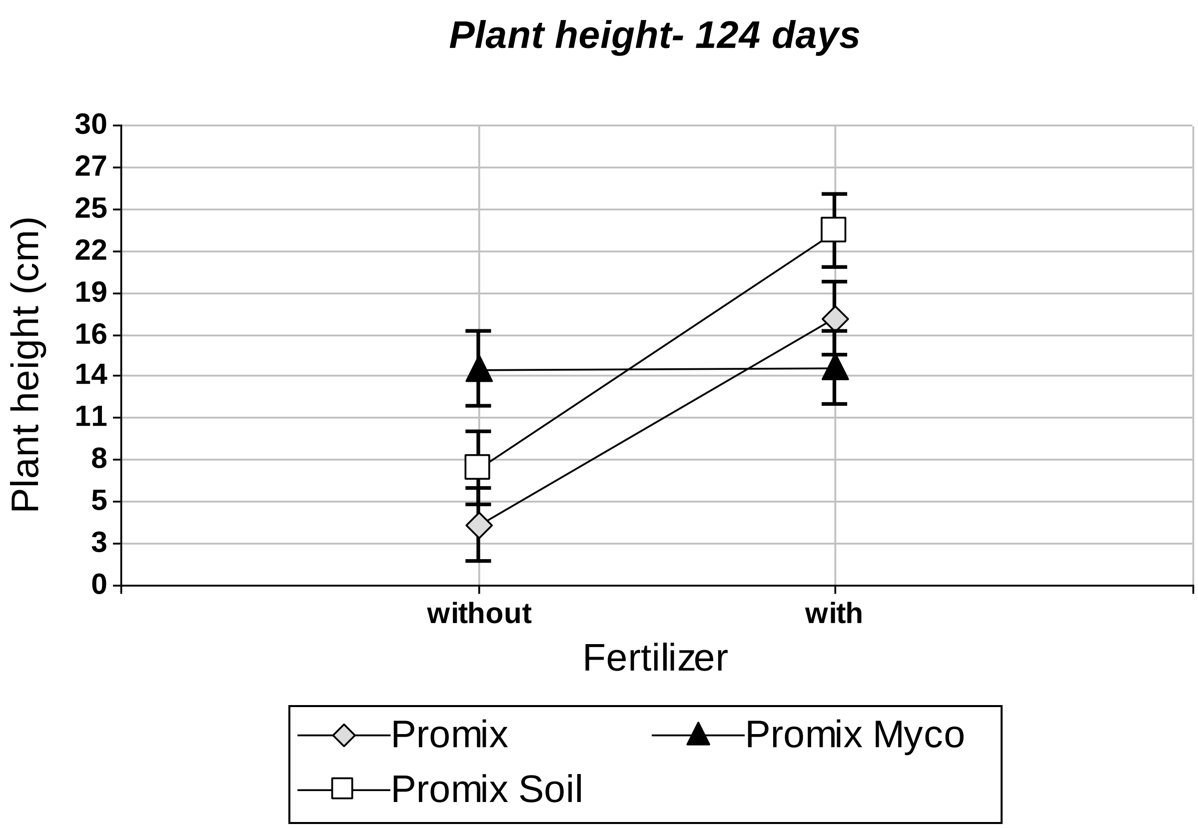


Figure 14. Mean plant height of 124 day old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

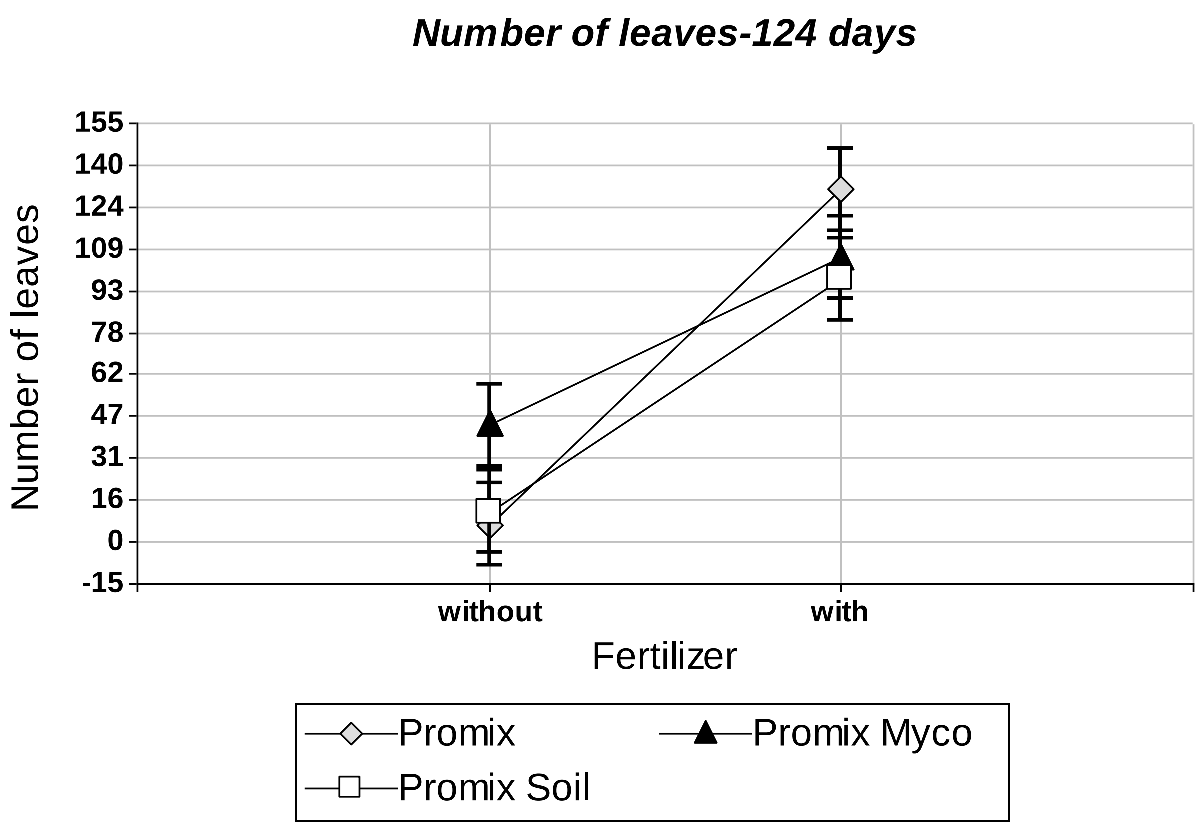


Figure 15. Mean for number of leaves of 124 day-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

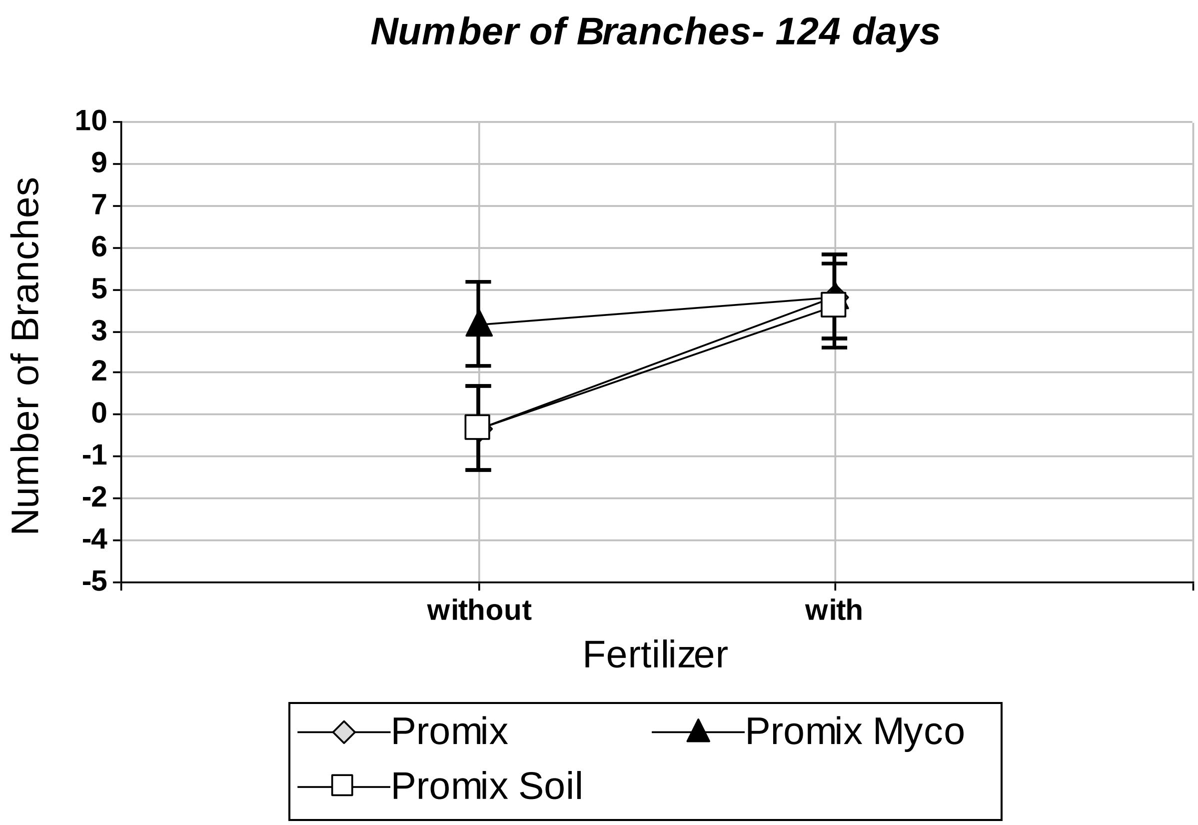


Figure 16. Mean for number of branches of 124 day-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

A

B

Figure 17: Experiment 2: *Capsicum chinenese* at 124 days after planting with (A) and without (B) fertilizer. Substrate treatments were Pro-mix® BX, Pro-mix® BX Mycorrhizae, and Pro-mix® BX + Soil (1:3 v/v) with native mycorrhizae.

At 145 days after planting, treatment response for plant height was the same as seen at 124 days after planting. There was a highly significant substrate x fertilizer interaction (Table 8). For plant height without fertilizer the one with higher plants was Promix- Myco between the three treatments, while Promix-Myco with fertilizer had the shortest plants (Figure 18). For plant height, the same trend continued at 165 and 188 days after planting (Tables 9 and 10; Figures 19 and 20).

Table 8. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height in *Capsicum chinense* 145 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height
Planting substrate (S)	2	<0.0001
Fertilizer (F)	1	<0.0001
S x F	2	<0.0001
Error	56	
LSD for S x F[1]		2.66927
C.V.		22.4%

[1]Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

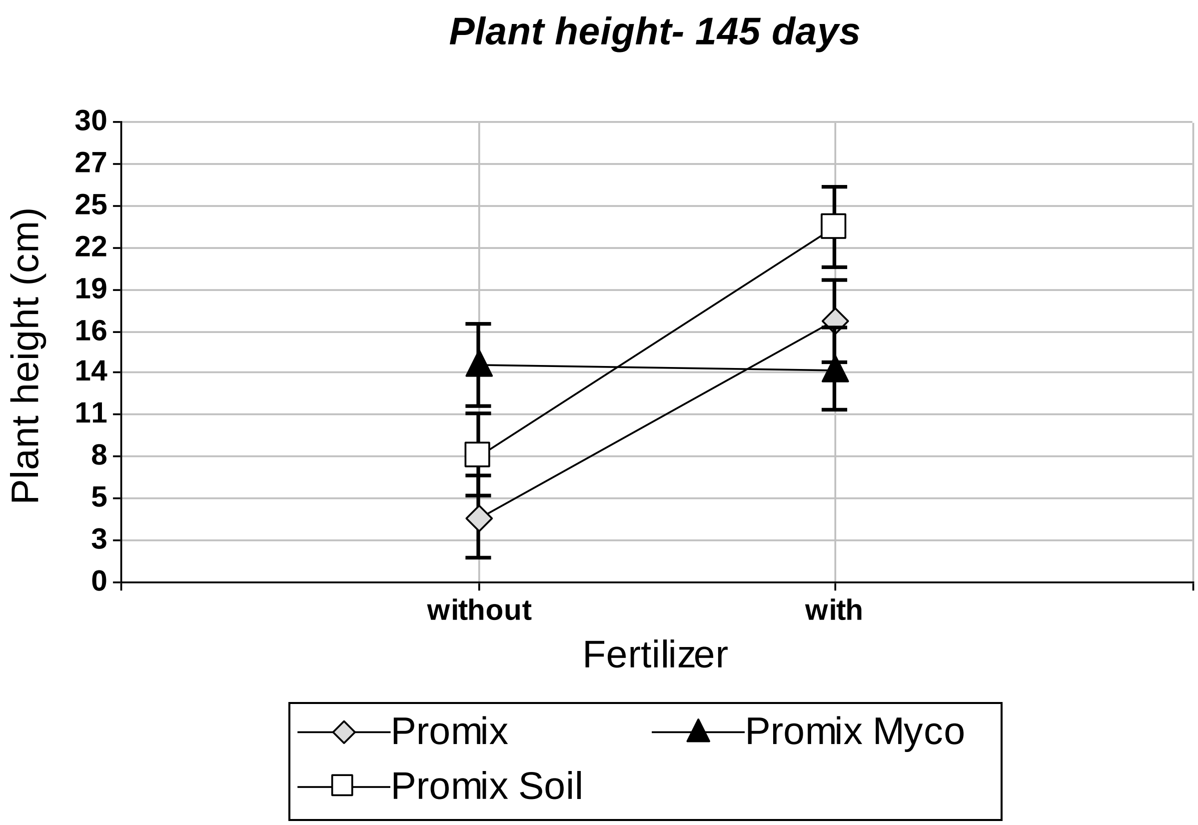


Figure 18. Mean plant height of 145 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

Table 9. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height in *Capsicum chinense* 165 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height
Planting substrate (S)	2	0.0007
Fertilizer (F)	1	<0.0001
S x F	2	<0.0001
Error	56[1]	
LSD for S x F[1]		2.79848
C.V.		22.4%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

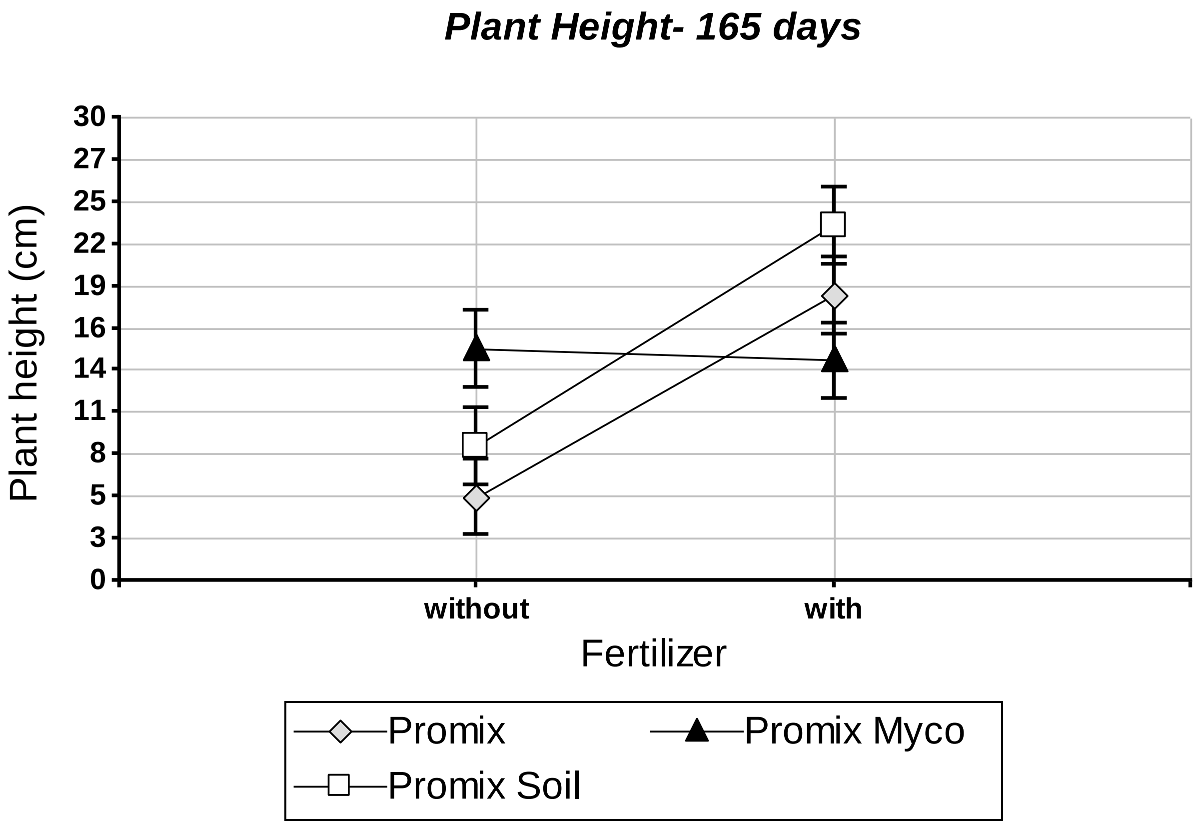


Figure 19. Mean plant height of 165 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

Table 10. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for plant height, number of leaves and number of branches in *Capsicum chinense* 188 days after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Plant height	Number of leaves	Number of branches
Planting substrate (S)	2	0.0006	0.0003	<0.0001
Fertilizer (F)	1	<0.0001	<0.0001	<0.0001
S x F	2	<0.0001	0.0001	<0.0001
Error	56[1]			
LSD for S x F[1]		2.56035	21.44865	1.63159
C.V.		19.6%	43.1%	49.0%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

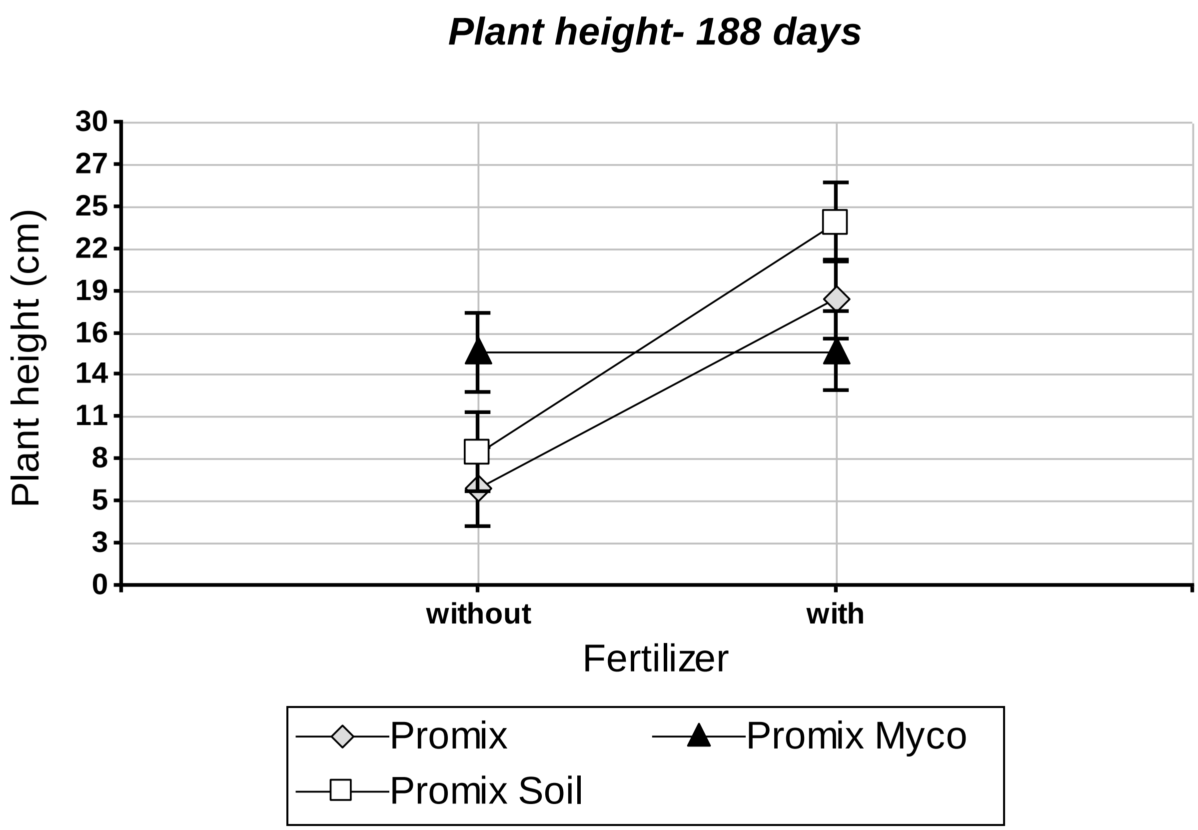


Figure 20. Mean plant height of 188 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

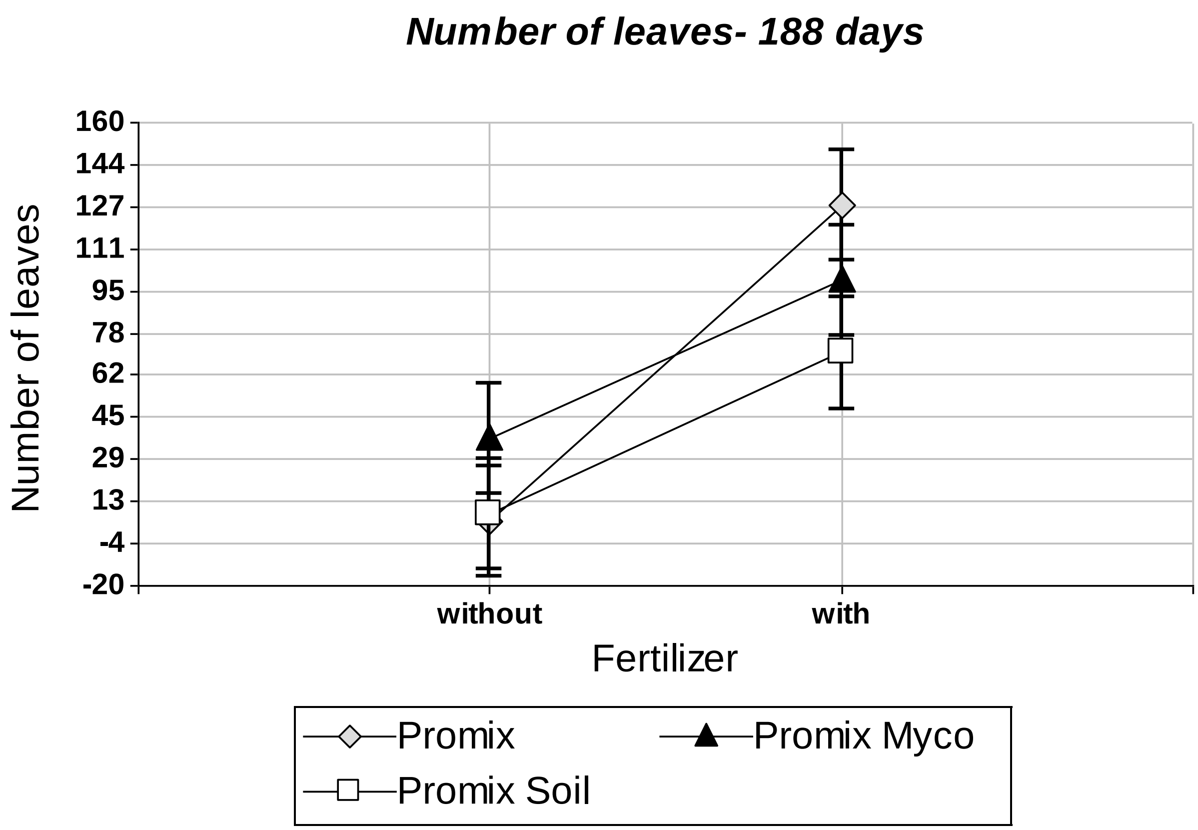


Figure 21. Mean for number of leaves of 188 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

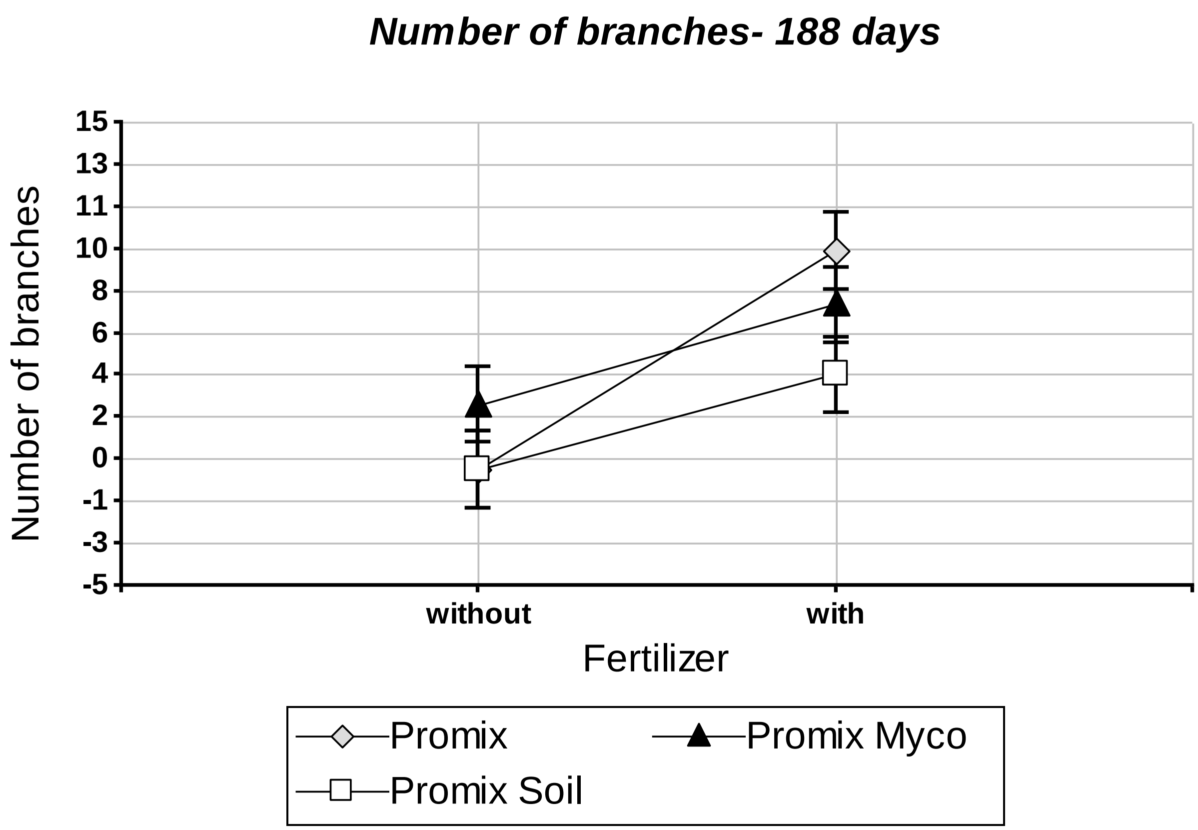


Figure 22. Mean for number of branches of 188 days-old plants of *Capsicum chinense* with and without fertilizer added to planting mixes Pro-mix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

Fruit harvest began at 103 days after planting (Figure 6). Data from all harvests were combined and analyzed (Table 11). The substrate x fertilizer interaction was highly significant. When no fertilizer was applied, only plants in Promix Myco yielded fruit (Figure 23 and 24). When fertilizer was added, production of fruit (fruit weight and number) increased for all substrates and there were no significant differences among substrates.Since no fruit were produced in unfertilized Promix and Promix+Soil, average fruit weight could only be measured in four of the six treatment combinations (Figure 25). Average fruit weight (size) was highest in unfertilized Promix Myco. There were no differences in average fruit size among plants grown in fertilized substrates.

Table 11. Sources of variation, degrees of freedom, F-test probability values, Fisher's Least Significant Difference (LSD) and coefficients of variation (C.V.) for total fruit yield (weight), total number of fruits, and average fruit weight in *Capsicum chinense* during a 188 day growing period, after planting in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Total weight	Total Number of fruits	Weight average
Planting substrate (S)	2	0.1032	0.6641	<0.0001
Fertilizer (F)	1	<0.0001	<0.0001	<0.0001
S x F	2	0.0059	0.0077	<0.0001
Error	56			
LSD for S x F[1]		23.60457	2.68571	1.35239
C.V.		53.6%	53.6%	23.2%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

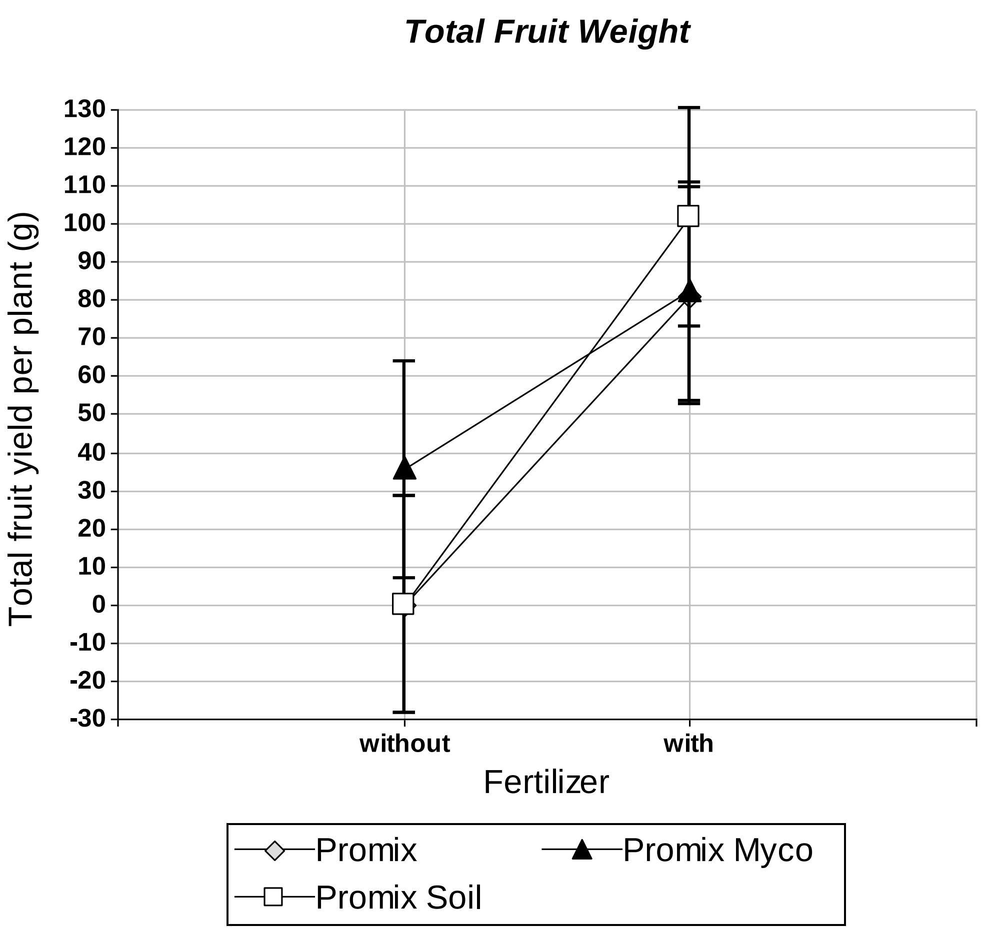


Figure 23. Total sum of weight for fruits for the period of 21 weeks of *Capsicum chinense* with and without fertilizer added to planting mixes Promix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent + the Least Significant Difference at the 0.05 probability level.

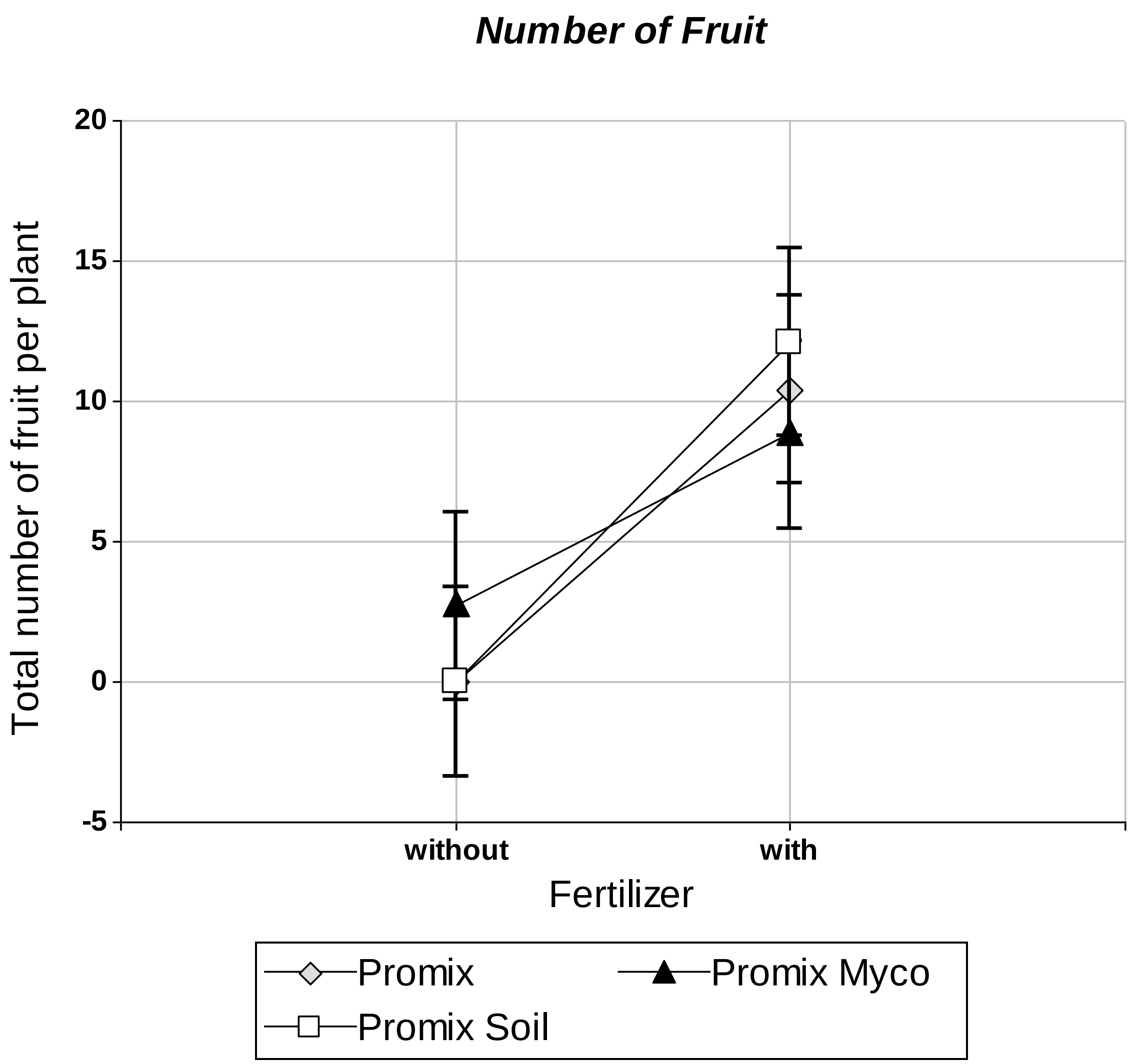


Figure 24. Average number of fruits per plant during a 188 days growing period of *Capsicum chinense* with and without fertilizer added to planting mixes Promix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent + the Least Significant Difference at the 0.05 probability level.

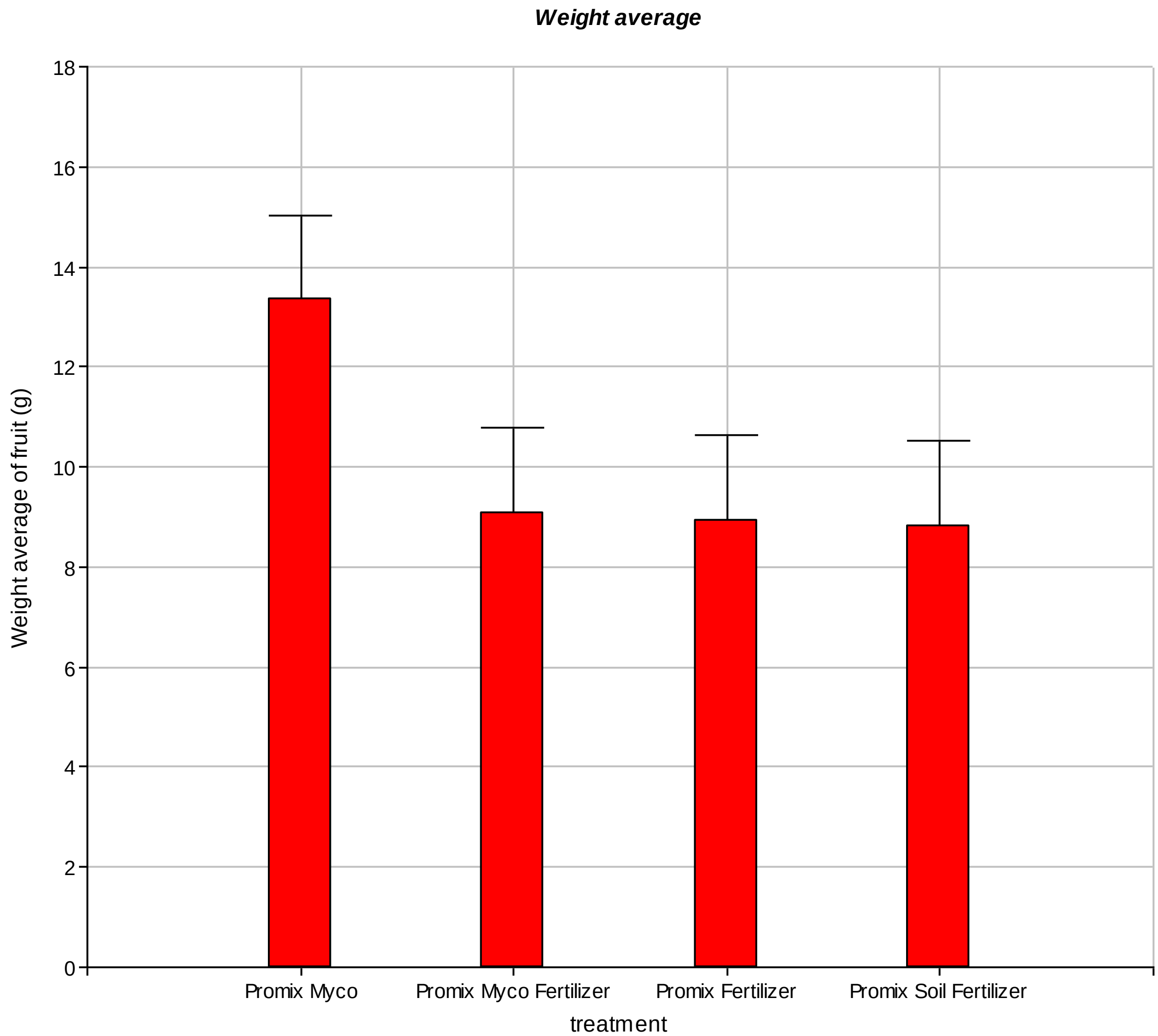


Figure 25. Average fruit weight during a 188 days growing period of *Capsicum chinense* with and without fertilizer added to planting mixes Promix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae [3/1 v/v] (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

3.3 Molecular identification of endomycorrhizae from local soil samples

Twenty samples with local soil and *C. chinense* roots were selected for DNA extractions. After nested PCR, cloning was done to obtain the DNA fragment of interest (a product of 500-600 bp for the 18S rDNA gene). The first sequencing attempt resulted in 6 samples of Glomeromycota members; therefore, only specific primers for Glomeromycota were used and cloning was eliminated. A total of 180 samples were sequenced with the following results: 13 were fungi (7.2%) and only 7 were part of the Glomeromycota (4.9%). These samples came only from roots of *Capsicum chinense*. Most of the samples were dominated by bacteria (142/180; 78.9%), with *Pseudomonas* as the most common, particularly *Pseudomonas koreensis* (86/180; 47.8%). Twenty four of the 180 samples (13.3%) contained Nematoda worms. The most commonly identified nematodes were *Acrobeloides nanus* and *Pratylenchus goodeyi* each found in 6.1% of the samples.

Figure 26 shows a maximum likelihood phylogenetic under the Tamura-3 parameter model with bootstrap consensus of 1000 pseudoreplicates. Samples PSRGLOM17 SP6, PSRGLOM 32 SP6, PSR GLOM16 SP6 and PSR GLOM13 SP6 form a well-supported monophyletic clade (BP 98%) that has a sister group with *Acaulospora* and *Diversispora* species. This clade is related to another group that contains *Paraglomus, Claroideoglomus, Gigaspora, Glomus*, *Cetraspora*, *Acaulospora* and *Ambispora*. Sample PSR1310A5 SP6 forms a separate lineage related to the previous clades, which might represent a new species of Glomeromycota. The genera *Funneliformis* and *Racocetra* are basal to the clade that contains all of the samples mentioned above. Sample PSR GLOM40 SP6 forms a well-supported clade with *Scutellospora* (BP 96%) sister to a clade mostly composed of *Glomus* species. Lastly, sample PS6R 1310giga Glom1310 G04 seems to be more closely related to *Glomus* and *Funneliformis* in a clade containing the odd *Geosiphon pyriformis* (97% similarity), a non-endomycorhizae member of the phylum Glomeromycota.

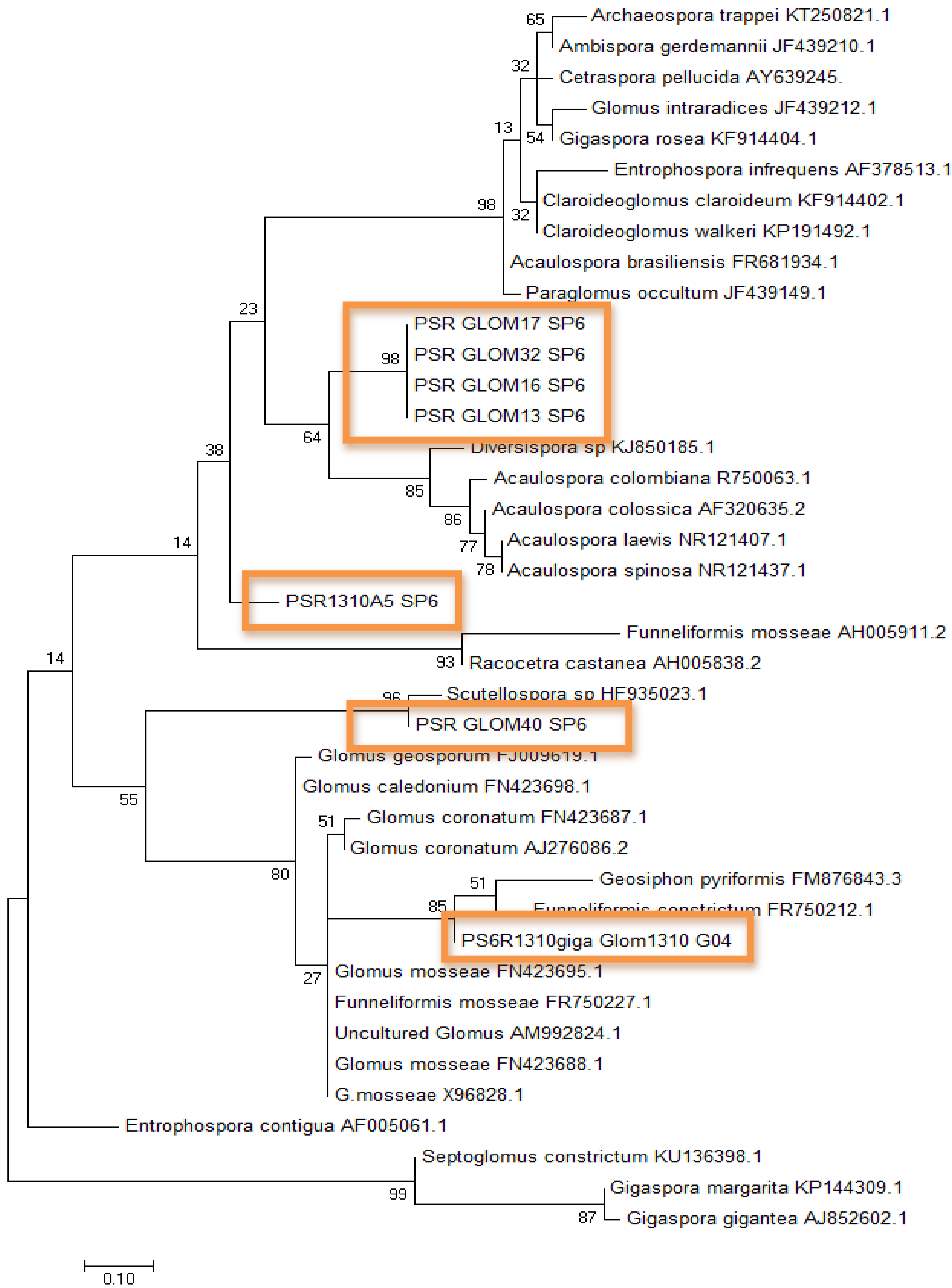
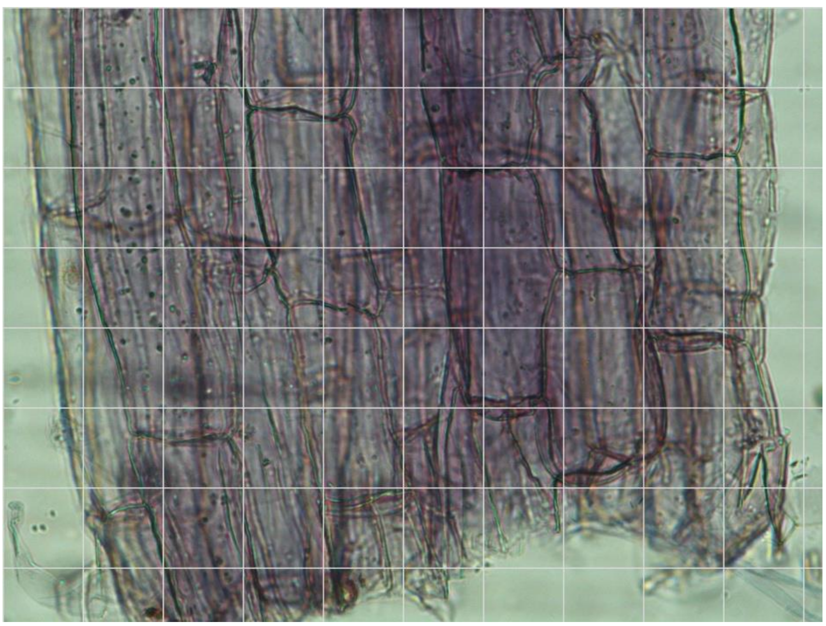


Figure 26: Maximum likelihood phylogenetic tree of endomycorrhizae 18S rDNA sequences from root samples of *Capsicum chinense* grown in Promix + Soil (soil collected behind the Piñero building, Department of Agroenvironmental Sciences, UPRM). The boxes represent samples that were sequenced and group with Glomeromycota in Genbank.

3.4 Percent root colonization

Percent of root colonization was assessed, after clearing and staining with 0.4% Trypan Blue as previously described. For counting, only the vesicles were considered as valid AMF structures (Figure 27). Data were analyzed using the factorial 3x2 analysis of variance. Significant differences in some of the treatments were found (Table 12). Infection percent was higher in treatment PSF with 21.85%, followed by PF (16.30%), PS (15.97%), PMF (13.27%) and PM (10.24%), respectively (Figure 28).

A



B

C

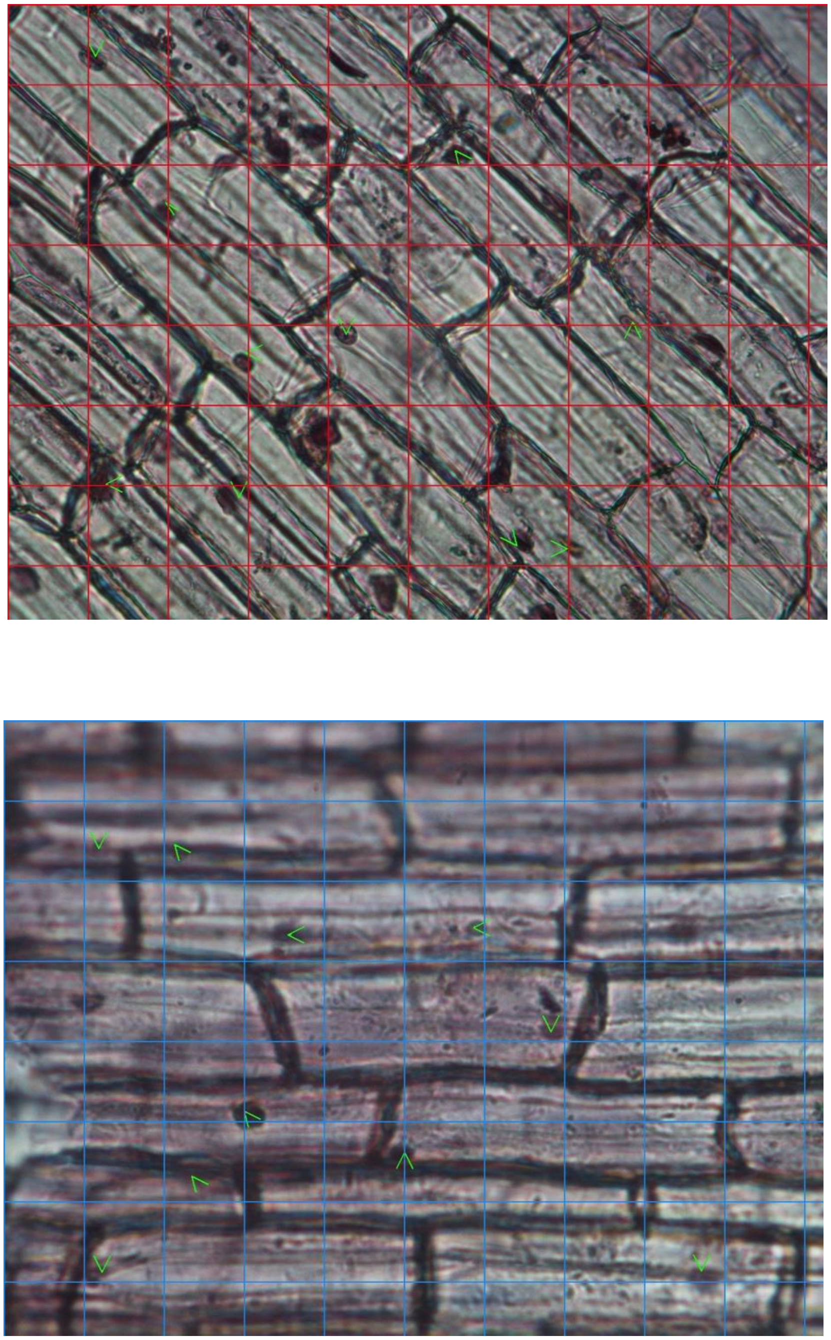


Figure 27: Roots cuts for plants with Pro-mix® BX (A), Pro-mix® BX with fertilizer (B), Pro-mix® BX Mycorrhizae (B), and Promix® BX +soil (1:3 v/v) with native mycorrhizae with fertilizer (C). Vesicle structures are found in B and C but not in A. Green arrows are showing vesicles.

Table 12. Sources of variation, degrees of freedom, F-test probability values and coefficients of variation (C.V.) of infection percent found in roots cuts for *Capsicum chinense* planted in six treatment combinations of planting substrates and fertilizer.

Source of variation	Degrees of freedom	Infection percent
Planting substrate (S)	2	0.0349
Fertilizer (F)	1	0.0171
S x F	2	0.5707
Error	56[1]	
LSD		8.85085
C.V.		70.4%

[1] Least significant difference for substrate x fertilizer treatment combinations at the 0.05 probability level.

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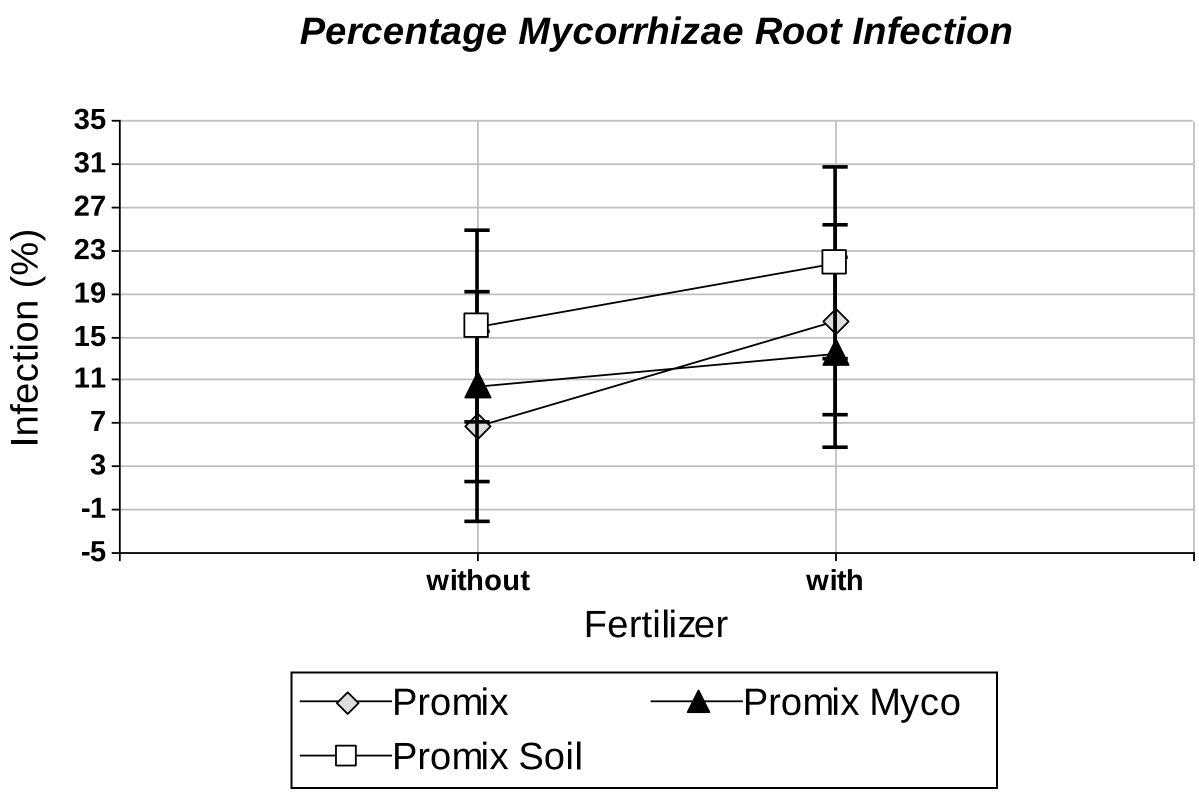

Figure 28. Infection percent from roots cuts of *Capsicum chinense* with and without fertilizer added to planting mixes Promix®-BX (Promix), Pro-mix®-BX Mycorrhizae (Promix-Myco) and Pro-mix®-BX + soil with mycorrhizae (75/25 v/v) (Promix-Soil). Bars represent ± the Least Significant Difference at the 0.05 probability level.

4 DISCUSSION

4.1Effects of mycorrhizae on **C. chinense** *growth and production*

To our knowledge, this is a first study in Puerto Rico to assess plant growth response between treatments involving mycorrhizae in a commercial crop. Our results showed that using Promix - Myco (with commercial *Glomus intraradices*) had a positive effect on plant height, number of leaves and branches, and fruit production, when no fertilizer was added. These results were consistent throughout the experimental period, although sometimes the differences were not statistically significant (Figures 3-6). Plants grown in Promix and Promix - Soil without fertilizer did not produce flowers, fruits, or branches. When fertilizer was added, Promix - Myco plants showed little or no response. In contrast, Promix and Promix - Soil usually exhibited a strong positive response to fertilizer (i.e. plant growth increase). It is important to mention that both mycorrhizae treatments (natural and commercial) were added only at the beginning and not re-inoculated throughout the study. During the first four weeks there were no significant differences among treatments in the variables under study.

Significant differences were found between fertilized and non-fertilized treatments, where the former showed greater averages in every variable studied (Figures 3-6). As an example, plant height after 24 days of planting (Figure 14) presented the highest values thereafter for Promix - Soil and Promix, both fertilized. Meanwhile, it seems that having *Glomus intraradices* in the soil did not significantly increase plant growth when combined with fertilizer treatments.

Fluctuation in flower and fruit production was observed during the experiments including a decline of them; this may have been caused by the insecticide used to control whitefly plague. Fertilizer addition was the main factor influencing fruit production significantly increasing total fruit number (Figure 24), but Promix-Myco treatment produced fruit with the highest average weight (Figure 25). Endomycorrhizae influencing fruit development under fertilizer treatments is not new. Previous work, established that when both are present they antagonise each other by significantly decreasing arbuscules and vesicle formation (Tanwar et al. 2013). We did not observe a decrease in endomycorrhizae structure formation; on the contrary, our highest infection percent occured in treatments with fertilizer. Our experiments were not conducted in a greenhouse; thus, complete environment control was not possible and may have influenced these results. Fruit production per plant (yield) was higher with fertilizer in our experiments, but fruit was produced in Promix-Myco without fertilized, while the other treatments have no production

(Figure 23). Based on these results alone we can conclude that using endomycorrhizae, *Glomus intraradices*, could help in what matters the most to an agriculturalist; a great quality of their product and recover more than their expenses in production. Endomycorrhizae have advantages over chemical fertilizers, such as, no need to be re-applying during the growth season (as in this experiment) and similar crop yields without fertilizer; thus reducing costs.

Although characterization of pathogens was not included, foliage and other plant structures look healthy for most of the study, except when the plague of whiteflies spread, which could indicate a reduction in pathogen load, which has been previously reported as another endomycorrhizae advantage (Salami 2002, Garmendia et al. 2004).

4.2 Molecular characterization of local mycorrhizae

There have been only two studies pertaining to endomycorrhizae in Puerto Rico: one on coffee cultivars (Lebrón et al. 2012) and another on tropical tree species ecology (Bachelot et al. 2017). The first study is more relevant to agriculture and thus closely related to this work. Lebrón et al. 2012 compared arbuscular mycorrhizal (AM) extraradical hyphae in the soil and diversity of AM fungi among three coffee cultivars, Caturra, Pacas, and Borbón, at three farms in Puerto Rico. In their findings, Caturra had significantly lower total extraradical AM hyphal length than Pacas and Borbón at all locations. Although the same morphotypes of mycorrhizal fungal spores were present in the rhizosphere of the three cultivars, frequencies of spore morphotypes differed significantly among them. Spore morphotypes were typical of *Glomus* and *Sclerocystis*.

Our study in the soil sampled in western Puerto Rico distinguished the genera *Scutellospora, Diversispora, Acaulospora, Racocetra, Funneliformis, Glomus* and *Paraglomus* (Figure 26). Previously, only a few genera of Glomeromycota had been reported for Puerto Rico (Lebrón et al. 2012) but with these new data the diversity present in the island soils has increased. Compared to other tropical environments the diversity of species for Puerto Rico is still unknown. Our current data only allows us to place the samples into genera because of the conserved marker used in the analysis. In Brazil, for example, many species have been reported: *Glomus glomerulatum, G. sinuosum, G. coremioides, G. fuegianum* and *G. taiwanensis* (Tomio Goto and Costa Maia 2005), *Acaulospora, Claroideoglomus, Gigaspora, Glomus, Rhizophagus* and *Scutellospora* (Barreto de Novais et al. 2013). For Panama, thirty arbuscular mycorrhizal types were reported; 23 belong to the Glomaceae, 3 to the Acaulosporaceae and 4 to the Gigasporaceae (Husband et al. 2002). If we compare Lebrón et al. (2012) findings with our results only *Glomus* is present in both studies. *Acaulospora, Glomus,* and *Scutellospora* are mentioned as part of tropical endomycorrhizae in other countries, but *Diversispora*, *Funneliformis* and *Racocetra* are new reports for Puerto Rico and the tropics in general.

We previously mentioned the majority of sequences recovered from our samples were bacteria, particularly *Pseudomonas;* Gamalero et al. (2004) found that *Pseudomonas* combined with AMF inoculants work synergistically increasing plant growth, thus it should not be surprising that the Promix - Soil with fertilizer treatment had a better outcome. Castillo et al. 2009 found similar results using *Capsicum annuum* L. with non-commercial inoculum, where fruit yield increase with *Glomus claroideum*. Also, in pepper Demre plants inoculated with *Glomus intraradices* shoots showed height increase versus untreated plants (Turkmen et al. 2005). Tanwar et al. (2013) mentioned that mycorrhizae treatments in *Capsicum annuum* contributed in early flowering.

4.3 Percentage of root colonization

For the purpose of this study, only vesicles were used to calculate infection percent. The results in these experiments are more difficult to interpret given that Promix-Soil with fertilizer, followed by Promix with fertilizer (treatment without endomycorrhizae) and Promix-Soil, presented the highest infection percent, unexpectedly. Promix-Soil with or without fertilizer had roots with the higher infection of vesicles, probably due to the exposure of increase endomycorrhizae diversity present in local soils. What was not expected was to find vesicles in the treatments with Promix only, which have no endomycorrhizae added. The reason of this occurrence is not well understood; a possibility may be because the Promix was not sterilized before potting. Another reason would be that because the experiments were conducted in an open environment, fungal spores were transferred from adjacent pots or the surroundings.

5 CONCLUSIONS

In this study, we have presented the positive outcome of using AMF in agriculture production of *C. chinense* and have established that a diverse community of AMF is part of the local soil in Western Puerto Rico.

1. First report using molecular identification of endomycorrhizal fungi present in soils of Western Puerto Rico. Including the genera *Scutellospora, Diversispora, Acaulospora, Racocetra, Funneliformis, Glomus,* and *Paraglomus.*
2. Evaluation using 3x2 Factorial ANOVA with LSD Fisher of sixth treatments in random plot for the variables related to plant growth: height, leaves, branches, fruits, and yield in *C. chinense*.

3. Use of AMF as biofertilizers improves plant growth leading to higher fruit yield with bigger and heavier products.
4. Higher mycorrhizae infection percentage was observed by using natural mycorrhizae.
5. The use of commercial or native mycorrhizae could potentially improve crop yield in *C. chinense*.

6 FUTURE PROJECTS

To optimize future work related to Arbuscular Mycorrhizal Fungi in this experiment, here we include some suggestions:

- Use a greenhouse to have better control over environmental conditions and insect pests.
- If plagues are unavoidable, use better insecticides since this project was affected by defoliation, and even by plant reduction (discarded).
- Further studies should increase characterization of AMF in more local soils of Western Puerto Rico to establish a key community of endomycorrhizae for *C. chinense* and other crops.
- More work in the mycorrhizae field should be done in Puerto Rico because there is limited information reported and it is relevant for agriculture.

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