UNIVERSIDAD AUTÓNOMA CHAPINGO

DEPARTAMENTO DE FITOTECNIA

DOCTORADO EN CIENCIAS EN HORTICULTURA

ESPECIES DE *Fusarium* ASOCIADAS A LA MARCHITEZ DEL TOMATE: IDENTIFICACIÓN, PATOGENICIDAD Y CONTROL BIOLÓGICO

Fusarium SPECIES ASSOCIATED WITH TOMATO WILTING: IDENTIFICATION, PATHOGENICITY AND BIOLOGICAL CONTROL

TESIS

Que como requisito parcial
para obtener el grado de:

DOCTOR EN CIENCIAS EN HORTICULTURA

Presenta:

MICAH ROYAN ISAAC

DIRECTOR: DR. JUAN ENRIQUE RODRÍGUEZ PÉREZ
CODIRECTOR: DR. SANTOS GERARDO LEYVA MIR

Chapingo, México, mayo de 2018

Investigación y Posgrado
Instituto de Horticultura

ESPECIES DE *Fusarium* ASOCIADAS A LA MARCHITEZ DEL TOMATE: IDENTIFICACIÓN, PATOGENICIDAD Y CONTROL BIOLÓGICO

***Fusarium* SPECIES ASSOCIATED WITH TOMATO WILTING: IDENTIFICATION, PATHOGENICITY AND BIOLOGICAL CONTROL**

Tesis realizada por Micah Royan Isaac bajo la supervisión del Comité Asesor indicado, aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS EN HORTICULTURA

DIRECTOR

DR. JUAN ENRIQUE RODRÍGUEZ PÉREZ

CODIRECTOR

DR. SANTOS GERARDO LEYVA MIR

ASESOR

DR. JAIME SAHAGÚN CASTELLANOS

LECTOR EXTERNO

DR. CRISTIAN NAVA DÍAZ

Chapingo, México, mayo de 2018

ACKNOWLEDGEMENTS

To the **National Council of Science and Technology (CONACyT),** for funding during my doctorate studies program.

To the **Universidad Autonoma Chapingo** and to the academic staff of the Doctorate degree in Sciences in Horticulture for the opportunity and the invaluable learning that they granted me.

To **D.Sc. Juan Enrique Rodríguez Pérez**, for his correct direction, availability, collaboration, experience and suggestions during the development of this work and especially for his kind support and friendship in various stages of my professional life.

To **D.Sc. Santos Gerardo Leyva Mir** for his valuable support, suggestions and observations for the improvement of this work and willingness to share his knowledge in the field, you are a source of admiration and motivation, thank you very much.

To **D.Sc. Jaime Sahagún Castellanos**, with all my respect and gratitude for accompanying me along the long journey on completing this project, for your contributions and observations made to the present investigation.

To **D.Sc. Cristian Nava Díaz** for his great support and tenacity, availability and friendship that he shows with all those who approach him.

To **D.Sc. Juan Manuel Tovar Pedraza and D.Sc. Moisés Camacho Tapia** for transmitting their knowledge about molecular biology and to be part of my training and professional development.

To **D.Sc. Kamila Câmara Correia and D.Sc. Jorge Sami Michereff** for the facilities provided during my visit to Brazil, as well as their advice in the analysis of sequences.

To my other collegues **Mariela Coutiño, Lucas Ibañez, Ilse-Michele, Christian Moncayo, Leticia Robles**, **Ismael Hernandez, Placido Facundo** and the **5th and 6th year students** of experimental designs, Horticultural science program during the (2014-2017).

To the workers **Mr. Eusebio Moreno Silva, Mr. Ángel Flores Estrada and Mr. Jorge Luis Sanchez Galicia** for their exceptional support during the establishment and management of the experiments in the greenhouse.

DEDICATIONS

To God, who gave me the faith, the strength to always go ahead despite the difficulties, to put me on the best path, illuminating every step of my life, and give me the health and hope to finish this work.

To my parents, **Lydia and Roystan** for their blessings, understanding, for teaching me to fight forward, for their support, no matter how difficult the times were, but above all for teaching me to be responsible what served as the basis to reach this goal.

To my wife, **Lorena Ángel Andrés**, I dedicate this work to my beloved wife, for her support and encouragement that she gives me day after day to reach new goals, both professional and personally.

To my children, **Tristan Nicolás Isaac Ángel** and **Karyna Quetzally Isaac Angel** that since they came into my life have been the best thing that has happened to me, they are the reason of my actions, of my daily struggle, motivation and overcoming.

To my brothers and sister, **Roland**, **Romic** and **Carla** for their support, affection, respect, understanding and for inspiring me to be better every day.

To my friends from the Doctorate degree program year 2014-2017 (**Mercedes Cuenca**, **Martin Gaona**, **Gisella**, **Uvalda**, **Felix**, **Ismael**, **Vicente**, **Rene Cano** and **Jose Francisco**), and with all those with whom I shared happy moments and all those people from whom I have received wise advice at some point, thank you for believing in me and also for those who have not. they did, because that gave me strength to achieve my goals.

To **D. Sc. Silvia Edith García Díaz** and **D. Sc. Jose. Tulio Méndez Montiel** for their friendship, support and love shown making me feel like one more member of their family, thanks for everything.

BIOGRAPHY

Mr. Micah Royan Isaac was born on February 17, 1983, in the town “Point salines,” belonging to the Parish of St. George's, on the beautiful island of Grenada. He completed his primary school studies at the “J.W. Fletcher Memorial R.C school” (1987-1994). His secondary school education at “Wesley College secondary school” (1994-1999), and technical studies at the “T. A. Marryshow Community College” (2000-2002). He has also provided professional services for two years at a government agency of Grenada as a Technical field supervisor, in the management various vegetable and fruit crops, at the Caribbean Agricultural Research and Development Institute.

He obtained a bachelor’s degree in plant science at the Universidad Autónoma Chapingo, Department of Plant Science (2006-2011). He also carried out his Master's degree studies at the Department of Agricultural Parasitology of the Universidad Autónoma Chapingo, (2012-2013) as a student of the Master's program in Plant Protection. He started his Doctorate studies in the Department of Plant Sciences Universidad Autónoma Chapingo, being a member of the group, year (2014-2017). He ended his studies as a student of the D. Sc. program in Horticulture Sciences, to be completed in February of 2018 with the presentation of this research work.

Email: mickie50@hotmail.com

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ESPECIES DE *FUSARIUM* ASOCIADAS A LA MARCHITEZ DEL TOMATE: IDENTIFICACIÓN, PATOGENICIDAD Y CONTROL BIOLÓGICO

FUSARIUM SPECIES ASSOCIATED WITH TOMATO WILTING: IDENTIFICATION, PATHOGENICITY AND BIOLOGICAL CONTROL

Micah Royan Isaac[1], Juan Enrique Rodríguez Pérez[2], Santos Gerardo Leyva Mir[2], Jaime Sahagún Castellanos

ABSTRACT

Fusarium oxysporum is responsible for the main disease of tomato. It causes significant losses in fruit yield. The objectives of this investigation were to collect and identify, by morphological and molecular techniques, Fusarium strains associated with tomato wilt, evaluate their pathogenicity in tomato seedlings and determine, *in vitro* conditions, the antagonistic effect of strains of *Trichoderma* sp. collected in the same places, against the pathogenic isolates of *F. oxysporum*, finally the biological effectiveness of Trichoderma in the greenhouse was evaluated. The isolates were obtained in 15 localities of three states of Mexico. The characterization of both fungi was carried out in PDA and CLA culture media, identified phylogenetically by the molecular markers ITS and TEF. The pathogenicity test was carried out on seedlings of four tomato varieties. Isolates were inoculated in spore suspension (10^6 conidia mL^{-1}), dual cultures were implanted in PDA culture medium in Petri dishes and two inoculation methods were used to perform greenhouse tests in order to determine the antagonistic capacity of *Trichoderma* against *Fusarium*. Analyses of variance, comparisons of means and linear regression were performed. We obtained 40 isolates identified morphologically and phylogenetically as *Fusarium oxsyporum*, *Fusarium andiyazi* and *Fusarium circinatum*. The strain of *F. oxysporum* MG557866 ITS-MG557873 TEF was the most pathogenic. The species *T. asperellum*, *T. stromaticum*, *T. koningiopsis* and *T. asperelloides* with antagonistic activity against the studied *Fusarium* isolates were identified. T. asperellum showed the highest inhibition of mycelial growth in 70% ($P \leq 0.05$). The predominant mechanism of the antagonistic activity of *Trichoderma* against *Fusarium* was mycoparasitism. The results obtained in *in vitro* and greenhouse showed the control capacity of *F. oxysporum* by *T. asperellum* in tomato culture.

Keywords: *Trichoderma asperellum*, *Fusarium circinatum*, ITS and TEF, mycoparasitism,

RESUMEN

Fusarium oxysporum es responsable de la principal enfermedad en le cultivo del tomate la cual causa pérdidas importantes en el rendimiento de fruto. Los objetivos de esta investigación fueron colectar e identificar, mediante técnicas morfológicas y moleculares, cepas de *Fusarium* asociados con la marchitez del tomate, evaluar su patogenicidad en plántulas de tomate y determinar, en condiciones *in vitro*, el efecto antagónico de cepas de *Trichoderma sp.* colectadas en los mismos lugares, contra los aislados patogénicos de *F. oxysporum*, al final se evaluó la efectividad biológica de *Trichoderma* en invernadero. Los aislamientos se obtuvieron en 15 localidades de tres estados de México. La caracterización de ambos hongos se realizó en los medios de cultivo PDA y CLA, y fueron identificados filogenéticamente mediante marcadores moleculares ITS y TEF. La prueba de patogenicidad se realizó en plántulas de cuatro variedades de tomate. Los aislados se inocularon en suspensión de esporas (10^6 conidios mL^{-1}), se implementaron cultivos duales en medio de cultivo PDA en cajas Petri y se usaron dos métodos de inoculación para realizar pruebas en invernadero con el fin de determinar la capacidad antagonista de *Trichoderma* contra *Fusarium*. Se realizaron análisis de varianza, comparaciones de medias y regresión lineal. Se obtuvieron 40 aislados identificados morfológica y filogenéticamente como *Fusarium oxsyporum*, *Fusarium andiyazi* y *Fusarium circinatum*. La cepa de *F. oxysporum* MG557866 ITS-MG557873 TEF fue la más patogénica. Se identificaron las especies *T. asperellum*, *T. stromaticum*, *T. koningiopsis* y *T. asperelloides* con actividad antagónica contra los aislados de *Fusarium* estudiados. *T. asperellum* mostró la mayor inhibición del crecimiento micelial en 70 % ($P \leq 0.05$). El mecanismo predominante de la actividad antagónica de *Trichoderma* contra *Fusarium* fue micoparasitismo. Los resultados obtenidos en condiciones *in vitro* e invernadero mostraron la capacidad de control de *F. oxysporum* mediante *T. asperellum*, en el cultivo de tomate.

Palabras claves: *Trichoderma asperellum, Fusarium circinatum,* ITS y TEF, micoparasitismo

[1] Tesista
[2] Director

1. GENERAL INTRODUCTION

In Mexico, tomato (*Solanum lycopersicum* L.) is of great socioeconomic importance, due to high export volumes, extended cultivated areas and continuous employment creation (Gonzalez and Martinez, 2002; Parke and Grünwald, 2012). In 2015, Mexico occupied the tenth position as the most significant tomato producer and was considered the second most considerable tomato exporter worldwide, with 3.53 million tons (FAOSTAT, 2015). The total production area sown in 2016 was of 161 thousand 328 hectares, reaching yields of 59.5 tons per hectare. Tomato is extremely important to the economies of the most abundant producing states of Mexico such as Sinaloa, Baja California, Michoacán, San Luis Potosí, Jalisco and Baja California Sur, all of which contributed 72 % of the nation's production (FAOSTAT 2016).

Tomato is affected by the incidence of diseases caused by soil fungi (Jiménez-Fernández *et al*., 2010), among this *Fusarium oxysporum* f. sp. *radicis-lycopersici* causing tomato wilt and is one of the principal diseases that affect tomato production (Thornton & Cramer, 2012; Inami *et al*., 2014), and is becoming an increasing problem globally (Pinaria *et al*., 2010; Macedo *et al*., 2017). This fungus was first observed in 1969 in Japan (Sato and Araki, 1974), and *Fusarium oxysporum* f. sp. *lycopersici*, described over 100 years ago in the UK (Massee, 1895), causes tomato wilting (Inami *et al*., 2014), resulting in low yields and high economic losses (Arie *et al*., 2007; Panthee and Chen, 2010), exceeding 50 % in production systems in Mexico (Apodaca *et al*., 2004).

The symptoms of *Fusarium oxysporum* includes the yellowing of the leaves, shoot, and branches, then slowly spread out and up the vine of the tomato plants. The vascular zone of the root will turn reddish brown causing root rot, which will eventually wilt, stunt and kill the plant (Panthee and Chen, 2010; Lopez-Berges *et al*., 2013). There have been reports that *Fusarium oxysporum* also affects plant roots, rapidly colonizes xylem tissue, restricting water and nutrient uptake, causing wilting, and eventually kills the plant (Gawehns *et al*., 2014). *Fusarium* produces chlamydospores (Leslie and Summerell, 2006) within the infected plant

and root tissues and can persist in the soil for up to 20 years or more (Alabouvette, 1986; Peng, *et al*., 1999), preventing the optimal development of the host plant (Recorbet, & Alabouvette, 1997).

Adequate identification of this pathogen is necessary to apply appropriate measures to reduce its harmful effects (Takken and Rep, 2010). Morphological characteristics based on shape and size microconidia and macroconidia, phialides and the formation of chlamydospores (Rodrigues and Menezes, 2005), are the primary form of identification and taxonomic description of *Fusarium* species (Leslie and Summerell, 2006). Additionally, modern molecular methods such as PCR and DNA sequence analysis of internal transcribed spacer (ITS) region and the translation elongation factor 1α (EF-1α), are faster and more reliable to obtain proper identification (El-Kazzaz *et al*., 2008; Singha, 2016).

The control of this fungus is mainly by the use of treatments based on fungicides. However, alternative treatments to fungicides are needed to ensure the efficacy of the limited number of fungicides available to control this disease is not lost through overuse and for integrated management of tomato wilt. In tomato trials in Mexico, adequate control of tomato wilt has been reported using the biological control agent *Trichoderma harzianum*. Consequently, the use of *Trichoderma* spp. as biocontrol could be an alternative to tomato wilt control (Jiménez and Sanabria, 2008).

One of the most popular microorganisms for biocontrol of *Fusarium oxysporum* is *Trichoderma* spp., (Bissett 1991a) that can be found in different types of soil, over a wide range of climatic zones (Zhang *et al*., 2005). This fungus can colonize under distinctive environmental conditions due to its high reproductive capacity (Bissett 1991b; Harman *et al*., 2004), low nutritional requirements and its growth capacity. The above is favored by organic matter, moisture content, and optimal temperature conditions of 25-30 °C (Srobar, 1978; Bhai & Dhanesh, 2008). *Trichoderma* spp., also can survive under extreme conditions such as low and high temperatures, pH and salty environments (Lewis and Papavizas, 1984; Asha *et al*., 2013). *Trichoderma* spp. have a symbiotic relationship with the roots of the plants, helping the nutrient flux, the development of the plants (Althaf and Srinivas,

2013), and in the synthesis and liberation of enzymes such as polysaccharoses, cellulose, xylanases, and quintanses (Verma *et al*., 2007). Some studies have also proven that the biological control can stimulate the secondary metabolism resulting in higher concentrations of bioactive substances (Mukherjee *et al*., 2012b; Abdelrahman *et al*., 2016). Many species of *Trichoderma*, if given optimal conditions, establish stable and long-lasting colonisations of root surfaces and even penetrate into the epidermis and a few cells below this level (Harmen *et al*., 2004).

The method of inoculation is essential for the success of the biological control strategy and can differ regarding different substrates (El Komy *et al*., 2015). The inoculation of the substrates with *Trichoderma* spp. has shown numerous positive results under hydroponics conditions (Mwangi *et al*., 2011). However, there is still considerable interest in finding more efficient mycoparasitic fungi of the Trichoderma spp., which can adapt to different soil and substrate conditions under greenhouse conditions. Most research is focused on the application of *Trichoderma* spp. to the conventional open field and the results in higher plant biomass, controlling the pathogen or improving the rooting systems of the plants (Ming *et al*., 2013). The secondary metabolism is not vital for the survival of the plants, but it has a defensive role against pathogens that are responding to improper growing conditions (Hartmann, 2007; Atkinson and Urwin, 2012; Rejeb *et al*., 2014).

1.2. GENERAL OBJECTIVES

1. Generate a sustainable control method to lower the environmental impact of *Fusarium oxysporum* using *Trichoderma* sp in tomato.
2. Identify species of *Trichoderma* with high effectiveness that controls *Fusarium oxysporum*, *in vitro* and under greenhouse conditions.
3. Study the interaction of the Plant - *Fusarium oxysporum* - *Trichoderma* spp. using different types of inoculation methods and substrates.

1.2.1. SPECIFIC OBJECTIVES

1. Determine the occurrence and incidence of Fusarium isolates in field and greenhouse conditions, in three states (Puebla, Morelos, and Tlaxcala) of Mexico.
2. Identify and characterize selected Fusarium isolates which causes tomato wilting, using morphological characteristics and sequence analysis of internal transcribed spacer (ITS) region of rDNA and the translation elongation factor 1α (EF-1α), and to verify the pathogenicity in four commercial tomato varieties.
3. Determine the occurrence and incidence of *Fusarium* isolate in field and greenhouse conditions, in the central growing region of Mexico.
4. Identify and characterize selected *Fusarium* species which causes tomato wilting, using morphological characteristics and sequence analysis of internal transcribed spacer (ITS) region of rDNA and the translation elongation factor 1α (EF-1α).
5. Identify of species of *Trichoderma* spp. concerning their antagonistic behavior by dual culture against isolates of *Fusarium oxysporum*.
6. Evaluate the effect of *Trichoderma* spp. in controlling *Fusarium oxsyporum* using two inoculation methods in the soil, coconut fiber, and peat moss substrates, in tomato plants grown under greenhouse conditions.

1.3. GENERAL HYPOTHESIS

2. The fungi of the genus Trichoderma would be able to exert a positive effect on plants inoculated with *Fusarium oxysporum*.

2.2.1. SPECIFIC HYPOTHESES

1. The Trichoderma isolates have the antagonistic and mycoparasitic capacity *in vitro* to *Fusarium oxysporum*.

2. The Trichoderma isolates are compatible in reducing the growth of *Fusarium oxysporum in vitro* and under greenhouse conditions
3. The different Trichoderma species has a positive effect on tomato plants inoculated *Fusarium oxysporum*.

1.3. LITERATURE REVIEW

The cultivated tomato, *Solanum lycopersicum* L., is a member of the large and diverse genus Solanum of the derived Asterid family Solanaceae. It belongs to a group of 13 closely related species all of which occur in arid habitats on the west coast of South America. The tomatoes are sister to the potatoes and began to diversify only very recently, after the rise of the Andes and the development of the arid western deserts. Tomatoes were probably brought to Europe by the Spanish from Mesoamerica and thence distributed worldwide (Weese and Bohs, 2007; Bauchet and Causse, 2012).

1.3.1. General description and taxonomy

1.3.1.1. General description

Tomato is a perennial herbaceous plant, but it is often grown as an annual crop even if biennial and perennial forms exist. Tomato is cultivated in tropical and temperate climates in open field or under greenhouse in a temperate climate. Greenhouses are often used for large-scale production. In the warm climate with the right light intensity for growth, around 45 days are necessary from germination to anthesis and 90-100 days to reach the beginning of fruit ripeness (Nuez, 2001). The end use of the crop, whether for the processing market or fresh market, will determine the cultivars sown, the time of harvest and harvest processes, which can be manual or mechanical (Nuez, 2001). The growth habit of the plant varies from indeterminate to determinate and may reach up to 3 meters (m) in height. The primary root may grow several meters in length. The stem is angular and covered by hairy and glandular trichomes that confer a characteristic smell.

Leaves are alternately arranged on the stem with a 137.5° phyllotaxy. Leaves range in shape from lobed to compound, with segments arranged pinnately. Compound leaves are typically comprised of five to nine leaflets. Leaflets are petiolated and dentated. All leaves are covered by glandular, hairy trichomes. The tomato fruit is globular or ovoid.

Botanically, the fruit exhibits all of the typical characteristics of berries; a simple fleshy fruit that encloses its seed in the pulp. The outer skin is a thin and fleshy tissue that comprises the remainder of the fruit wall as well as the placenta. The color of the fruit is derived from the cells within the fleshy tissue. Tomato fruits can be either bilocular or multilocular. Between 50 and 200 seeds are located inside the locular cavities and are enclosed in gelatinous membranes. On average, the seeds are small (5 x 4 x 2 mm) and lentil-shaped. The seed contains the embryo and the endosperm and is covered by a durable seed coat, called the testa. The development of the fruit takes seven to nine weeks after fertilization. The many end uses of tomato fruit, as well as food and feed safety considerations, including the composition of essential food and feed nutrients, anti-nutrients, allergens, and toxicants, are detailed in the “OECD consensus document on compositional considerations for new varieties of tomato” (OECD, 2008).

1.3.1.2. Taxonomy

The cultivated tomato is a member of the genus Solanum within the family Solanaceae. The Solanaceae, commonly known as the nightshade family, also includes other notable cultivated plants such as tobacco, chili pepper, potato, and eggplant. Tomato classification has been the subject of much discussion, and the diversity of the genus has led to the reassessment of earlier taxonomic treatments. Tomato was initially named *Solanum lycopersicum* by Linnaeus in 1753; *Lycopersicon lycopersicum* (L.) Karsten has also been used (Valdes and Gray, 1998). Miller (1768) in The Gardener’s Dictionary used *Lycopersicon esculentum*. Rick (1979) included nine species in the Lycopersicon genus. For a long time, tomatoes were known as *L. esculentum*, but recent research has shown

that they are part of the genus Solanum and are now again broadly referred to as *Solanum lycopersicum* (Spooner, Anderson, and Jansen, 1993; Bohs and Olmstead, 1997; Olmstead and Palmer, 1997; Knapp, 2002; Spooner et al., 2005, 2003; Peralta *et al*., 2008).

1.3.1.3. Origin and domestication of tomato (*Solanum lycopersicum* L.)

Tomato (*Solanum lycopersicum* L.) originated from the Andean region and is now included as part of the regions of Chile, Bolivia, Ecuador, Colombia, and Peru. The time and place of domestication of tomato are not known with certainty. The tomato had reached a relatively advanced stage of domestication before being taken to Europe in the 16th century, and further domestication on a much more intense level occurred throughout Europe in the 18th and 19th centuries (Sims, 1980). Since the 20th century, human beings have created a massive array of morphologically different cultivars and forms from the single species *Solanum lycopersicum* via plant breeding. Through domestication, research and breeding activities that were implemented by scientists and breeders worldwide, modern tomato varieties (mostly hybrids) have been developed with all shapes, colours, and sizes (Foolad, 2007).

Tomatoes were domesticated in America; however, the original site of domestication and the early events of domestication are mainly obscure (Peralta and Spooner, 2007). Two hypotheses have been advanced for the original place of tomato domestication, one Peruvian and the other Mexican. Although definite proof for the time and place of domestication is lacking, Mexico is presumed to be the most probable region of domestication, with Peru as the center of diversity for wild relatives (Larry and Joanne, 2007). *Solanum lycopersicum* var. *cerasiforme* is thought to be the ancestor of cultivated tomato, based on its extensive presence in Central America and the presence of a shorten style length in flower (Cox, 2000). However, recent genetic investigations have shown that the plants known as ‘cerasiforme’ are a mixture of wild and cultivated tomatoes rather than being ‘ancestral’ to the cultivated tomatoes (Nesbitt and Tanksley, 2002).

1.3.1.4. Importance of tomato

The cultivated tomato, *Solanum lycopersicum* L., is the world's most highly consumed vegetable due to its status as an essential ingredient in a large variety of raw, cooked or processed foods. Tomato is grown worldwide for local use or as an export crop. In 2016, the global area cultivated with tomato was 5 million hectares with a production of 161 million tonnes, the major tomato-producing countries being China and India (FAOSTAT, 2017). Tomato can be grown in a variety of geographical zones in open fields or greenhouses, and the fruit can be harvested by manual or mechanical means. Under certain conditions (e.g., rejuvenation pruning, weeding, irrigation, frost protection), this crop plant can be perennial or semi-perennial, but commercially it is considered an annual (Geisenberg and Stewart, 1986). Although there are many types of growing systems for greenhouse tomatoes, the two major cropping systems are two crops per year and one crop per year. Its importance lies not only in profit but also in the income generated in local economies for farmers and agricultural workers (Villarreal, 1982; Coll-Hurtado and Godínez Calderón, 2003). Protected agriculture is a broad category of production methods providing some degree of control over various environmental factors. This category includes production technologies such as greenhouses, glasshouses, tunnels and covered fields (Nieves-García, van der Valk, and Elings, 2011).

Although there is no quantitative data about the world's vegetable production in greenhouses, some calculations have been made. For example, in 2012, the greenhouse vegetable production was about 81 million kilograms (kg), of which 40 million kg was tomato, and 37 million kg was cucumber. More specifically, in 2012, the tomato production in greenhouses in North America accounted for the 52 % of the market in Canada and the 22 % of the market in the United States (Farm Credit Canada, 2012). The commercially valuable tomato fruit can vary in color, size, and shape (Vaughan and Geissler, 1997). The fruit contains a large quantity of water, vitamins and minerals, low amounts of proteins and fats, and some carbohydrates. It also contains carotenes, such as lycopene (which gives

the fruit its predominantly red color) and beta-Carotene (which gives the fruit its orange color). Modern tomato cultivars produce fruits that contain up to 3 % sugar of fresh fruit weight. It also contains tomatine, an alkaloid with fungicidal properties. The concentration of tomatine decreases as the fruit matures and tomatine concentration contributes to determining the taxonomy of the species. Thus it can be useful in crop breeding for cultivated tomatoes (OECD, 2008; Spooner, Anderson and Jansen, 1993). Cultivated tomato is related to wild tomatoes originating from Peru, Ecuador and other parts of South America including the Galapagos Islands. The center of its domestication and diversification is Mexico (Rick, 1978; Jenkins, 1948; Peralta, Spooner and Knapp, 2008). Wild relatives of tomato and intermediate forms (landraces or creoles) harbor a wealth of genetic diversity and are important sources of genetic material in crop improvement and conservation programmes (Sánchez-Peña *et al*., 2004). Tomato is one of the best studied cultivated dicotyledonous plants at the molecular level and has been used as a model species for research into gene mapping, gene characterization (e.g., plant pathogen resistance genes) and gene transfer approaches. It is also useful to study other plant traits such as fruit ripening, hormone function and vitamin biosynthesis (Gebhardt *et al*., 1991; Chetelat and Ji, 2006; Ji and Scott, 2006).

1.3.1.5. *Fusarium oxysporum* Schlecht

Fusarium oxysporum Schlecht (Fo) is a free-living ascomycete fungus with no known sexual state. Fo is a complex species comprised of ubiquitous soil-borne plant pathogens, with ca. 120 formae speciales (ff. spp.) based on host specificity (Michielse and Rep, 2009; Arie, 2010). The different ff. spp. show considerable genetic diversity and have polyphyletic origin (O'Donnell *et al*., 1998; Nirmaladevi *et al*., 2016). Fo causes significant economic losses of many crops including tomato (*Solanum lycopersium* L.), which is one of the most worldwide cultivated vegetable crops. Fo diseases in tomato are mainly caused by f. sp. radicis-lycopersici Jarvis and Shomaker (FORL), responsible for crown and root rot, and

by f. sp. *lycopersici* (Sacc.) Snyder and Hansen (FOL), responsible for vascular wilt disease (Edel-Hermann *et al*., 2012), and although both ff. spp. infect the same host plant, FOL and FORL have strict host specificity. Three physiological races of FOL (1, 2, and 3) have been differentiated depending on their ability to infect tomato cultivars carrying different resistance loci (Mes *et al*., 1999). The use of resistant cultivars and resistant rootstocks remains the most appropriate way to prevent Fo diseases in tomato production. Hence, determining which pathogens are emerging in the field is important in order to select the most suitable tomato cultivar. Since pathogenic strains of Fo cannot be identified morphologically, pathogenicity tests are commonly used on different tomato cultivars. However, these methods are very time-consuming and expensive (Baysal *et al*., 2009), and also the results of these types of biological tests can be affected by variations in temperature (Boix-Ruíz *et al*., 2015).

The gene sequence variability found in polygalacturonases, major enzymes involved in Fo-plant interactions (Di Pietro and Roncero, 1998), has been useful to study the genetic diversity in populations of this fungus (Kawabe *et al*., 2005). A PCR-based technique, using a set of primers specific to the sequences of the endo-polygalacturonase gene pg1 and the exo-polygalacturonase gene pgx4 of Fo isolates from Japan, allowed FOL and FORL, and the races of FOL (Hirano and Arie, 2006) to be differentiated. However, discrepancies have been observed in identifying some isolates collected from tomato crops in other areas of the world, such as those from the Mediterranean coast of Turkey, with pathogenicity tests and PCR carried out using this set of primers (Baysal *et al*., 2009; Çolak and Biçici, 2013).

FOL isolates appear to have horizontally transferred accessory chromosomes (Ma, 2014) which encode a number of putative effectors, including the set of the secreted in xylem (SIX) proteins (Houterman *et al*., 2009). Several SIX genes have been associated with the three races of FOL, and the molecular markers developed for these genes provide a robust PCR-based method for identifying the host specificity of FOL isolated from plant tissues (Lievens *et al*., 2009; Jelinski *et al*., 2017). In addition, the presence of SIX1 can be used to identify FOL isolates.

Moreover, SIX4 allows the identification of race 1 isolates, and SIX3 variations can serve to differentiate race 2 from race 3 isolates (Lievens *et al*., 2009). A previous study exploring the genetic diversity of Fo and other *Fusarium* spp. pathogenic on tomato in different Mediterranean countries, which combine the use of the presence of SIX1, intergenic spacer (IGS) DNA typing and vegetative compatibility grouping (VCG), identified 27 out of 27 Fo Algerian isolates as FORL (Edel-Hermann *et al*., 2012). Unfortunately, cultivars of tomato with resistance to FORL are not yet commercially available. And, crown and root rot disease caused by FORL is widely present in most of the African and Asian Mediterranean countries, including Algeria, where tomato production is economically important, occupying second place after potato.

1.3.1.6. Importance of *Fusarium oxysporum*

This Fusarium disease is caused by the fungus *Fusarium oxysporum* Schlechtend: Fr. f. sp. *radicis-lycopersici* W.R. Jarvis & Shoemaker, a close relative of the Fusarium wilt pathogen *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *Lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. The pathogen damages mostly tomato but it has also been reported in eggplant and pepper and can be particularly severe in greenhouse production systems. (Di Pietro *et al*., 2001; Asha *et al*., 2011; Ajigbola *et al*., 2013). This wilt is caused by the soil borne fungus which is one of the most devastating diseases of tomato (Sudhamoy *et al*., 2009). It affects greenhouse and field grown tomatoes in warm vegetable production areas. The disease is characterized by yellowed leaves and wilted plants with minimal or absent crop yield. There may be a 30 to 40 % yield loss due to the disease and this may go up to 80 % under favorable weather conditions (Kapoor,1988; Kirankumar *et al*., 2008). High Fusarium wilt incidence in tomato of 25–55 % has been recorded from various parts of Mexico. The pathogen invades the root epidermis and extends into the vascular tissue. It colonizes the xylem vessels producing mycelium and conidia. The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging

(Beckman, 1987). Three physiological races (1, 2, and 3) of the pathogen are distinguished by their specific pathogenicity to tomato cultivars (Kawabe *et al*., 2005). Since *F. oxysporum* f. sp. *lycopersici* (Fol) is an asexual fungus, genetic exchange occurs via somatic fusion and hetreokaryon formation between vegetative compatible strains.

1.3.1.7. Distribution of *Fusarium oxysporum*

Tomato wilt has been found in many countries in all continents where tomatoes are grown both in the open tropical and 22 subtropical climates (Walker, 1971). Massee (1895) was the first who described the disease of tomato. Jones and Woltz (1981) listed the disease in over 30 countries and probably occurs in many unreported locations where tomatoes are grown. Of the three presently known races of the fungus, Walker (1971) and Jones *et al*. (1991) reported that 1 and 2 probably have a worldwide distribution. Race 1 caused significant resistance to disease problems on tomato throughout the USA until identified by Bohn and Tucker (1939; 1940) and incorporated into tomato cultivars (Porte and Walker, 1941). Race 2 was first reported in Ohio in 1945 (Alexander and Tucker, 1945) and later elsewhere (Gerdemann and Finley, 1950; Goode, 1966) but was not of much concern until it was reported in Florida in 1961 (Stall, 1961; Jones, 1966; Strobel, 1969). Race 3 was first reported in Brazil (Tokeshi *et al*., 1966) and has subsequently been identified in Australia (Grattidge and O'Brien, 1982), Mexico (Valenzuela-Ureta *et al*., 1996) and Florida (Volin and Jones, 1982), California (Davis *et al*., 1988), Georgia (Chelemi and Dankers, 1992), Arkansas and North Carolina (Marllatt *et al*., 1996) in the USA There are species that are also associated with crops such as sorghum, in Africa and USA (Marasas *et al*., 2001), with rice bakanae, in Asia and Africa (Wulff *et al*., 2010), and with corn in Siria (Madamia *et al*., 2013).

1.3.1.8. Conditions necessary for *Fusarium* growth and development

Factors such as temperature, humidity and other environmental factors have a great importance for the incidence and severity of certain *Fusarium* species (Vigier *et al*., 1997). Optimum temperature of approximately 25 °C and could tolerate a temperature as high as 30 °C and relative humidity between 60-80 %for growth. These factors are also important in mycotoxicosis epidemiology, because the production of mycotoxins by the different species is differentially affected by some environmental factors such as temperature (Di Menna *et al*., 1991; Jimenéza et al., 1996). Host range and climatic factors influence the growth, survival, spreading and hence the incidence of *Fusarium* species and the crop damage. The influence of origin and climatic factors on *Fusarium* diseases is complicated by the fact that *Fusarium* species are able to cause the diseases individually or in complex. There are some reports on how the *Fusarium* species differentially respond to different environmental variations, mostly temperature, isolate origin and humidity (Conrath *et al*., 2002). Las condiciones ambientales, tales como alta humedad, tipo de suelo, disponibilidad de nutrientes, entre otros, a menudo promueven la aparición y diseminación de patógenos en el suelo; además, el manejo de la enfermedad a menudo se basa en la aplicación de fungicidas como fumigantes para el tratamiento de suelos, y para la prevención de enfermedades foliares y de tallo (Aleandri *et al*., 2015).

1.3.1.9. The process of tomato infection by *Fusarium oxysporum*

The process consists of several steps, including root surface attachment and colonization, penetration and colonization of the root cortex and, in the case of wilt inducing formae speciales, mycelia proliferation inside the xylem vessels (Di Pietro *et al*., 2003). Working with *Fusarium oxysporum*, Di Pietro *et al*. (2001) observed conidial germination on roots, growth in the tomato root cortex and colonization of the xylem by the pathogen. Chlamydospores (thickwalled, survival spores) of green fluorescent proteinlabeled Fol were also observed on and in

tomato roots 7 and 22 days post-inoculation, respectively. However, neither conidiophores nor microconidia were detected in xylem vessels (Van der Does *et al*., 2008). The role of microconidia in xylem colonization has been shown to be trivial in a study of Ren1, a putative transcription factor essential for micro-and macroconidia formation. A mutant disrupted in this gene produces only chlamydospores and abnormal rod-shaped, conidium-like cells, but is not affected in pathogenesis, suggesting that microconidia and macroconidia are not important for pathogenicity (Ohara *et al*., 2004).

Following penetration, Czymmek *et al*. (2007) observed that fungal growth was initially intercellular but, ultimately, became intracellular, and the collapse of plant cells was observed at sites of fungal penetration, presumably as a consequence of a loss of turgor pressure. Plant cells that were not in direct contact with mycelium were also subjected to changes such as the loss of autofluorescent vacuole content and changes in the appearance of the endoplasmic reticulum.

1.3.1.10. Role of cell wall-degrading enzymes (cwde) and nutrient metabolism in pathogenicity

The contribution part of CWDE in the process of infection is not completely elucidated. To penetrate and colonize plant tissues, pathogenic F. oxysporum, like most fungi, secrete an arsenal of CWDE, such as polygalacturonases, pectatelyases, xylanases and proteases. However, shutting down individual cell-wall degrading enzymes- or protease-encoding genes did not show any impact on virulence (Di Pietro *et al*., 2003). The reason for this might be functional redundancy of these genes.

Carbon metabolism can have an impact on pathogenesis through its effects on the expression of CWDE-encoding genes as it was shown in the analysis of *Fusarium oxysporum* f. sp. *lycopersici* Frp1 disruptant (frp1). Frp1 is a gene absolutely required for pathogenicity and frp1 is impaired in root colonization capacity (Duyvesteinj *et al*., 2005). Jonkers *et al*. (2009) have shown that mutation in the Frp1 results in reduced assimilation of organic acids.

1.3.1.11. Tomato responses to infection by Fol

Recent development in the fields of plant genomics and transcriptomics driven by advances in computational methods has expanded our understanding of plant-microbe interactions and their outcomes at the molecular level.

Investigation of plant-expressed molecules following pathogen infection provides valuable insights into mechanisms that underlie plant defense. Such mechanisms involve the regulation of gene expression, cascade signaling activation, hormone balancing and synthesis of defensive metabolites (Mithofer and Boland, 2012).

Substantial body of work has addressed tomato-Fol interactions and provided accumulating evidence of specific responses of tomato plants to Fol attack. In a recent study (Andolfo *et al*., 2014) genome-wide transcriptional analysis evidenced the overexpression of 2392 genes (about 64% of the differentially expressed genes during infection) in resistant tomato plants infected by Fol, indicating considerable gene activation upon inoculation.

The upregulated genes are associated to maintenance of cellular structures and cellular homeostasis. These are very important metabolic activities required by plants to survive fungus-inflicted stresses. For example, the master gene of inflammation was one of the up-regulated genes in tomato-Fol interaction. This gene is a key player in antiapoptotic (anti programmed cell death) signaling and is able to prevent apoptotic signaling pathway by inhibiting map-kinases (Paul *et al*., 2011). Since *Fusarium oxysporum* is a necrotrophic fungus (Trusov *et al*., 2006) that kills host cells prior to infection, through the predicted deployment of cell death inducing toxins and enzymes, the overexpression of the anti-apoptosis gene could confer resistance to Fol.

The expression of plant resistance genes leads also to the chemical modification of plant cell wall. In tomato plants, extract of Fol induces an increase in cell wall strengthening via the deposition of lignin, and an increased concentration of phenolic compounds, such as ferulic acid, 4-hydrobenzoinc acid and 4-coumaric acid (Mandal and mitra, 2007). Such reactions build strong physical barriers at the infection sites and pose major hurdle for the pathogen to overcome for

successful infection. Another way of plant defense strategies against pathogen attack is the release of anti-microbial compounds to counteract pathogen ingression upon infection. For instance, Fol-infected tomato plants secrete the steroidal glycoalkaloid saponin α-tomatine that forms complexes with sterol in the pathogen fungal membrane. These structures affect membrane plasticity and cause pores in fungal cell wall that lead to leakage of cellular contents (Rodick, 1977; Ghanem *et al*., 2011), contributing thereby to resistance.

1.3.2. Biological Control of Tomato wilt

1.3.2.1. *Trichoderma* spp.

Trichoderma species (teleomorph Hypocrea) are cosmopolitan filamentous fungi frequently found in agricultural habitats because of their ability to colonize the rhizosphere and progress in different soils (Hermosa *et al*., 2004; Rubio *et al*., 2005). The biocontrol capability of these fungi is well recognized since they are antagonists of phytopathogenic fungi, oomycetes and nematodes (Lorito *et al*., 2010; Medeiros *et al*., 2017). The biocontrol mechanisms of Trichoderma are at least based on competition for nutrients, the production of hydrolytic enzymes and/or antibiotics (Harman *et al*., 2004). In addition, a systemic activation of plant defense responses against biotic and abiotic damages has been observed for selected rhizosphere-competent Trichoderma strains (Hermosa *et al*., 2012; Ruocco *et al*., 2015; Rubio *et al*., 2017a), and the ability of Trichoderma spp. to reduce Fusarium wilt in tomato has been previously described (Cotxarrera et al., 2002; Taghdi *et al*., 2015).

The formulation of *T. harzianum* Th-10 and fungicide treatment recorded only 40.1% and 18.1% reduction of the disease respectively compared to control. In the Fusarium wilt-nematode interaction system also, soil application of biocontrol agents reduced significantly the wilt incidence and also the root lesion and root knot index. In addition to this, 50 to 82% of reduction in nematode population viz., *Pratylenchus coffeae* and Meloidogyne incognita was also noted due to application of bioagents and the maximum reduction was due to *T. harzianum*

treatment (Thangavelu, 2002). Raghuchander *et al*. (1997) reported that *T. viride* and *P. fluorescens* were equally effective in reducing the wilt incidence. Inoculation of potted abaca plants with *Trichoderma viride* and yeast showed 81.76 and 82.52% reduction of wilt disease severity respectively in the antagonist treated plants. (Bastasa and Baliad, 2005).

Similarly, soil application of *T. viride* NRCB1 as chaffy grain formulation significantly reduced the external (up to 78%) and internal symptoms (up to 80 %) of Fusarium wilt disease in tissue cultured as well as sucker derived plants of banana cv. *Rasthali* (Silk-AAB) and increased the plant growth parameters significantly as compared to the talc powder formulation under pot culture and field conditions (Thangavelu and Mustaffa, 2010). The possible mechanisms involved in the reduction of Fusarium wilt severity due to *Trichoderma* spp. treatment might be the mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defence system. The mycoparasitism involves in coiling, disorganization of host cell contents and penetration of the host (Papavisas, 1985). During the mycoparasitism, *Trichoderma* spp. parasitizes the hyphae of the pathogen and produce extracellular enzymes such as proteolytic enzymes, β -1, 3- glucanolytic enzymes and chitinase etc., which cause lysis of the pathogen. The toxic metabolites such as extracellular enzymes, volatiles and antibiotics like gliotoxin and viridin which are highly fungistatic substances (Weindling, 1941) are considered as elements involved in antibiosis. In addition, *Trichoderma* spp. could compete and sequester ions of iron (the ions are essential for the plant pathogen,) by releasing compounds known as siderophores (Srinivasan *et al*., 1992).

1.3.2.2. *Pseudomonas* spp.

Pseudomonas spp. are particularly suitable for application as agricultural biocontrol agents since they can use many exudates compounds as a nutrient source (Lugtenberg *et al*.,1999a); abundantly present in natural soils, particularly on plant root systems (Sands & Rovira, 1971); high growth rate, possess diverse

mechanisms of actions towards phytopathogens including the production of a wide range of antagonistic metabolites (Lugtenberg *et al*., 1991; Dowling & O'Gara, 1994; Dunlap *et al*.,1996; Lugtenberg *et al*., 1999b), easy to grow in vitro and subsequently can be reintroduced into the rhizosphere (Lugtenberg *et al*., 1994; Rhodes & Powell, 1994) and capable of inducing a systemic resistance to pathogens (Van Loon *et al*., 1998; Pieterse *et al*., 2001). Several studies have investigated the ability of *P. fluorescens* to suppress Fusarium wilt disease of banana. Fluorescent pseudomonad species such as *Pseudomonas fluorescens* (Sakthivel and Gnanamanickam, 1987), *Pseudomonas putida* (de Freitas and Germida, 1991), *Pseudomonas chlororaphis* (Chin-A-Woeng *et al*., 1998) and *Pseudomonas aeruginosa* (Anjaiah *et al*., 2003) have been used to suppress pathogens as well as to promote growth and yield in many crop plants. Sivamani and Gnanamanickam (1988) reported that the seedlings treated with *P. fluorescens* showed less severe wilting and internal discoloration due to Foc infection in green house experiments. The bacterized seedlings also showed better root growth and enhanced plant height (Kamou *et al*., 2015).

Fishal *et al*. (2010) assessed the ability of two endophytic bacteria originally isolated from healthy oil palm roots, *Pseudomonas* sp. (UPMP3) and Burkholderia sp. (UPMB3) to induce resistance in susceptible Berangan banana against *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) under glasshouse conditions. The study showed that pre-inoculation of banana plants with *Pseudomonas* sp UPMP3 recorded 51% reduction of Fusarium wilt disease severity, whereas the combined application of UPMP3+UPMB3 and single application of UPMB3 alone recorded only 39 and 38% reduction of Fusarium wilt disease severity respectively. Ting *et al*. (2011) reported that among six endobacteria isolates, only two isolates (*Herbaspirillum* spp and *Pseudomonas* spp.) produced volatile compounds which were capable of inhibiting the growth of Foc race 4.

The compounds were identified as 2- pentane 3-methyl, methanethiol and 3-undecene. They found that the isolate *Herbaspirillum* spp. recorded 20.3% inhibition of growth of Foc race 4 as its volatile compounds contained all the three compounds whereas *Pseudomonas* isolate AVA02 recorded only 1.4% of growth

inhibition of race 4 Foc as its volatile compounds contained only methanethiol and 3- undecene. They concluded that the presence of all these three compounds especially 2- pentane 3-methyl and also in high quantity is very important for the antifungal activity.

1.3.2.3. *Bacillus* spp.

Bacillus subtilis has been identified as a potential biological control agent. These strains could produce a wide range of antifungal compounds, such as subtilin, TasA, subtilosin, bacilysin, mycobacillin and some enzymes, which can degrade fungal cell wall (Berg *et al*., 2001). It was suggested that these antibiotic production plays a major role in plant disease suppression (Knox *et al*., 2000; Leelasuphakul *et al*., 2006). In addition, some antagonistic mechanisms of these *Bacillus* species involves in the competition for nutrients and space, the induction of plant resistance, entre otros (Guerra-Cantera *et al*., 2005; Van loon *et al*., 1998).

Sun *et al.* (2011) isolated an antagonistic *Bacillus* strain, KY-21 from the soil of banana's rhizosphere and tested against Foc both under *in-vitro* and *in-vivo* conditions. Under lab condition, mycelium growth of the pathogen was seriously inhibited after treatment with the fermentation filtrate of KY-21. The microscopic examination of mycelium revealed that the tips of the hypha were deformed into spherical structures that were remarkably constricted by dual culture. Besides, the inoculation of banana plants with Bacillus strain, KY-21 also increased the activities of polyphenol oxidase (PPO) and peroxidase (POD) significantly compared to control. The *in-vivo* biocontrol assays showed that at 60 days after Foc inoculation, the plantlets treated with KY-21 exhibited 35% severe wilt symptom and 18.3% severe vascular discoloration as against 68.4% and 48.3% of severe wilt symptom and severe vascular discoloration respectively in control plantlets. Besides, plantlets inoculated with KY-21 showed significantly reduced development of disease as compared to the control. 5. Actinomycetes Actinomycetes particularly *Streptomyces* spp. are important soil dwelling

microorganisms, generally saprophytic, spend majority of their life cycle as spores and are best known for their ability to produce antibiotics. They may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Crawford *et al*., 1993).

1.3.2.4. *Streptomyces* spp.

Streptomyces species have been used extensively in the biological control of several formae speciales of *F. oxysporum*, which caused wilt disease in many plant species (Reddi and Rao, 1971; Lahdenpera and Oy 1987; Smith *et al*., 1990; Li *et al*., 2010). *Streptomyces violaceusniger* strain G10 isolated from a coastal mangrove (*Rhizophora apiculata* (Blume)] stand, was shown to exhibit strong in-vitro antagonism toward several plant pathogenic fungi including Foc race 4. Under *in-vivo* bioassay, treating the planting hole and roots of tissue-culture-derived 'Novaria' banana plantlets with *Streptomyces* sp. strain g^{10} suspension (108 cfu/ml), resulted in 47% reduction of leaf symptom index (LSI) and 53% of rhizome discoloration index (RDI) with reduced wilt severity when the plantlets were inoculated with 10^4 spores/ml Foc race 4 compared to untreated plantlets. However, the reduction in disease severity was not significant when plantlets were inoculated with a higher concentration (10^6 spores/ml) of Foc race 4 (Getha *et al*., 2005). Getha and Vikineswary (2002) studied the interaction between *Streptomyces violaceusniger* strain g^{10} and *F. oxysporum* f.sp. *cubense* and demonstrated the production of antifungal metabolites especially antibiotics by the antagonists which caused swelling, distortion, excessive branching and lysis of hyphae and inhibition of spore germination of Foc pathogen by the antagonist. Among 242 actinomycete strains, isolated from the interior of leaves and roots of healthy and wilting banana plants, *Streptomyces griseorubiginosus*-like strains were the most frequently encountered strains. The screening of these strains for antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* revealed that 50 % of the *Streptomyces* strains isolated from healthy trees especially from the roots had antagonistic activities against Foc and only 27% of strains isolated from wilting trees showed the same activity (Cao *et al*., 2004). Similarly, in 2005, out of

131 endophytic actinomycete strains isolated from banana roots, the most frequently isolated and siderophore producing endophytic *Streptomyces* sp. strain S96 was found to be highly antagonistic to Foc. The subsequent in vivo biocontrol assays carried out showed that the disease severity index of *Fusarium* wilt was significantly reduced and mean fresh weight of plantlets increased compared to those grown in the absence of the biocontrol strain S96 (Cao *et al*., 2005; Elango *et al*., 2015).

1.4. REFERENCES

Abdelrahman, Mostafa & Abdel-Motaal, Fatma & El-Sayed, Magdi & Jogaiah, Sudisha & Shigyo, Masayoshi & Ito, Shin-ichi & Tran, Lam-Son. (2016). Dissection of *Trichoderma longibrachiatum*-induced defense in onion (*Allium cepa* L.) against *Fusarium oxysporum* f. sp. *cepa* by target metabolite profiling. Plant Science. 246: 128–138.

Ajigbola, C.F. and Babalola, O.O. (2013). Integrated Management Strategies for Tomato Fusarium Wilt. Biocontrol Sciences. 18:117–127.

Aleandri M. P., G. Chilosi, N. Bruni, A. Tomassini, A. M. Vettraino, A. Vannini. (2015). Use of nursery potting mixes amended with local *Trichoderma* strains with multiple complementary mechanisms to control soil-borne diseases. Crop Protection, 67:269–278.

Alabouvette C. (1986). Fusarium-wilt suppressive soils from the Châteaurenard region: review of a 10-year study. Agronomie, EDP Sciences, 6: 273–284.

Alexander, L.J. and Tucker, C.M. (1945). Physiological specialization in the tomato wilt with fungus. Journal of Agricultural Research 70:303–314.

Althaf HSK, Srinivas P (2013). Association of arbuscular mycorrhizal fungi and other rhizosphere microbes with different medicinal plants. Research Journal of Biotechnology 8:24-28.

Amsellem, Z., Zidack, N. K., Quimby, P. C., Jr., & Gressel, J. (1999). Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. Crop Protection, 18: 643–649.

Apodaca SMA, Zavaleta ME, Osada KS, García ER, Valenzuela UJG (2004). Hospedantes asintomáticos de *Fusarium oxysporum* Schlechtend. f. sp. *radicis-lycopersici* W.R. Jarvis y Shoemaker en Sinaloa, México. Revista Mexicana de Fitopatología 22: 7–13

Arie T, Takahashi H, Kodama M, Teraoka T (2007). Tomato as a model plant for plant-pathogen interactions. Plant Biotechnology 24:135–147.

Asha, B. B., Chandra Nayaka, S., Udayashankar, A. C., Srinivas, C. & Niranjana, S. R. (2011). Selection of effective bio-antagonistic bacteria for biological control of tomato wilt caused by *Fusarium oxysporum*. BioScan.6: 239–244

Asha N.N. Raghunandan B.L. and. Shivaprakash M.K (2013). Survival and population studies of *Trichoderma* spp. Madras Agric. J., 100: 554–558.

Atkinson NJ, Urwin PE (2012). The interaction of plant biotic and abiotic stresses: from genes to field. Research Journal of Biotechnology 8:24–28.

Bastasa, G.N, and Baliad, AA (2005). Biological control of Fusarium wilt of abaca (*Fusarium oxysporum*) with *Trichoderma* and yeast. Philippine Journal of Crop Science (PJCS) 30: 29–37

Bauchet G, Causse M. (2012). Genetic diversity in tomato (*Solanum lycopersicum* L.) and its wild relatives. In: Caliskan M, editor. Genetic diversity in plants. InTech 978-953-51-0185-7:133–62.

Baysal, T., Ersus, S., & Apaydın, E. (2009). The effect of corn zein edible film coating on quality of intermediate moisture tomatoes. Gida, 34: 359–366.

Beckman, C. H. (1987) The nature of wilt diseases of plants. American Phytopathological Society. 175–176.

Berg. G., Fritze. A., Roskot. N., Smalla. K., (2001). Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. Journal of Applied Microbiology 91: 963-971.

Bhai RS, Dhanesh J, (2008). Occurrence of fungal diseases in vanilla (*Vanilla planifolia* Andrews) in Kerala. Journal of Spices and Aromatic Crops 17: 140–8.

Bissett, J. (1991a): A revision of the genus Trichoderma. II. Infrageneric classification. Can. J. Bot. 69, 2357 – 2372.

Bissett, J. (1991b). The revision of the genus Trichoderma III. Section Pachybasidium. Canadian. Journal of Botany, 69: 2357–237.

Bohn, G.W., Tucker, C.M. (1939). Immunity to Fusarium wilt in the tomato. Science, 89: 603–604.

Bohs, L. and Olmstead R. (1997). Phylogenetic relationships in S. (Solanaceae) based on ndhF sequences, Systematic Botany. 22:5–17.

Cao, L., Qiu, Z., Dai, X., Tan, H., Lin, Y., Zhou, S. (2004). Isolation of endophytic actinomycetes from roots and leaves of banana (*Musa acuminata*) plants and their activities against *Fusarium oxysporum* f. sp. *cubense*. World Journal of Microbiology & Biotechnology 20: 501–504.

Cao, L., Qiu, Z., You, J., Tan, H., Zhou, S. (2005). Isolation and characterization of endophytic streptomycete antagonists of Fusarium wilt pathogen from surface-sterilized banana roots. FEMS Microbiology Letters 247:147–152

Chellemi Do, Dankers, H.A. (1992). First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on tomato in north west Florida and Georgia. Plant Disease, 76: 861.

Chetelat, R.T. and Y. Ji (2006). Cytogenetics and evolution, in: Razdan, M. and A.K. Matoo (eds.), Genetics Improvement of Solanaceous Crops. Tomato, Science Publishers, New Dehli, India 2: 120

Chin-A-Woeng, T.F.C., Bloemberg, G.V., Vander Bij, A.J., Vander Drift, K.M.G.M., Schripsema, J., Kroon, B., Scheffer, R.J., Keel, C. (1998). Biocontrol by phenazine-1- carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici*. Mol Plant Microbe Interact 11: 1069–1077.

Coll-Hurtado, A. and M. de L. Godínez Calderón (2003). La agricultura en México: Un atlas en blanco y negro, Colección Temas Selectos de Geografía en México, Instituto de Geografía, UNAM, Mexico City.

Conrath U., Pieterse C.M.J., Mauch-Mani B. (2002). Priming in plant-pathogen interactions. Trends in Plant Science, 7: 210–216.

Cotxarrera L, Trillas-Gay MI, Steinberg C, Alabouvette C. (2002). Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biology and Biochemistry 34, 467–76.

Cox S. (2000) I Say Tomayto, You Say Tomahto, 2000 http://lamar.colostate.edu/~samcox/Tomato.html.

Crawford, D.L., Lynch, J.M., Whipps, J.M., Ousley, M.A., 1993. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Applied Environmental Microbiology 59, 3899–3905.

Czymmek K. J., Fogg M., Powell D. H., Sweigard J., Park S.-Y., Kang S. (2007). *In vivo* time-lapse documentation using confocal and multi-photon microscopy reveals the mechanisms of invasion into the Arabidopsis root vascular system by *Fusarium oxysporum*. Fungal Genet. Biol. 44: 1011–1023.

Davis, R.M.; Farrar, J.J. (1988). A third race of *Fusarium oxysporum* f.sp. *lycopersici* identified in California. Plant Disease, 72: 453.

de Freitas, J.R., Germida, J.J. (1991). *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizoctonia solani*. Canadian Journal of Microbiology 37: 780–784.

Di Menna M.E., Lauren D.R., Smith W.A. (1991): Effect of incubation temperature on zearalenone production by strains of *Fusarium crookwellense*. Mycopathologia, 116: 81–86.

Di Pietro, A., and Roncero, M.I. (1998) Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol Plant–Microbe Interact 11: 91–98.

Di Pietro A, Garcia-Maceira FI, Meglecz E, Roncero MIG (2001). A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Molecular Microbiology 39, 1140–1152

Di Pietro A., Madrid M.P., Caracuel Z., Delgado-Jarana J., Roncero M.I.G. (2003). *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology 4: 315–326

Dowling, D.N., O'Gara, F., 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends in Biotechnology 3, 121–141.

Dunlap, C., Delaney, I., Fenton, A., Lohrke. S., Moënne-Loccoz, Y., O'Gara, F., (1996). The biotechnology and application of *Pseudomonas* inoculants for the biocontrol of phytopathogens, 441– 448. In: Stacey, G., Mullin, B., Gresshoff, P.M., eds. Biology of plant microbe interactions. St Paul, MN, USA: International Society for Molecular Plant–Microbe Interactions.

Duyvesteijn R.G., van Wijk R., Boer Y., Rep M., Cornelissen B.J., and Haring, M.A. (2005). Frp1 is a *Fusarium oxysporum* F-box protein required for pathogenicity on tomato. Mol. Microbiol. 57: 1051–1063.

Edel-Hermann V., Gautheron N., Steinberg C. (2012). Genetic diversity of *Fusarium oxysporum* and related species pathogenic on tomato in Algeria and other mediterranean countries. Plant Pathol. 61: 787–800

Elango V., Manjukarunambika K., Ponmurugan P., Marimuthu S. (2015). Evaluation of *Streptomyces* spp. for effective management of Poria hypolateritia causing red root-rot disease in tea plants. Biological Control 89: 75–83.

EL Komy, M. H.; Saleh, A. A.; Eranthodi, A.; Molan, Y. Y. (2015). Characterization of novel *Trichoderma asperellum* isolates to select effective biocontrol agents against tomato Fusarium wilt. The Plant Pathology Journal, v. 31, n. 1, p. 50-60.

El-Kazzaz MK, El-Fadly GB, Hassan MAA, El-Kot GAN (2008). Identification of some Fusarium spp. using Molecular Biology Techniques, Egypt Journal of Phytopathology 36:57–69.

FAO. (2016). FAO stat. Annual Crop Production Statistics. Food and Agriculture Organization of the United Nations. Statistics Division. Retrieved from http://faostat3.fao.org/download/Q/QC/E

FAOSTAT. (2015). FAOSTAT Database on Agriculture. Retrieved from http://faostat3.fao.org/home

FAOSTAT (2017). “Production – Crops – Area harvested/ Production quantity – Tomatoes – 2014”, FAO Statistics online database, Food and Agriculture Organization, Rome, www.fao.org/faostat/en (accessed 22 Sept. 2017).

Farm Credit Canada (2012), “Update on the North American greenhouse vegetable industry”, Canada.

Fishal, E.M.M., Meon, S., Yun, W.M., 2010. Induction of Tolerance to Fusarium Wilt and Defense-Related Mechanisms in the Plantlets of Susceptible Berangan Banana PreInoculated with *Pseudomonas* sp. (UPMP3) and Burkholderia sp. (UPMB3). Agricultural Sciences in China 9, 1140–1149.

Foolad MR. 2007. Genome mapping and molecular breeding of tomato. Int J Plant Genom 2007.

Fletcher JT, Smewin BJ, Cook RTA (1988) Tomato powdery mildew. Plant Pathology 37, 594–598

Fry WE, Goodwin SB (1997) Re-emergence of potato and tomato late blight in the United States. Plant Disease 81, 1349–1357

Gawehns F., Houterman P.M., Ait Ichou F., Michielse C.B., Hijdra M., Cornelissen B.J.C., Rep M., and Takken F.L.W. (2014). The *Fusarium oxysporum* Effector Six6 Contributes to Virulence and Suppresses I2-Mediated Cell Death. MPMI 27: 336–348.

Gebhardt, C. et al. (1991), RFLP maps of potato and their alignment with the homologous tomato genome, Theoretical and Applied Genetics, Vol. 83: 49–57.

Geisenberg, C. and K. Stewart (1986). Field crop management, in Atherton, J.G. and J. Rudich (eds.), The Tomato Crop: A Scientific Basis for Improvement, Chapman & Hall, London, pp. 511–557.

Gerdemann, J.W. and Finely A.M. (1950). The pathogenicity of race 1 and 2 of *Fusarium oxysporum* f.sp. *lycopersici.* Phytopathology 41:238-244.

Getha, K., Vikineswary, S., (2002). Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f.sp. *cubense* race 4: Indirect evidence for the role of antibiosis in the antagonistic process. Journal of Industrial Microbiology & Biotechnology. 28, 303 – 310.

Getha K., Vikineswary, S., Wong, W., Seki, T., Ward, A., Goodfellow, M., (2005). Evaluation of Streptomyces sp. strain G10 for suppression of Fusarium wilt and rhizosphere colonization in pot grown banana plantlets. Journal of Industrial Microbiology and Biotechnology 32: 24-32.

Ghanem ME, Albacete A, Smigocki AC, Frébort I, Pospíšilová H, Martínez-Andújar C, et al. (2011). Root-synthesizedcytokinins improveshoot growth and fruityield in salinized tomato (*Solanum lycopersicum* L.) plants. J Exp Bot 2011;62: 125–4

González SRF, M A Martínez D. (2002). Dumping: El caso del jitomate, México 1996. Com. Socio econ. Estad. Inform. 6:1-30.

Goode, M.J. (1966). New race of tomato Fusarium wilt fungus. Arkansas Farm Research, 15:12.

Grattidge, R.; O'Brien, R.G. (1982). Occurrence of third race of Fusarium wilt of tomatoes in Queens-land. Plant Disease, 66:165-166.

Guerra-Cantera MARV, Raymundo, A.K., (2005). Utilization of a polyphasic approach in the taxonomic reassessment of antibiotic and enzyme-producing *Bacillus* spp. isolated from the Philippines. World. J. Microb. Biot., 21: 635–644

Harman G E, Howell C R, Viterbo A, Chet I and Lorito M. (2004). *Trichoderma* species–opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2:43-56.

Hartmann T (2007). From waste products to ecochemicals: fifty-year research of plant secondary metabolism. Phytochemistry 68:2831–2846.

Hermosa M. R., Keck E., Chamorro I., Rubio B., Sanz L., Vizcaíno J. A., *et al*. (2004). Genetic diversity shown in Trichoderma biocontrol isolates. Mycol. Res. 108, 897–906.

Hermosa R., Viterbo A., Chet I., Monte E. (2012). Plant-beneficial effects of Trichoderma and of its genes. Microbiology 158, 17–25.

Hirano Y., Arie T. (2006). PCR-based differentiation of *Fusarium oxysporum* f. sp. *lycopersici* and *radicis- lycopersici* and races of *F. oxysporum* f. sp. *lycopersici*. J. Gen. Plant Pathol. 72: 273–283.

Houterman P. M., Cornelissen B. J., Rep M. (2009). Suppression of plant resistance gene-based immunity by a fungal effector. PLoS Pathog. 4

Inami K, Kashiwa T, Kawabe M, Onokubo-Okabe A, Ishikawa N, Pérez R E, … Arie T (2014). The tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* shares common ancestors with nonpathogenic *F. oxysporum* isolated from wild tomatoes in the Peruvian Andes. Microbes and Environments 29:200–210.

Jelinski NA, Broz K, Jonkers W, Ma L-J, Kistler HC (2017). Effector gene suites in some soil isolates of *Fusarium oxysporum* are not sufficient predictors, of vascular wilt in tomato. Phytopathology 107:842–851.

Jenkins, J.A. (1948), "The origin of the cultivated tomato", Economic Botany: 2:79–392.

Ji, Y. and J.W. Scott (2006), "Tomato", in: Singh, R.J. (ed.), Genetic Resources, Chromosome Engineering, and Crop Improvement Series IV: Vegetable Crops, CRC Press, Boca Raton, Florida, 59–113.

Jiménez, C. y N. Sanabria. (2008). Población final de *Trichoderma harzianum* en el control de la marchitez vascular en tomate causado por *F. oxysporum* f. sp. *lycopersici*. Fitopatología Venezolana 21: 29–30.

Jiméneza M., MáÑez M., Hernández E. (1996): Influence of water activity and temperature on the production of zearalenone in corn by three Fusarium species. International Journal of Food Microbiology, 29: 417–421.

Jiménez-Fernández D, Montes-Borregob M, Navas Cortés J, Jiménez-Díaz R, Landab, BB (2010). Identification and quantification of *Fusarium oxysporum* in plants and soil by means of an improved specific and quantitative PCR assay, Applied Soil Ecology 46. 372–362.

Jones, J.P. and Woltz, S.S. (1981). Fusarium-incited diseases of tomato and potato and their control. In: Fusarium: Disease, Biology and Taxonomy. Nelson P.E. Toussoun. TA, Cook, R.J., (eds). Pennsylvania State University Park. PP 157-168.

Jones, J.P. (1966). Distribution of race 2 of *Fusarium oxysporum* f.sp. *lycopersici* in Florida. Plant Disease Report, 50:707-708.

Kamou, N.N., Karasali, H., Menexes, G., Kasiotis, K.M., Bon, M.C., Papadakis, E.N., Tzelepis, G.D., Lotos, L., Lagopodi, A.L. (2015). Isolation screening and characterization of local beneficial rhizobacteria based upon their ability to suppress the growth of *Fusarium oxysporum* f. sp. r*adices lycopersici* and tomato foot and root rot. Biocontrol Science and Technology. 25, 928-949.

Kapoor, I. J.1988 Fungi involved in tomato wilt syndrome in Delhi, Maharashtra and Tamilnadu. Indian Phytopathol. 41, 208–213.

Kawabe, M. et al. (2005) Three evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, based on sequences of IGS, MAT1, and pg1, are each composed of isolates of a single mating type and a single or closely related vegetative compatibility group. J. Gen. Plant Pathol. 71, 263–272.

Kirankumar, R., Jagadeesh, K. S., Krishnaraj, P. U. & Patil, M. S. (2008) Enhanced growth promotion of Tomato and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of *Tobacco Mosaic Virus* pathogen. J. Agric. Sci.21, 309–311.

Knapp S. (2002). Tobacco to tomatoes: a phylogenetic perspective on fruit diversity in the Solanaceae, Journal of Experimental Botany, 53: 2001–2022.

Knox O.G.G., Killham, K., Leifert, C., (2000). Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. Appl. Soil Ecol 15, 227–231.

Lahdenpera, M. L., Oy, K., (1987). The control of Fusarium wilt on carnation with a Streptomyces preparation. Acta Horticult 216, 85– 92.

Larry R, Joanne L. Razdan MK, Mattoo AK. (2007). Genetic resources of tomato, Genetic improvement of solanaceous crops, Enfield, NH Science Publishers Tomato vol 2.

Leelasuphakul, W., Sivanunsakul, P., Phongpaichit, S. (2006). Purification, characterization and synergistic activity of β-1,3- glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. Enzym. Microb. Technol 38: 990–997.

Leslie JF, Summerell BA (2006). The Fusarium Laboratory Manual. 369 p. Blackwell Publishing, Iowa, USA.

Lewis, J.A. and Papavizas, G.C. 1984. Chlamydospore formation by Trichoderma spp.in natural substrates. Can. J. Microbiol., 30: 1-7.

Li Q., Ning P., Zheng L., Huang J., Li G., Hsiang T. 2010. Fumigant activity of volatiles of *Streptomyces globisporus* JK-1 against *Penicillium italicum* on *Citrus microcarpa*. Postharvest Biology and Technology 58: 157–165

Lopez-Berges MS, Hera C, Sulyok M, Schafer K, Capilla J, Guarro J, Di Pietro A. (2013). The velvet complex governs mycotoxin production and virulence of *Fusarium oxysporum* on plant and mammalian hosts. Mol Microbiol.;87: 49–65.

Lorito, M., Woo, S. L., Harman, G. E. & Monte, E. (2010). Translational research on Trichoderma: from 'omics to the field. Annu Rev Phytopathol 48, 395–417.

Lugtenberg, B.J.J., de Weger, L.A., Bennett, J.W., (1991). Microbial stimulation of plant growth and protection from disease. Current Opinions in Biotechnology 2, 457–464.

Lugtenberg, B.J.J., Dekkers, L.C., Bansraj, M., Bloemberg, G.V., Camacho, M., Chin-AWoeng, T.F.C., van den Hondel, C., Kravchenko, L., Kuiper, I., Lagopodi, A.L., Mulders, I., Phoelich, C., Ram, A., Tikhonovich, I., Tuinman, S., Wijffelman, C., Wijfjes A., 1999b. Pseudomonas genes and traits involved in tomato root colonization. In: De Wit PJGM, Bisseling, T., Stiekema, W.J., eds 1999. IC-MPMI Congress Proceedings: biology of plant–microbe interactions, Vol. 2. St Paul, MN, USA: International Society for Molecular Plant–Microbe Interactions, 324–330.

Ma, L. J.; Geiser, D. M.; Proctor, R. H.; Rooney, A. P.; O'Donnell, K., Trail, F. y Kazan, K. (2014). Fusarium Pathogenomics. Annual review of microbiology, 67, 399–416.

Macedo R, Sales LP, Yoshida F, Silva-Abud LL, Lobo M Junior. 2017, Potential worldwide distribution of Fusarium dry root rot in common beans based on the optimal environment for disease occurrence. PLoS One;12(11)

Madania, A., M. Altawil, W. Naffaa, P.H. Volker, M. Hawat, (2013). Morphological and molecular characterization of Fusarium isolated from maize in Syria. Journal of Phytpathology 161: 452-458.

Mandal, S. and Mitra, A. (2007). Reinforcement of cell wall in roots of Lycopersicon esculentum through induction of phenolic compounds and lignin by elicitors. Physiological and Molecular Plant Pathology, 71: 201–209

Marllat, M.L.; Correl, J.C.; Kaufmann, P. and Copper, P.E. (1996). Two genetically distinct populations of *Fusarium oxysporum* f.sp. *lycopersici* race 3 in the United States. Plant Disease, 12:1342.

Massee, G. (1895). The “Sleepy disease” of tomatoes. Garden Chronicles Series 3, 17:707-708.

Miller, P. (1768) The Gardeners Dictionary, abridged 8th edition, London.

Ming Q, Su C, Zheng C, Jia M, Zhang Q, Zhang H, Qin L (2013). Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis. Journal of Experimental Botany 64:5687-5694.

Mithoefer A, Boland W. (2012). Plant defense against herbivores: Chemical aspects. Annual Review of Plant Biology 63, 431–450.

Mukherjee et al., P.K. Mukherjee, B.A. Horwitz, C.M. (2012b) Kenerley Secondary metabolism in Trichoderma–a genomic perspective Microbiology (Reading, England), 158, pp. 35-45

Mwangi, Margaret W., Monda, Ethel O., Okoth, Sheila A., & Jefwa, Joyce M. (2011). Inoculation of tomato seedlings with *Trichoderma harzianum* and Arbuscular Mycorrhizal Fungi and their effect on growth and control of wilt in tomato seedlings. Brazilian Journal of Microbiology, 42(2), 508-513.

Nesbitt TC, Tanksley SD. (2002) Comparative sequencing in the genus Lycopersicon: implication for the evolution of fruit size in the domestication of cultivated tomatoes, Genetics, vol. 162 (pg. 365-379)

Nieves-García, V., O. van der Valk and A. Elings (2011), Mexican Protected Horticulture, Wageningen University and Research, Netherlands.

Nuez, F. (2001), El Cultivo del Tomate, Ediciones Mundi-Prensa.

OECD (2008), “Consensus document on compositional considerations for new varieties of tomato: Key food and feed nutrients, toxicants and allergens”, Series on the Safety of Novel Foods and Feeds, No. 17, OECD, Paris, www.oecd.org/env/ehs/biotrack/46815296.pdf.

Ohara T, Inoue I, Namiki F, Kunoh H, Tsuge T. 2004. REN1 is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. Genetics 166: 113–124

Olmstead, R.G. and J.D. Palmer (1997), “Implications for the phylogeny, classification, and biogeography of Solanum from cpDNA restriction site variation”, Systematic Botany, 22; 19–29.

Panthee, D. R., & Chen, F. (2010). Genomics of fungal disease resistance in tomato. Current Genomics, 11(1), 30–39. https://doi.org/10.2174/138920210790217927

Papavizas, G.C., 1985. Trichoderma and Gliocladium: biology, ecology and potential for biocontrol. Annu Rev Phytopathol 23, 23–54.

Parke, J. L., & Grünwald, N. J. (2012). A Systems Approach for Management of Pests and Pathogens of Nursery Crops. Plant Disease, 96(9), 1236–1244. https://doi.org/10.1094/PDIS-11-11-0986-FE

Peng, H.X., Sivasithamparama, K., Turner, D.W. 1999. Chlamydospore germination and Fusarium wilt of banana plantlets in suppressive and conducive soils are affected by physical and chemical factors. Soil Biology and Biochemistry 31: 1363–1374.

Peralta IE, Spooner DM. Razdan MK, Mattoo AK. (2007). History, origin and early cultivation of tomato (Solanaceae), Genetic improvement of solanaceous crops, Enfield, NH Science Publishers, 2:1-27

Peralta IE, Knapp S, Spooner DM (2006). Nomenclature for wild and cultivated tomatoes, Tomato Genetics Cooperative Report, vol. 56 (pg. 6-12)

Peralta, I.E., D.M. Spooner and S. Knapp (2008), Taxonomy of Wild Tomatoes and Their Telatives (Solanum sect. Lycopersicoides, sect. Juglandifolia,

sect. Lycopersicon; Solanaceae), Systematic Botany Monographs, The American Society of Plant Taxonomists, Vol. 84, pp. 186.

Pieterse, C.M.J., van Pelt J.A., van Wees S.C.M., Ton, J., Leon-Kloosterziel K.M., Keurentjes J.J.B., Verhagen B.W.M., van Knoester, M,, DSI, Bakker, P.A.H.M., van Loon, L.C., 2001. Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. European Journal of Plant Pathology 107,51–61.

Pinaria AG, Liew E, Burgess LW, 2010. Fusarium species associated with vanilla stem rot in Indonesia. Australasian Plant Pathology 39, 176– 83.

Porte, W.S., Walker, H.B. (1941). The pan American tomato, a new red variety highly resistant to Fusarium wilt, United States. Department of Agricultural Circular, 611:

Raguchander, T., Jayashree, K., Samiyappan, R., 1997. Management of Fusarium wilt of banana using antagonistic microorganisms. Journal of Biological Control 11, 101– 105.

Recorbet, G. & Alabouvette, C. (1997) Adhesion of *Fusarium oxysporum* conidia to tomato roots. Letters in Applied Microbiology 25: 375-379

Reddi, G. S., Rao. A. S., 1971. Antagonism of soil actinomycetes to some soil - borne plant pathogenic fungi. Indian Phytopathol 24, 649–657.

Rejeb IB, Pastor V, Mauch-Mani B (2014). Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. Plants 3(4):458-475.

Rhodes, D.J., Powell, K.A., 1994. Biological seed treatments – the development process. BCPC Monograph 57, 303–310.

Rick, C.M. (1979), “Biosystematic studies in Lycopersicon and closely related species of Solanum”, in: Hawkes, J., R. Lester and A. Skelding (eds.), The Biology and Taxonomy of the Solanaceae, Academic Press, New York, pp. 667–697.

Rick, C.M. (1978), “The tomato”, Scientific American, 239: 77–87.

Rodrigues AAC, Menezes M (2005). Identification and pathogenic characterization of endophytic Fusarium species from cowpea seeds. Mycopathologia. 159:79–85.

Sakthivel, N., Gnanamanickam, S.S., 1987. Evaluation of Pseudomonas fluorescens for suppression of sheath rot disease and for the enhancement of grain yields in rice (Oryza sativa L.). Applied Environmental Microbiology 53, 2056–2059.

Sánchez-Peña, P. et al. (2004), “Sources of resistance to whitefly (*Bemisia* spp.) in wild populations of *S. lycopersicum* var. *cerasiforme* (Dunal) Spooner G.J. Anderson et R.K. Jansen in Northwestern Mexico”, Genetic Resources and Crop Evolution, 53:711-719.

Sands, D.C., Rovira, A.D., (1971). *Pseudomonas fluorescens* biotype G, the dominant fluorescent pseudomonas in South Australian soils and wheat rhizospheres. Journal of Applied Bacteriology 34, 261–275.

Sato, R. and Araki, T. (1974): On the tomato root rot disease occurring under vinyl house conditions in southern Hokkaido. Ann. Rep. Soc. Plant Prot. North Jpn., 25:5-13.

Sims WL (1980). History of tomato production for industry around the world, Acta Horticulturae, vol. 100 (pg. 25-26)

Singha MI, Kakoty Y, Unni GB, Das J, Kalita CM (2016). Identification and characterization of *Fusarium* sp. using ITS and RAPD causing Fusarium wilt of tomato isolated from Assam, North East India. Journal of Genetic Engineering and Biotechnology 14:99–105.

Sivamani, E., Gnanamanickam, S. S., 1988. Biological control of *Fusarium oxysporum* f.sp. *cubense* in banana by inoculation with *Pseudomonas fluorescens*. Plant Soil 107, 3 9

Smith, J., Putnam, A., Nair, M. (1990). In vitro control of Fusarium diseases of *Asparagus officinalis* L. with a Streptomyces or its polyene antibiotic, faeriefungin. J Agric Food Chem 38, 1729–1733.

Spooner, D.M. et al. (2003), “Plant nomenclature and taxonomy – an horticultural and agronomic perspective”, Horticultural Reviews, Vol. 28, pp. 1-60.

Spooner, D.M., I.E. Peralta and S. Knapp (2005), “Comparison of AFLPs with other markers for phylogenetic inference in wild tomatoes [*Solanum* L. section *lycopersicon* (Mill.) Wettst.]”, Taxon, Vol. 54/1, pp. 43-61.

Spooner, D., G. Anderson and R. Jansen (1993), “Chloroplast DNA evidence for the interrelationships of tomatoes, potatoes and pepino (Solanaceae)”, American Journal of Botany, Vol. 80/6, pp. 676-698.

Srinivasan, U., Staines, H. J., Bruce, A.,1992. Influence of media type on antagonistic modes of *Trichoderma* spp. against wood decay basidiomycetes, Mater. Org. 27, 301–321.

Srobar, S. (1978) The influence of temperature and pH on the growth of mycelium of the causative agents of Fusarioses in wheat in Slovakia Czechoslovakia. UVTI (Ustav Vedeckotechnickych Informaci) Ochrana Rostlin 14: 269-274

Stall, R.E. (1961). Development of Fusarium wilt resistant varieties of tomato caused by strain different from race 1 isolates of *Fusarium oxysporum* f.sp. *lycopersici*. Plant disease Report, 45:12-15.

Strobel, J.W. (1969). A determinate tomato resistant to race 1 and 2 of the Fusarium wilt pathogen. Fla. Agr. Exp. Sta. Cric. S 202.

Sudhamoy, M., Nitupama, M. & Adinpunya, M. (2009) Salicylic acid induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Plant Physiol. Biochem. 47, 642–649

Sun, J.B., Peng, M., Wang, Y.G., Zhao P.J., Xia Q.Y., 2011. Isolation and characterization of antagonistic bacteria against Fusarium wilt and induction of defense related enzymes in banana. African Journal of Microbiology Research 5, 509-515.

Taghdi Y., Hermosa R., Domínguez S., Rubio M. B., Essalmani H., Nicolas C., et al. (2015). Effectiveness of composts and *Trichoderma* strains for control of Fusarium wilt of tomato. Phytopathol. Mediterr. 54, 232–240.

Takken F, Rep M (2010). The arms race between tomato and Fusarium oxysporum. Molecular Plant Pathology 11:309–314.

Thangavelu, R., and Mustaffa M.M. (2010). A Potential isolate of *Trichoderma viride* NRCB1and its mass production for the effective management of Fusarium wilt disease in banana. Tree and Forestry Science and Biotechnology 4; 76-84.

Thangavelu, R., Palaniswami, A., Velazhahan, R., (2004). Mass production of *Trichoderma harzianum* for managing Fusarium wilt of banana. Agriculture, Ecosytems and Environment 103, 259–263.

Thangavelu, R. 2002. Characterization of Fsarium oxysporum schlecht. f.sp. cubense (e.f. smith) snyd. & hans. and Molecular Approaches for the Management of Fusarium Wilt of Banana. Ph.D. thesis. Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India, 254

Thornton, P., & Cramer, L. (2012). Impacts of climate change on the agricultural and aquatic systems and natural resources within the CGIAR's mandate. CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS), CCAFS Work, 23: 1–201.

Ting, A.S.Y., Mah, S.W., Tee, C.S., (2011). Detection of potential volatile inhibitory compounds produced by endobacteria with biocontrol properties towards Fusarium oxysporum f. sp. cubense race 4. World J Microbiol Biotechnol. 27, 229–235.

Tokeshi, H., Galli, F.; Kurozawa, C. (1966). A new race of tomato Fusarium in Sao Paulo (translate little). Anais Esc. Sup-Agr. "Luiz Queiroz" 23:217- 227.

Trusov, Y., Rookes, J.E., Chakravorty, D., Armour, D., Schenk, P.M., and Botella, J.R. (2006). Heterotrimeric G-proteins facilitate Arabidopsis resistance to necrotrophic pathogens and are involved in jasmonate signaling. Plant Physiol. 140 210–220.

Valdes, V.M. and D. Gray (1998), "The influence of stage of fruit maturation on seed quality in tomato (*Lycopersicon lycopersicum* [L.] Karsten)", Seed Science and Technology, Vol. 26/2, pp. 309-318

Valenzuela-Ureta, J.G., Lawn D.A., Heisey, R.F., Zamudio Guzman V. (1996). First report of Fusarium wilt race 3, caused by *Fusarium oxysporum* f.sp. *lycopersici* of tomato in Mexico. Plant Disease, 80:105.

Van loon, L.C., Bakker, P.A., Pieterse, C.M., (1998). Systemic resistance induced by rhizospere bacteria. Annu. Rev. Phytopathol 36, 453-483.

Vaughan, J.G. and C.A. Geissler (1997), The New Oxford Book of Food Plants, Oxford University Press.

Verma M., Brar S. K., Tyag, R.D., Surampalli, R.Y., Valero, J.R. 2007. Review: Antagonistic fungi, Trichoderma spp.: Panoply of biological control. Biochemical Engineering Journal 37: 1-20

Vigier B., Reid L.M., Seifert K.A. (1997): Distribution and prediction of Fusarium species associated with maize ear rot in Ontario. Canadian Journal of Plant Pathology, 19: 60–65.

Villarreal, R.L. (1982), Tomates, Instituto Interamericano de Cooperación para la Agricultura, San José, Costa Rica.

Volin, R.B.; Jones, J.P. (1982). A new race of Fusarium wilt of tomato in Florida and sources of resistance. Proceedings Florida State. Horticultural Society, 95:268-270.

Walker, J.C. (1971). Fusarium wilt of tomato. Monograph No. 6. St Paul Minnesota, U.S.A. American Phytopathological Society.

Weese TL, Bohs L. (2007). A three-gene phylogeny of the genus Solanum (Solanaceae). Syst Bot; 32:445–63

Weindling, R. 1941. Experimental consideration of the mold toxin of *Gliocladium* and *Trichoderma*. Phytopathology 31, 991-1003

Wulff, E.G, Sorensen J.L, Lubeck M., Nielsen K.F., Thrane U., Torp J. (2010). Fusarium spp. associated with rice bakanae: ecology, genetic diversity, pathogenicity and toxigenecity. Environmental Microbiology 12: 649–657.

Zhang, X., G. Hegerl, F. W. Zwiers, and J. Kenyon (2005), Avoiding inhomogeneity in percentile based indices of temperature extremes, J. Clim., 18, 1641–1651.

2. OCCURRENCE, IDENTIFICATION, AND PATHOGENICITY OF *Fusarium* SPP. ASSOCIATED WITH TOMATO WILT IN MEXICO.

2.1. ABSTRACT

Fusarium wilt is considered as one of the most important diseases that affects tomato (*Solanum lycopersicum* L.) cultivation. The aim of this study was to identify and characterize Fusarium species with the potential to cause tomato wilt using morphological and molecular approaches, to generate the necessary information to achieve adequate control of this disease. Fusarium isolates were found associated with commercial grown cultivars with disease incidence ranging from 10 to 85%. Forty isolates were identified by morphological characteristics as *Fusarium oxysporum* (38) and as *Fusarium* sp. (2). The isolates were evaluated for their pathogenicity on healthy tomato seedlings, which presented root rot at 20–35 days after inoculation. Fifteen of the most pathogenic isolates were analyzed with the internal transcribed spacer (ITS) region of DNA and the partial sequence of the translation elongation factor 1α (EF-1α). Isolates associated with tomato wilt were identified molecularly as *Fusarium oxysporum* (13), *Fusarium circinatum* (1), and *Fusarium andiyazi* (1). Both analysis revealed that the primary agent of tomato wilt in Mexico was *F. oxysporum*. This finding provides relevant information on tomato wilt in Mexico to decide the proper control methods for the pathogen.

Keywords: characterization, *Fusarium oxysporum* complex, genotypes, ITS and EF-1α, phylogenetic tree, tomato.

2.2. RESUMEN

La marchitez causada por *Fusarium* spp. es considerada como una de las enfermedades más importantes que afecta el cultivo del tomate (*Solanum lycopersicum* L.). El objetivo de este estudio fue identificar y caracterizar las especies de *Fusarium* con el potencial de causar la marchitez del tomate mediante técnicas morfológicos y moleculares, a fin de generar información necesaria para lograr un control eficaz de esta enfermedad. Los aislamientos de Fusarium se encontraron asociados con cultivares comercialmente cultivados provocando incidencias que varía entre 10 y 85%. Cuarenta aislamientos fueron identificados por características morfológicas como *Fusarium oxysporum* (38) y como *Fusarium* sp. (2.) Los aislamientos se evaluaron por su patogenicidad en plántulas de tomate sanas, que presentaron pudricion de la raíz a los 20-35 días después de la inoculación. Quince de los aislados más patógenos se analizaron mediante el uso de secuenciación con los marcadores ITS (espacio interno transcripto) y EF-1α TEF (Factor de elongación de la traducción). Los aislados asociados con la marchitez del tomate se identificaron molecularmente como *Fusarium oxysporum* (13), *Fusarium circinatum* (1) y *Fusarium andiyazi* (1). Ambos análisis revelaron que el agente principal de la marchitez del tomate en México es *F. oxysporum*. Este hallazgo proporciona información relevante sobre la marchitez del tomate en México para decidir que métodos son más apropiados para el control patógeno.

Palabras clave: caracterización, complejo de *Fusarium oxysporum*, genotipos, ITS y EF-1α, árbol filogenético, tomate.

2.3. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a high-value horticultural crop worldwide. The overall tomato production estimates 161 million ton and total production area of 8.5 million ha, in 2015. Mexico has shown remarkable development in tomato production and has been recognized as the second largest tomato exporter (FAOSTAT 2015). However, tomato production has been tremendously limited by a high number of biotic and abiotic factors (Parke and Grünwald, 2012), among them, fungal soil-borne pathogens such as *Fusarium oxysporum* f. sp. *radicis-lycopersici*, first observed in 1969 in Japan (Sato and Araki, 1974), *and Fusarium oxysporum* f. sp. *lycopersici*, described over 100 years ago in the UK (Massee, 1895), causes tomato wilting (Inami *et al*., 2014), resulting in low yields and high economic losses(Arie *et al*., 2007; Panthee and Chen, 2010), exceeding 50 % in production systems in Mexico (Apodaca *et al*., 2004).

Adequate identification of this pathogen is necessary to apply appropriate measures to reduce its negative effects (Takken and Rep, 2010). Morphological characteristics based on shape and size microconidia and macroconidia, phialides and the formation of chlamydospores (Rodrigues and Menezes, 2005), are the main form of identification and taxonomic description of Fusarium species (Leslie and Summerell, 2006). Additionally, modern molecular methods such as PCR and DNA sequence analysis of internal transcribed spacer (ITS) region and the translation elongation factor 1α (EF-1α), are faster and more reliable to obtain proper identification (El-Kazzaz *et al*., 2008; Singha, 2016).

Hence, the objectives of the present study were to determine the occurrence and incidence of Fusarium isolates in field and greenhouse conditions, in the central growing region of Mexico. Identify and characterize selected Fusarium isolates which causes tomato wilting, using morphological characteristics and sequence analysis of internal transcribed spacer (ITS) region of rDNA and the translation elongation factor 1α (EF-1α), and to verify the pathogenicity in four commercial tomato varieties. Despite some studies which have addressed these problems (Leyva-Mir *et al*., 2013), scarce information is available concerning occurrence,

growth rates and phylogenetic relation of this pathogens found in fields and greenhouses production in Mexico. Thus, the finding in this study would provide relevant information on *Fusarium* species that affects tomato production in Mexico, to help decide the proper control methods to improve fruit yields.

2.4. MATERIALS AND METHODS

2.4.1. Sampling of plant tissue and isolation of fungi

In 2014 and 2015, samples of tomato plants showing symptoms of wilting and chlorosis were obtained from a total of 12 different geographical locations in central Mexico, distributed in the states of Morelos, Puebla, and Tlaxcala (Figure 1). Symptomatic fragments were taken from the infected parts of the plant showing necrosis, dark brown and/or reddish coloration, sterilized in a 5 % sodium hypochlorite solution, rinsed with sterilized water and placed on potato dextrose agar (PDA) (Difco, USA) supplemented with 0.5 gL^{-1} of streptomycin sulphate (Sigma-Aldrich, USA) and 1 ml L^{-1} of lactic acid. The Petri dishes were incubated at 26 °C for 3–5 days. Fungal colonies were transferred to fresh PDA medium and one colony per sample was re-isolated using a single spore (Leslie and Summerell, 2006). Forty of the isolates were stored in glycerol at 15% in cryogenic tubes at -80°C for further investigations. The isolates used in this study were deposited in the Culture Collection of Phytopathogenic Fungi at the Universidad Autónoma Chapingo as UACH–202 to UACH–241. The disease incidence (%) of stem and root infections was calculated as the ratio of total number of infected plants divided by the total number of examined plants, multiplied by 100.

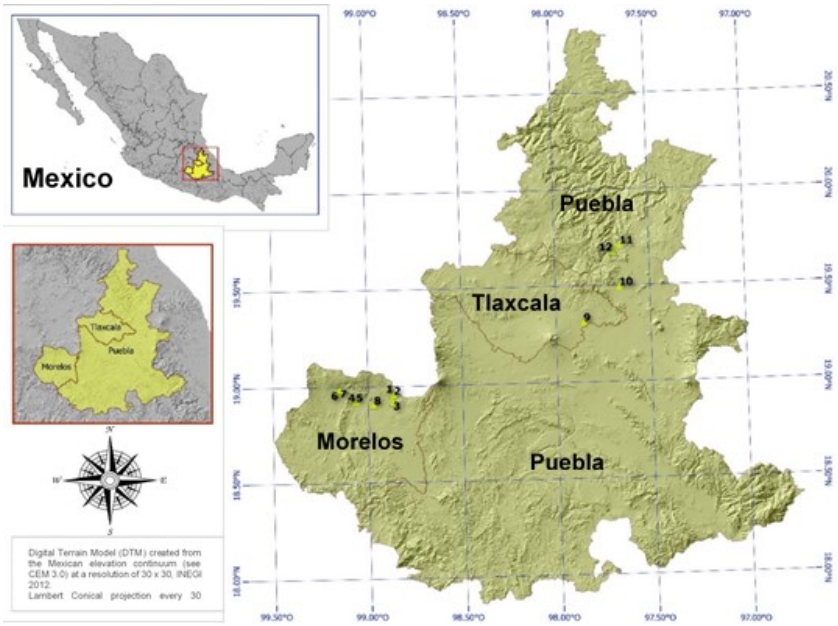


Figure 1 Tomato growing areas sampled in central Mexico (Morelos, Puebla and Tlaxcala), in the growing season of 2014 and 2015.

2.4.2. Morphological characterization

For the morphological identification, pure cultures obtained from a single spore of each isolate were grown on PDA to examine the mycelium growth and appearance (Leslie and Summerell, 2006).To differentiate among isolates the presence or absence of chlamydospores, formation of sporodochia, width and length(µm) of fifty random macroconidia and one hundred microconidia, were measured with the Motic Image Plus v. 4.0 (Motic Group®,China). Isolates were grown on PDA and carnation leaf agar (CLA) medium. Wet chambers were used to observe the development of monophialides and/or polyphialides (Rodrigues and Menezes, 2005). Species of *Fusarium* were identified using the keys and descriptions reported by Booth (1971), Nelson *et al*. (1983), and Leslie and Summerell (2006). For characterization on culture medium, a 5-mm disc in diameter of each single spore isolate previously grown for 5-day-old on PDA medium, were placed at the center of each Petri dish and were incubated at 26 ± 2 °C under darkness.

2.4.3. Pathogenicity test

The pathogenicity of 40 *Fusarium* isolates was verified on healthy 35-days-old tomato seedlings of four commercial tomato cultivars (Riogrande, Moctezuma F_1, Floradade, and DRW 7744 F_1) under greenhouse conditions. Conidial suspension of each fungal isolate was prepared using sterile distilled water and conidia of 7-days-old colonies to inoculate each plant. The concentration of the conidial suspension was adjusted to 10^6 spores·ml^{-1} including macroconidia and microconidia, using a hemocytometer. The inoculated plants were transplanted in styrofoam cups (1L) as described by Shahnazi *et al*. (2012). Data collection were carried out at 5, 10, 15, 20, 25, 30, and 35 days after inoculation (DAI). To determine the severity a visual scale was designed based on the percentage of affected plants, where 1= no symptoms; 2= initial symptoms or 10-20% chlorosis of leaves; 3= 20-50% chlorosis of leaves; 4= >50% chlorosis of leaves and initial symptoms flaccidity of the top leaves; 5= completely or the major part of the plant wilted or death.

Dry weight of the plants following oven drying at 80 °C for 48 h, along with internal lesion was registered. The experimental design of pathogenicity test was a completely randomized block with four replicates. The experimental unit was five styrofoam cups. Area under the disease progress curve (AUDPC) was calculated according to Campbell and Madden (1990). The AUDPC values were used to classify the level of pathogenicity among the different *Fusarium* species. Analysis of variance (ANOVA) of the data was performed with the SAS v. 9.3 (2012). The mean values of the treatments were compared using the Tukey test ($p \leq 0.05$).

2.4.4. Radial growth rate

The radial growth (cm) of the fungal isolates was recorded in 24 h intervals during seven days. The experiment was performed twice, each one with three replicates. The radial growth of the isolates was estimated with the respective slopes of the simple linear regressions, obtained using the time (days) as independent variable and radial growth as the dependent variable (Lamrani, 2009). The radial growth

was classified as: fast ($\beta_1 > 12$), moderate ($7 \leq \beta_1 \leq 12$) and slow growth ($\beta_1 < 7$). The final radial growth measurement and area under disease progress curve (AUDPC), were calculated and submitted to an analysis of variance procedure. The mean values of the isolates were compared with the Tukey test ($P \leq 0.05$).

2.4.5. DNA extraction, PCR amplification and sequencing

DNA extraction was performed only for 15 isolates with seven-day-old mycelial colony growth on PDA medium in Petri dishes at 26 °C. Mycelium and conidia of each isolate was scrapped and ground to a fine powder under liquid nitrogen using a mortar and pestle. DNA extraction was done by using The DNeasy Plant Mini Kit (Qiagen®, USA) according to the manufacturer's instructions. DNA was visualized on 1 % agarose gels, with TAE 1 X buffer and 5 µL of the PCR product at 90 Volts. The gel was analysed in a Transilluminator Gel Doc-It TM 300 (UVP®, UK) and stored at -20 °C. For *Fusarium* species identification, the internal transcribed spacer (ITS) region of DNA was amplified using the primers ITS5/ITS4 (White *et al*., 1990), and the partial sequence of the translation elongation factor 1α (EF-1α) gene using the primers EF1-728F/EF1-986R (O'Donnell *et al*., 1998). PCR products were prepared to a final volume of 50 µL, containing PCR buffer (1x), 2.5 µL $MgCl_2$, 0.2 mM of 1 µL dNTP, 0.8 µL of each primer, 1U of DNA polymerase (Promega®, USA) and 100 ng of DNA and was carried out in a C-1000TM Thermal Cycler (Biorad®, USA). The amplified products were sent to MACROGEN (Korea) for sequencing.

2.4.6. Phylogenetic analysis

The phylogeny was reconstructed by analyses from sequences of the ITS region and EF-1α gene. Forward and reverse sequences were assembled using the Staden Package (Staden *et al*., 1998). Sequences generated in this study were supplemented with additional sequences obtained from GenBank, based on BLASTn searches and the literature. Sequences of the isolates were aligned using ClustalX v. 1.81 (Thompson *et al*., 1997) and manual adjusted. Individual

alignments of ITS and EF-1α dataset were analysed with Maximum Likelihood and Maximum Parsimony using MEGA 5 (Tamura *et al*., 2011) with partial deletion of gaps, substitution models proposed by this program and 1000 bootstrap replicates. The obtained sequences were deposited in the NCBI database.

Table 1. List of isolates used in this study obtained from tomato plants (*Solanum lycopersicum* L.)

Species	Isolate	Locality	GenBank	
			ITS	EF1-a
Fusarium oxysporum f. sp. *melonis*	UACH-217	Atlatlahucan, Morelos	MG557869	MG557870
Fusarium circinatum	UACH-218	Almincingo, Morelos	MG557868	MG557871
Fusarium oxysporum	UACH-219	Huamantla, Tlaxcala	MG557867	MG557872
Fusarium oxysporum f. sp. *melonis*	UACH-220	Huamantla, Tlaxcala	MG557866	MG557873
Fusarium oxysporum f. sp. *melonis*	UACH-221	Almincingo, Morelos	MG557865	MG557874
Fusarium oxysporum f. sp. *passiflorae*	UACH-222	Santa Catarina, Morelos	MG557864	MG557875
Fusarium oxysporum f. sp. *melonis*	UACH-223	Huamantla, Tlaxcala	MG557863	MG557876
Fusarium andiyazi	UACH-224	Apanquetzalco, Morelos	MG557862	MG557877
Fusarium oxysporum f. sp. *asparagi*	UACH-225	Apanquetzalco, Morelos	MG557861	MG557878
Fusarium oxysporum f. sp. *melonis*	UACH-226	Apanquetzalco, Morelos	MG557860	MG557879
Fusarium oxysporum	UACH-227	Apanquetzalco, Morelos	MG557859	MG557880
Fusarium oxysporum f. sp. *melonis*	UACH-228	Apanquetzalco, Morelos	MG557858	MG557881
Fusarium oxysporum f. sp. *melonis*	UACH-229	Libres, Puebla	MG557857	MG557882
Fusarium oxysporum f. sp. *passiflorae*	UACH-230	Libres, Puebla	MG557856	MG557883
Fusarium oxysporum f. sp. *cubense*	UACH-231	Atlatlahucan, Morelos	MG557855	MG557884

2.5. RESULTS AND DISCUSSION

2.5.1. Field and greenhouse sampling

Fusarium wilting was observed in the 120 samples (Figure 1) obtained from tomato plants (two to five months old) of twelve locations in three states of Mexico

(Fig. 2a-b) in field and greenhouse conditions; typical symptoms were more prominent in the field. The disease incidence varied from 10-85%; the highest was observed in Apanquetzalco, Morelos (82%), grown in the field. Meanwhile, the lowest was observed in a greenhouse located in Tlaxcala, with 12%. Pony Express was the most affected cultivar followed by Tisey-DRD8551, Serengheti, and Reserva, with intermediate incidence; while El Cid and Moctezuma were less affected (Table 1). Those results were similar to those reported by Hernández-Martinez *et al*. (2014), indicating that Fusarium wilt is one of the most devastating disease, resulting in 40-70% in economic losses around the world.

The severity and incidence of this pathogen is conditioned according to the geographical location, climatic factors, cultural practices (Daami-Remadi, 2006), and physiology of the host plant (Tivoli *et al*., 1986). According to Nirmaladevi *et al*. (2016) *Fusarium* wilt occurs in most cultivated soils and is isolated mainly from contaminated plants tissues. Many of these plants grown in these soils may become infected to some degree during their life cycle, suggesting their potential involvement in the spread and incidence of the disease (Rajput *et al*., 2008). Our results confirm that observation. The effect of *Fusarium* wilt is most apparent during the flowering stage when the plant and its productivity are more sensitive to stress (Gargouri-Kammoun *et al*., 2009; Panthee & Chen, 2010; Siddique *et al*., 2014).

The fields where tomato plants were cultivated are often subjected to crop rotation of other Gramineae (millet, corn, sorghum, rice and sugarcane), and Solanaceous crops (potato, pepper, and eggplant), suggesting the source of transmission of *Fusarium* spp. (Trabelsi *et al*., 2017). Thus, these crops can increase the pathogen population in the soil in a very efficient way (Cai *et al*., 2011). However, wilting caused by *Fusarium* spp. in Mexico has not been fully documented, despite its significant impact on commercial production and yields of tomato plants. This could be explained because Mexico is the centre of domestication for tomato, indicating the widespread variability of different tomato cultivars (Marin-Montes *et al*., 2016), therefore, increasing the distribution, variability and evolution of *Fusarium* spp. (Inami *et al*., 2014).

Table 2. Mean incidence of tomato (*Solanum lycopersicum* L.) wilting in different field and greenhouse systems in Central Mexico.

State	Place of sample collection	GPS Coordinates	Altitude (msl)	Production System type	Plant age (months)	Tomato cultivar	Tomato growth type	% Mean incidence
Morelos	Atlatlahucan	20.971°N 51.372°E	1817	Field	2.5	Serengheti	Determinate	27 b
Morelos	Atlatlahucan	20.959°N 51.419°E	1785	Field	2	Pony Express	Determinate	22 c
Morelos	Yecapixtla	20.917°N 51.388°E	1774	Field	3.5	Tisey-DRD 8551	Determinate	20.83 c
Morelos	Almincingo	20.922°N 49.281°E	1254	Field	4	Riogrande	Determinate	46.67 ab
Morelos	Apanquetzalco	20.923°N 49.325°E	1250	Field	3	Pony Express	Determinate	82 a
Morelos	Santa Catarina	20.981°N 48.245°E	1676	Field	3	Tisey-DRD 8551	Determinate	26 b
Morelos	Santa Catarina	20.993°N 48.451°E	1746	Field	3	Pony Express	Determinate	29.1 b
Morelos	Oaxtepec	20.904°N 50.312°E	1250	Field	3	Pony Express	Determinate	39.44 b
Tlaxcala	Huamantla	21.360°N 62.026°E	2422	Greenhouse	4	El Cid	Indeterminate	12 e
Puebla	Libres	21.562°N 64.044°E	2376	Greenhouse	5	Moctezuma	Indeterminate	27 b
Puebla	San Miguel	21.802°N 64.107°E	2107	Greenhouse	4	El Cid	Indeterminate	18 d
Puebla	Zautla	21.761°N 63.703°E	2037	Greenhouse	4	Reserva	Indeterminate	29 b

Different letters in each column indicates significant difference (Tukey, $P<0.05$).
Msl: meters above sea level

2.5.2. Morphological characteristics and growth rate of the *Fusarium* species

The *Fusarium* isolates exhibited colonies on PDA medium that were red, white, purple, brown and pink in colour. The colonies showed diversity according to the morphological characteristics based on macroconidia, microconidia, clamydospores and phialides (Figure 2 e-l), described by Nelson *et al*. (1983) and Leslie and Summerell (2006). Thirty-eight isolates were classified as *Fusarium oxysporum* and two as *Fusarium* sp. (Isolates 1 and 2) which varied in shape and size and were similar to those defined by Zainudin *et al*. (2010).

Mycelia of *Fusarium oxysporum* isolates were sparse to abundant, while the colours ranged from white to pink, and often pale violet. Macroconidia presented were also sparse to abundant, are formed from monophialides on branched conidiophores or on the surface of sporodochia (Figure 2d), ranging from orange to brown. Three-septate macroconidia were most common although four thin walled septate were also presented. The macroconidia measured 26.5 to 36.1 × 2.6 to 4.6 µm. The microconidia presented 0-septate, oval, elliptical or reniform (kidney-shaped), and were formed abundantly in false heads on short monophialides. The microconidia measured 3.4 to 12 × 2 to 3.3 µm. Chlamydospores were formed abundantly in hyphae, singly or in pairs, terminally or on an intercalary basis presenting both smooth and rough walls. In carnation leaf agar (CLA) medium some of the isolates presented chlamydospores after 21 days. The *Fusarium oxysporum* isolates exhibited a high level of diversity presenting a wide range of colours and morphological structures. All 38 isolates were consistent with reports by Leslie and Summerell (2006). This species is considered as the principal causal agent of tomato wilt and *Fusarium* crown rot (Tanyolac and Akkale, 2010) in vegetable plants.

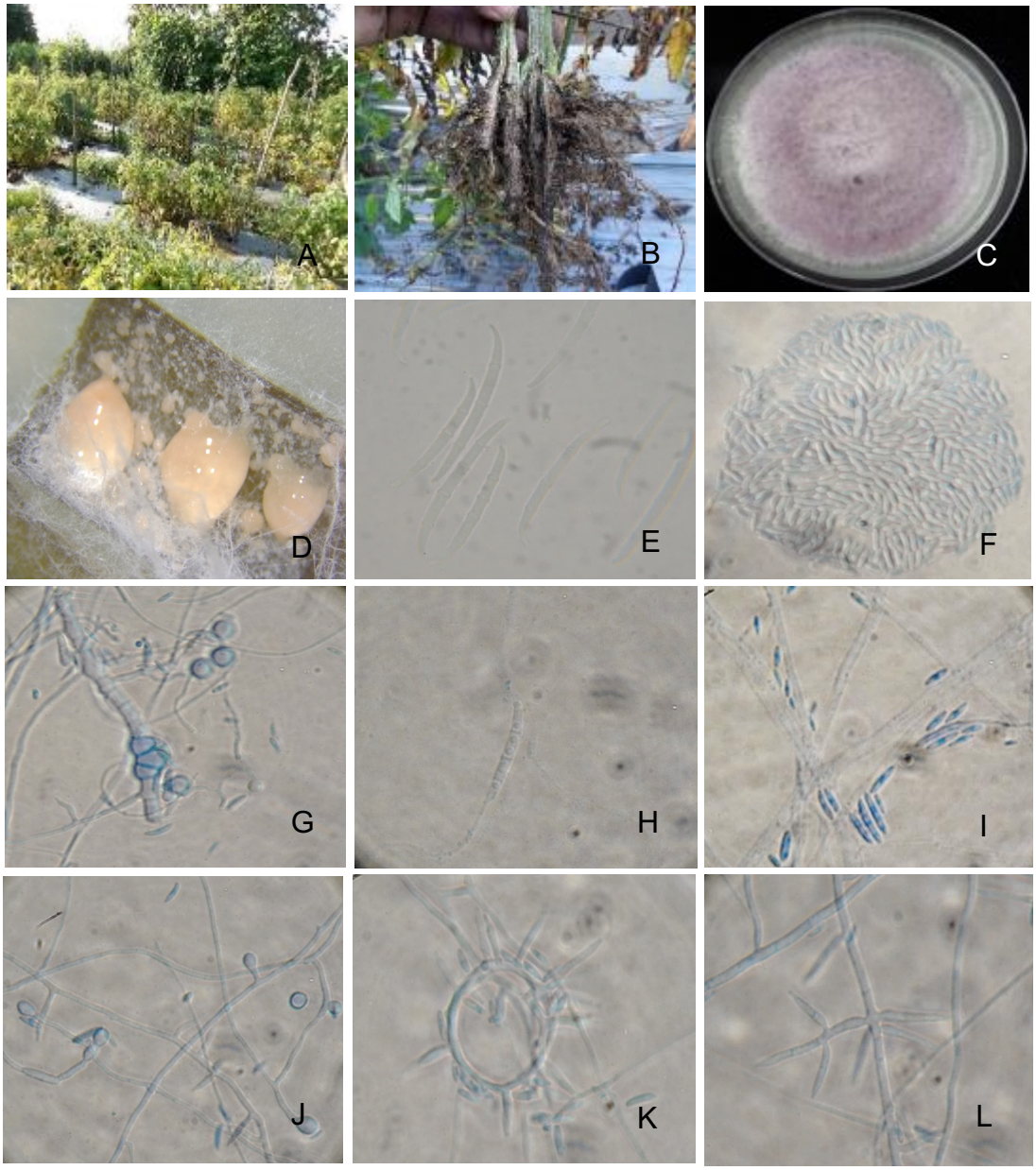


Figure 2. Symptoms, colony, and morphological characteristics of *Fusarium* spp. (A). Yellowing symptoms of tomato plants in open field conditions. (B). Dark lesions and discoloration of tomato stems. (C). Violet colour of *Fusarium oxysporum* colony grown in PDA medium. (D). Mass of sporodochia *F. oxysporum* (E). Macroconidia of *F. oxysporum*. (F). Mass of microconidia of *F. oxysporum*. (G). Pseudochlamydospores of *Fusarium andiyazi*. (H). Macroconidia of *F. andiyazi*. (I). Microconidia of *F. andiyazi*. (J). Chlamydospores of *F. oxysporum*. (K) Coiled hyphae of *Fusarium circinatum*. (L). Monophialides of *F. circinatum*.

The isolate classified as *Fusarium* sp. (Isolate 1), exhibited abundant aerial mycelium, the color of the colony was initially white then turned to red and violet. The macroconidia were straight or slightly curved, with 3-4 septate, 38.6 to 46 × 2.7 to 3.4 µm (Figure 2h). Sporodochia were rarely observed on CLA medium. The microconidia were oval, with a flat base, without septa, of 9.2 to 10.9 × 2.6 to 2.8 µm, formed in monophialide in conidiophores sometimes branched, in false heads or long chains of more than 12 conidia. After 3-4 weeks in PDA medium, pseudochlamydospores in hyphae with smooth walls, and in short chains were observed (Figure 2g). These observations were similar to those of *Fusarium andiyazi* which have been associated with sorghum, in Africa and USA (Marasas *et al*., 2001), with rice bakanae, in Asia and Africa (Wulff *et al*., 2010), and with corn in Siria (Madamia *et al*., 2013). *Fusarium andiyazi* is a major pathogen of sorghum (Klittich *et al*., 1997; Marasas *et al*., 2001) and can be distinguished from other *Fusarium* spp., mainly *Fusarium verticillioides*, *Fusarium thapsinum*, and *Fusarium musae,* based on its production of pseudochlamydospores in PDA medium (Van Hove *et al*., 2011). These structures are distinguishable from true chlamydospores because they have no surface ornamentation and they are not thick- or double-walled (Leslie and Summerell 2006). According to Klaasen and Nelson (1998), pseudochlamydospores differ from 'swollen hyphal cells' because they do not have transverse septa (Marasas *et al*., 2001).

On the other hand, the isolate classified as *Fusarium* sp. (Isolate 2) presented aerial mycelium was white, or slightly violet. The colony exhibited growth rate relatively rapidly. In CLA, coiled hyphae (Figure 2k) were observed, but these characters are shared with species like *F. mexicanum*, *F. pseudocircinatum*, *F. sterilihyphosum,* and *F. tupiense* (Nirenberg and O'Donnell, 1998; Britz *et al*., 2002; Lima *et al*., 2012). Macroconidia were typically 3-septate, with slightly curved walls, measuring 32 to 42 × 3.3 to 3.8 µm. Microconidia were typically single-celled, ovoid (or nearly oval), were borne in false heads on aerial polyphialides. The microconidia measured 7.6 to 11.8 × 1.8 to 3.5 µm. Presented proliferation of microconidiophores, coupled with a slight twisting of the aerial mycelium on which they are borne, differentiating the morphology of the colony. Chlamydospores were absent. These observations were similar to those of *Fusarium circinatum* (Leslie and Summerell 2006), which was recently reported in

association with grass species as symptomless hosts in the proximity of *Pinus* stands (Sweet and Gordon, 2012) in which it has the ability to survive in alternative hosts which is relevant for establishment and subsequent dissemination of the pathogen to new areas. The fungus is also associated with significant losses in yield and productivity, making it one of the most important limitations to commercial forestry (Wingfield *et al*., 2008; Mitchell *et al*., 2011). According to Steenkamp *et al*. (2014), it is widely believed that Central America and Mexico represents the centre of origin of *F. circinatum* this based on the high levels of genetic diversity among species and the wide number of Pinus trees produced in Mexico, generating coevolution of both (Britz *et al*., 2005).

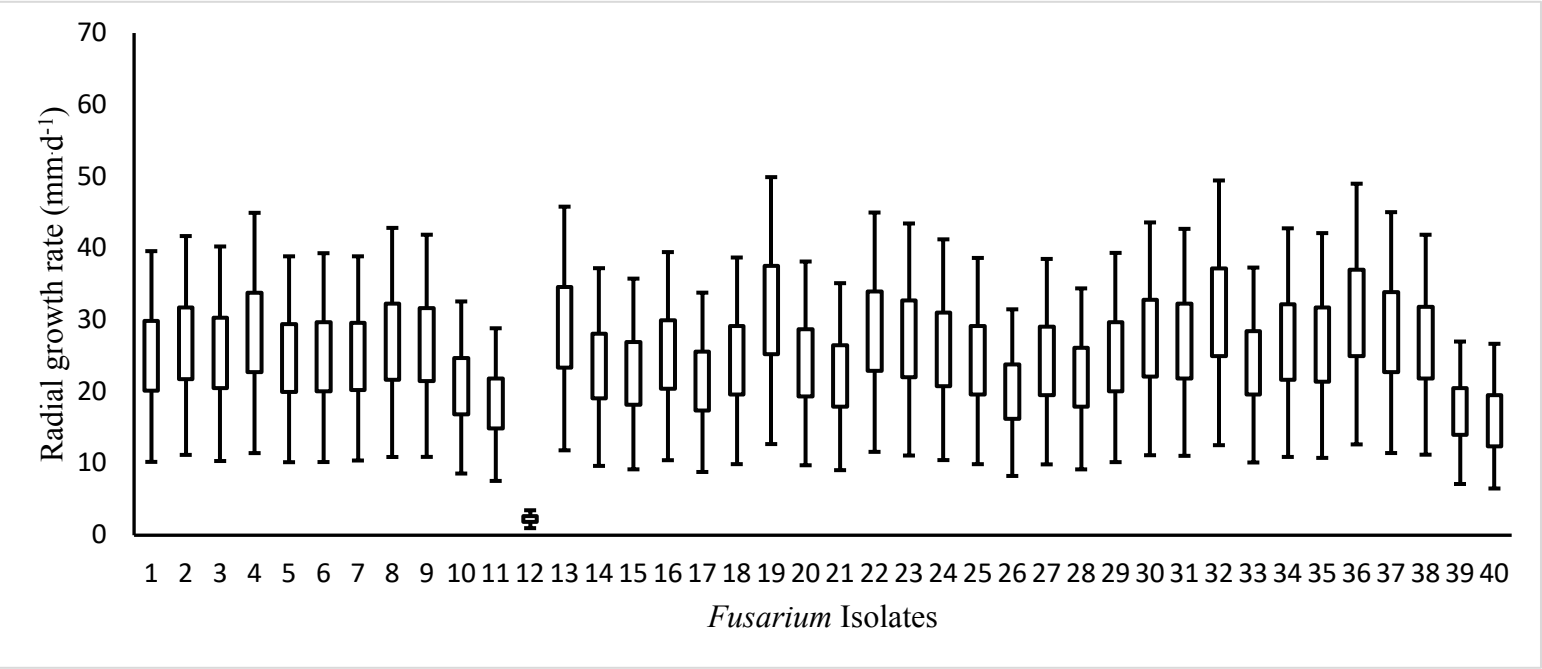


Figure 3 .Radial growth rate values (β1, cm.d-1) of 40 Fusarium isolates (lines represent the 95 % confidence intervals).

The radial growth (β_1, mmd^{-1}) for the 40 *Fusarium* isolates, obtained by linear regression in a period of five to seven days in PDA medium (Figure 3), showed R^2's above 0.8 and all the β_1 values were different to zero ($P< 0.01$). Three isolates (UACH-216, UACH-234, and UACH-238) showed fast growth (12.0–12.3 mmd^{-1}) which reached maximum growth on the 5th day of incubation; while 34 isolates exhibited moderate growth (7.6–11.2 mmd^{-1}), and three (UACH-213, UACH-241, and UACH-212) presented slow growth (0.8–6.9 mmd^{-1}). The isolates with maximum growth covered the Petri dish by a 100% on the 7th day; while the isolates with minimal growth only covered by 50%. Chehri *et al*. (2015) mentioned that the growth rate of *Fusarium solani* were 5.5–8.6 mmd^{-1}, this were slightly under the results obtained in the study 6.9–12 mmd^{-1} (Figure 3), this probably due to difference in temperature and light intensity used during the experiment. According to Brock *et al*. (1994) and Trabelsi *et al*. (2017), fungal growth under natural conditions is very slow due to the low intake of substrates and to the variation of nutrient distribution among microbial habitats. Growth also depends on other factors such the antagonistic interactions with other species like *Trichoderma* spp. competing for the space and nutrients (Marzano *et al*., 2013), drought and moisture conditions, stress caused by nutrient depletion, and changes in temperature and soil pH (Trabelsi *et al*., 2017). This helps to explain the capacity of the *Fusarium* species in adapting and growing under different a wide range of environmental conditions.

2.5.3. Pathogenicity test and the response of the tomato cultivars

The pathogenicity test showed considerably differences among isolates in terms of their ability to cause disease as mentioned by Joshi *et al*. (2013). In this study, 15 isolates were highly pathogenic, 15 showed moderate pathogenicity, and 10were weak. Symptoms were presented 21–35 days after inoculation in which the leaves presented yellowing, flaccidity and wilting. Necrosis and a brownish discoloration were also observed on roots and stem as well as a reddish coloration of vascular tissues as shown in Figure 2. Furthermore, the inoculated fungi were re-isolated from plants that showed witling symptoms, while no symptoms were found on control plants, for this reason the Koch´s postulates were confirmed. ANOVA results of disease severity of *Fusarium* spp. were variable where AUDPC, internal lesion, dry weight, and fresh

weight values indicated highly significant differences among the inoculated isolates and cultivars (Table 2) as well as for their interaction ($P \leq 0.01$).

Table 3. Means comparisons of four varieties of tomatoes during pathogenicity test

Variety	Severity (AUDPC)	Internal lesion (cm)	Dry weight (g)	Fresh weight (g)
Riogrande	948.05 a	32.112 b	1.627 c	86.219 c
Moctezuma	439.02 d	39.005 a	4.2751 a	210.098 a
Floradade	833.66 b	32.372 b	1.4255 c	83.96 c
7744	712.93 c	33.501 b	2.6434 b	138.935 b
HSD	15.892	3.2628	0.2901	15.053

Note: HSD: Honest significant difference
Different letters in each column indicates significant difference (Tukey, P<0.05).
AUDPC: Area under the disease progress curve

The means comparison for cultivars indicated that Moctezuma showed higher resistance to *Fusarium* by presenting the lowest AUDPC, and higher fresh and dry weight (Table 2); although the internal lesion was high. In contrast, RioGrande, Floradade, and 7744 presented similar performance showing high susceptibility. According to Steinkellner *et al*. (2005) the pathogenicity of *Fusarium* in tomato could be affected by the *Fusarium* race, internal factor such as enzymes, growth-regulating compounds, toxins, gummosis; and environmental and growing conditions among themnutrition, phenological stage, and cultural management. Jiménez-Fernández *et al*. (2010) mentioned *F. oxysporum* are responsible for wilt diseases on many plants of economic importance other than tomato. Pathogenic strains show high levels of host specificity and are classified on this basis into *formae speciales* and races. Ajit *et al*. (2006) also reported that *F. oxysporum* has approximately 80 *formae speciales* divided into pathotypes specific to species, and subdivided into races specific to cultivar within a species.

Table 4. Results of pathogenicity test of *Fusarium* spp. isolates

Locality	Code	Severity (AUDPC)	Severity	Internal lesion (cm)	Dry weight (g)	Fresh weight (g)
Atlatlahucan	UACH-202	400 op	Weak	38.8 c-k	1.7 h-k	85 ijk
Santa Cat. Monte	UACH-203	985 d-f	High	12.4 op	1.4 k	68.5 jk
Apanquetzalco	UACH-204	415 op	Weak	24.4 k-p	1.7 h-k	87 h-k
Atlatlahucan	UACH-205	760 f-i	High	17.6 nop	1.7 h-k	86 h-k
Atlatlahucan	UACH-206	880 efg	High	25.6 j-o	1.7 h-k	95 e-k
San Miguel	UACH-207	428 op	Weak	41.1 b-i	1.8 g-k	85 ijk
Libres	UACH-208	548 i-n	Moderate	48 b-e	2.8 c-j	138 b-j
San Miguel	UACH-209	350 p	Weak	51.8 a-d	3.4 b-e	190 b
Zuautla	UACH-210	843 e-h	High	25.1 k-o	1.7 h-k	90.5 f-k
Santa Catarina	UACH-211	765 f-g	High	46.2 c-g	3.5 b-e	190 b
Oaxtepec	UACH-212	828 e-h	High	36.4 d-l	1.5 jk	81 ijk
Apanquetzalco	UACH-213	465 k-p	Moderate	45.1 b-g	4.5 ab	185 b
San Miguel	UACH-214	783 f-h	High	22.1 l-p	2.3 e-k	133 b-k
San Miguel	UACH-215	1395 ab	High	12.4 op	1.4 jk	76 ijk
Yecapixtla	UACH-216	435 n-p	Weak	26.4 l-o	1.6 h-k	102 c-k
Huamantla	UACH-217	605 g-p	Moderate	52.3 abc	3 c-h	163 b-f
Atlatlahucan	UACH-218	478 j-l	Moderate	67.1 a	3.2 b-f	172.9 bc
Almincingo	UACH-219	470 l-p	Moderate	36.7 c-l	3.5 b-d	200 b
Huamantla	UACH-220	1698 a	High	50.3 cbd	3.7 bcd	200 b
Huamantla	UACH-221	640 h-o	Moderate	31.7 f-l	3.1 b-g	162 b-g
Almincingo	UACH-222	590 i-p	Moderate	46.6 b-f	3 c-h	170 bcd
Santa Catarina	UACH-223	710 f-l	Moderate	24.4 k-p	1.8 f-k	107 c-k
Huamantla	UACH-224	1218 bcd	High	29.2 h-l	1.8 f-k	97.8 f-k
Apanquetzalco	UACH-225	743 f-l	Moderate	27.8 i-l	2.4 d-k	129 b-k
Apanquetzalco	UACH-226	663 j-l	Moderate	29.7 i-n	3.4 b-e	161 b-g
Apanquetzalco	UACH-227	1073 c-d	High	29.8 i-n	1.4 kj	79 ijk
Apanquetzalco	UACH-228	730 f-l	Moderate	26.6 j-o	2.5 c-k	147 b-i
Apanquetzalco	UACH-229	973 def	High	24.7 k-p	1.6 ijk	103.3 c-k

Libres	UACH-230	715	f-l	Moderate	34.3	e-m	1.8	g-k	83.6	ijk
Libres	UACH-231	1268	bc	High	33.9	f-l	1.9	f-k	99	d-k
Apanquetzalco	UACH-232	650	h-l	Moderate	30.5	i-m	2.1	e-k	133	b-k
Apanquetzalco	UACH-233	753	f-j	Moderate	25	k-o	1.7	h-k	106	c-k
Huamantla	UACH-234	1430	ab	High	51.4	a-d	1.7	h-k	85.2	ijk
Oaxtepec	UACH-235	603	h-p	Moderate	37.5	c-k	3.6	bcd	183	b
Huamantla	UACH-236	735	f-l	Moderate	33.5	f-n	1.5	jk	89.5	g-k
Oaxtepec	UACH-237	748	f-l	Moderate	27.9	i-n	2.7	c-k	159	b-h
Oaxtepec	UACH-238	745	f-l	Moderate	57.1	ab	3.9	bc	173	bc
Oaxtepec	UACH-239	655	g-l	Moderate	50.5	bcd	3.4	b-e	165.2	b-e
Huamantla	UACH-240	403	op	Weak	18.5	m-p	1.4	k	65	jk
Apanquetzalco	UACH-241	473	k-p	Moderate	42.9	b-h	1.7	h-k	64	k
Control		30	q		8.89	p	5.6	a	333	a
HSD		277			15.9		1.4136		73.362	

Note: HSD: Honest significant difference
Different letters in each column indicates significant difference (Tukey, $p < 0.05$
AUDPC: Area under the disease progress curve

2.5.4. Molecular characterization of the *Fusarium* isolates

Molecular characterization based on analyses of ITS region and EF-1α gene sequences confirmed the findings of the morphological observations, hence the importance of combining both morphological and genetic data for fungal identification (Correia *et al*., 2013). The phylogenetic tree resulting in the formation of three major clusters. According to Aoki *et al*. (2014), primers used in any study must be specific for the correct amplification of *Fusarium* spp. This situation proves that the majority of the *Fusarium* isolates pertains to the *Fusarium oxysporum* complex (95%), one as *Fusarium andiyazi* (2.5%), and one as *Fusarium circinatum* (2.5%). The last two isolates forms part of the *Gibberella fujikuroi* complex (Fig. 3). Several studies have shown that phylogenetic species criterion is most appropriate and congruent (O'Donnell *et al*., 1998; Cai *et al*., 2011; Bashyal and Aggarwal, 2013).

For example, phylogenetic analyses have revealed the existence of several cryptic species (Steenkamp *et al*., 2002) such as *Fusarium subglutinans*, using the genes translation elongation factor 1α gene (EF-1α) and the DNA sequences of ITS regions for specie recognition, thus, offering a finer resolution, separating strains of most *Fusarium* complex species (Hsuan *et al*., 2011; Irzykowska *et al*., 2012). The identification of the genera *Fusarium* on a species level is essential because species are used as the basic units of analysis and for global biodiversity assessments (Sites and Marshall, 2004) based on monophyletic grouping at higher levels and discover lineages at lower levels.

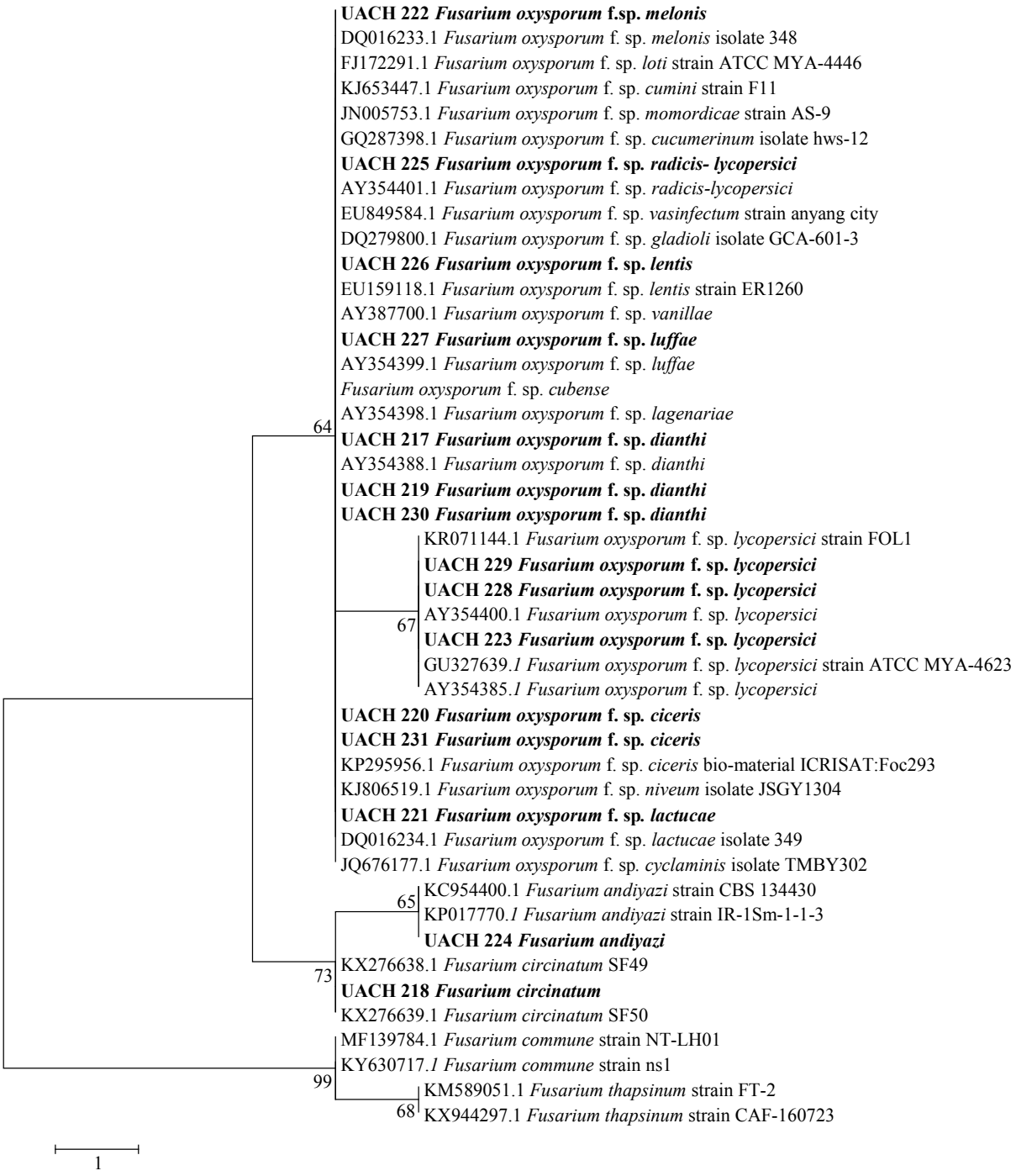


Figure 4. Maximum parsimony tree generated from sequence analysis of the ITS region dataset. Designated out group taxon is Fusarium thapsinum and Fusarium. proliferatum. Bootstrap support values for maximum parsimony (MP) and maximum likelihood (ML) are shown above the nodes. The isolated characterized in this study are in boldface.

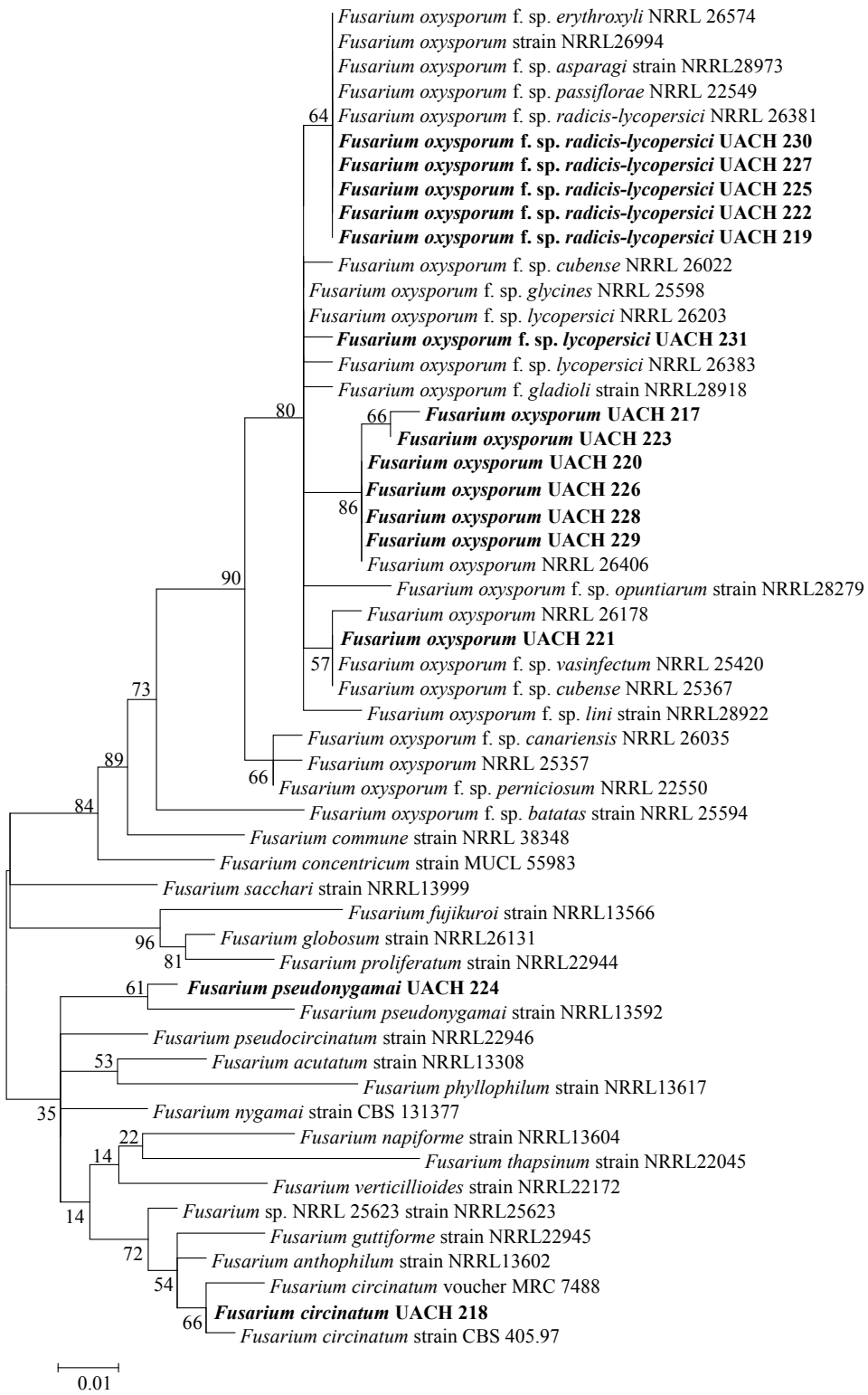


Figure 5. Molecular Phylogenetic anaylsis by Maximum Likelihood Method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei (2011) model. The tree with the highest log likelihood (-1042.5922) is

shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 54 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 210 positions in the final dataset.

2.6. CONCLUSIONS

In conclusion, in the present study three *Fusarium* spp. (*Fusarium oxysporum*, *Fusarium andiyazi* and *Fusarium circinatum*) associated with wilting disease of tomato in Mexico were identified, which will be very helpful information for developing new strategies for the adequate control of this pathogen. We didn't find any relationship between the identified *Fusarium* species with the sampled areas and the production system; this shows the high capacity of this pathogen to affect tomato established under any condition due to the genetic variability of *Fusarium*, which affected the high variability of the infection period and growth rate as observed in this study. This situation can also explain the presence of the different species such as the *Fusarium oxysporum* complex, *Fusarium andiyazi* and *Fusarium circinatum* in tomato; this is of significant important to continue monitoring and evaluating crop diseases development to avoid high losses in tomato production.

2.7. REFERENCES

Ajit NS, Verma R, Shanmugam V (2006). Extracellular chitinases of fluorescent Pseudomonads antifungal to *F. oxysporum* f.sp. dianthi causing carnation wilt. Current Microbiology 52, 310–316.

Akanmu AO, Abiala MA, Odebode AC (2013). Pathogenic effect of soil-borne Fusarium species on the growth of millet seedlings. World Journal of Agricultural Sciences 9: 60–68.

Aoki T, O'Donnell K, Geiser DM (2014). Systematics of key phytopathogenic Fusarium species: Current status and future challenges. Journal of General Plant Pathology 80: 189–201.

Apodaca SMA, Zavaleta ME, Osada KS, García ER, Valenzuela UJG (2004). Hospedantes asintomáticos de *Fusarium oxysporum* Schlechtend. f. sp. radicis-lycopersici W.R. Jarvis y Shoemaker en Sinaloa, México.Revista Mexicana de Fitopatología 22: 7–13

Arie T, Takahashi H, Kodama M, Teraoka T (2007). Tomato as a model plant for plant-pathogen interactions. Plant Biotechnology 24:135–147.

Bashyal BM, Aggarwal R (2013). Molecular identification of Fusarium species associated with bakanae disease of rice (*Oryza sativa*) in India, Indian Journal of Agricultural Sciences 83:71–6.

Booth C (1971). The genus Fusarium. Commonwealth Mycological Institute, Kew, Surrey, England. pp 237.

Britz H, Coutinho TA, Wingeld MJ, Marasas WFO (2002) Validation of the description of *Gibberella circinata* and morphological differentiation of the anamorph *Fusarium circinatum*. Sydowia 54:9–22.

Cai L, Giraud T, Zhang N, Begerow D, Cai G, Shivas GR (2011). The evolution of species concepts and species recognition criteria in plant pathogenic fungi. Fungal Diversity. 50:121.

Campbell CL, Madden LV (1990). Introduction to plant disease epidemiology, ed. John Wiley and Sons, Inc., New York, NY, USA. 1990.

Chehri K, Salleh B, Zakaria L (2015). Morphological and Phylogenetic Analysis of Fusarium solani Species Complex in Malaysia. Microbial Ecology 69:457–471.

Correia CK, CâmaraSM, Barbosa GM, Sales RJ, Brisach AC, Gramaje D, León M, García-Jiménez J, Abad-Campos P, Armengol J, Michereff S J (2013). Fungal trunk pathogens associated with table grape decline in Northeastern Brazil. Phytopathologia Mediterranea 53:380–388.

Daami-Remadi M (2006) Etude des fusarioses de la pomme de terre. [Study of fusariosis of the potato]. Thèse, Institut Supérieur 424 Agronomique de Chott-Mariem, Tunisie, p 238

El-Kazzaz MK, El-Fadly GB, Hassan MAA, El-Kot GAN (2008). Identification of some Fusarium spp. using Molecular Biology Techniques, Egypt Journal of Phytopathology 36:57–69.

FAOSTAT (2015). Database on Agriculture. FAO - Food and Agriculture Organization of the United Nations.

Gargouri-Kammoun L, Gargouri S, Rezgui S, Trifi M, Bahri N, Hajlaoui M R (2009). Pathogenicity and Aggressiveness of Fusarium and Microdochium on Wheat Seedlings under Controlled Conditions. Tunisian Journal of Plant Protection, 4(2), 135–144.

Hernández-Martínez HR, Benítez LA, Escalante BF, Velázquez EJ, Aspeytia SD, Mendoza MIE, Ochoa LAL (2014). Razas de *Fusarium oxysporum* f. sp. *lycopersici* en predios tomateros en San Luis Potosí. Revista mexicana de ciencias agrícolas 5: 1169-1178.

Hsuan HM, Salleh B, Zakaria L (2011). Molecular Identification of Fusarium Species in *Gibberella fujikuroi* Species Complex from Rice, Sugarcane and Maize from Peninsular Malaysia. International Journal of Molecular Sciences 12: 6722–6732.

Inami K, KashiwaT, Kawabe M, Onokubo-Okabe A, Ishikawa N, Pérez RE, Hozumi T, Caballero LA, Cáceres de Baldarrago F, Roco MJ, Madadi KA, PeeverTL, Teraoka T, Kodama M, Arie T (2014). The Tomato Wilt Fungus *Fusarium oxysporum* f. sp. *lycopersici* shares Common Ancestors with Nonpathogenic *F. oxysporum* isolated from Wild Tomatoes in the Peruvian Andes. Microbes and environments / JSME 29:200–210.

Irzykowska L, Bocianowski J, Waśkiewicz A, Weber Z, Karolewski Z, Goliński P, Kostecki M, Irzykowski W (2012). Genetic variation of Fusarium oxysporum isolates forming fumonisin B1 and moniliformin. Journal of Applied Genetics 53:237–247.

Jiménez-Fernández D, Montes-Borregob M, Navas Cortés J, Jiménez-Díaz R, Landab, BB (2010). Identification and quantification of *Fusarium oxysporum* in plants and soil by means of an improved specific and quantitative PCR assay, Applied Soil Ecology 46. 372–362.

Joshi, M, Srivastava R, Sharma Prakash AK (2013). Isolation and characterization of *Fusarium oxysporum*, a wilt causing fungus, for its pathogenic and non-pathogenic nature in tomato (*Solanum lycopersicum*). Journal of Applied and Natural Science 5:108–117.

Klaasen JA, Nelson PE (1996). Identification of a mating population, *Gibberella nygamai* sp. nov., within the *Fusarium nygamai* anamorph. Mycologia 88:965–969.

Klittich CJR, Leslie JF, Nelson PE, Marasas WFO (1997). *Fusarium thapsinum* (*Gibberella thapsina*): a new species in section Liseola from sorghum. Mycologia 89:643–652.

Lamrani K (2009). Etude de la biodiversite´des moisissures nuisibleset utiles isole´es a`partir des Maaˆsra du Maroc. [Study of the biodiversity of harmful and beneficial fungi isolated from the Maasra of Morocco.] The`se en Microbiologie, Universite´Mohamed V-Agdal Faculte´des Sciences Rabat. N°d'ordre: 2461

Leslie JF, Summerell BA (2006). The Fusarium Laboratory Manual. 369 p. Blackwell Publishing, Iowa, USA.

Leyva-Mir, SG, González-Solano CM, Rodríguez-Pérez JE, and Montalvo-Hernández D (2013). Comportamiento de líneas avanzadas de tomate (*Solanum lycopersicum* L.) a fitopatógenos en Chapingo, México. Rev ChapSerie horti. 19:301–313.

Lima CS, Pfenning LH, Costa SS, Abreu LM, Leslie JF (2012) *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* complex that causes mango malformation in Brazil. Mycologia 104:1408–1419.

Marzano M, Gallo A, Altomare C (2013). Improvement of biocontrol efficacy of *Trichoderma harzianum* vs. *Fusarium oxysporum* f. sp. *lycopersici* through UV-induced tolerance to fusaric acid. Biological Control, 67: 397–408.

Marín-Montes IM, Rodríguez-Pérez JE, Sahagún-Castellanos J, Hernandez-Ibañez L, Velasco-García AM (2016). Variación morfológica y molecular de 55 colectas de tomate nativo de México. Revista Chapingo Serie horticultura 22:117-132.

Massee G (1895). The "Sleepy disease" of tomatoes. Garden Chronicles Series3. 17:707–708.

Marasas WFO, Lamprecht SC, Zeller KA, Leslie JF (2001). *Fusarium andiyazi* sp. nov., a new species from sorghum. Mycologia 93: 1203–1210.

Morales-Rodríguez I, Yañez-Morales M, Silva-Rojas HV, García de los Santos G, Guzmán de Peña D (2007). Biodiversity of Fusarium species in Mexico associated with ear rot in maize, and their identification using a phylogenetic approach, Mycopathologia. 163. 31–9.

Nelson PE, Toussoun TA, Marasas WFO (1983). Fusarium Species: An Illustrated Manual for Identification. Pennsylvania State University Press, University Park, Pennsylvania, USA. 193 p.

Nirenberg HI, O'Donnell K (1998) New Fusarium species and combinations within the *Gibberella fujikuroi* species complex. Mycologia 90:434–458

Nirmaladevi D, Venkataramana M, Srivastava R K, Uppalapati SR., Gupta VK, Yli-Mattila T, Tsui KMC, Srinivas C, Niranjana SR, Chandra, NS (2016). Molecular phylogeny, pathogenicity and toxigenicity of *Fusarium oxysporum* f. sp. lycopersici. Scientific Reports, 6: 21367.

O'DonnellK, Cigelnik E, Casper HH (1998). Molecular phylogenetic, morphological, and mycotoxin data support re-identification of the Quorn mycoprotein fungus as Fusarium venenatum. Fungal Genetics and Biology 23: 57–67.

O'Donnell K, Cigelnik E, Nirenberg HI (1998). Molecular Systematics and Phylogeography of the Gibberella fujikuroi Species. Mycologia, 90:465–493.

Panthee DR, Chen F (2010). Genomics of fungal disease resistance in tomato. Currentgenomics, 11:30–39.

ParkeJL, Grünwald NJ (2012). A Systems approach for management of pests and pathogens of nursery Crops. Plant Diseases 96:1236–1244.

Rodrigues AAC, Menezes M (2005). Identification and pathogenic characterization of endophytic Fusarium species from cowpea seeds. Mycopathologia. 159:79–85.

SAS Institute Inc (2012). Base SAS® 9.3 Procedures Guide. Cary, NC: SAS Institute Inc.

Sato R, Araki T (1974). On the tomato root-rot disease occurring under vinyl-house conditions in southern Hokkaido. Annual Report of the Society of Plant Protection of North Japan 25:5–13.

ShahnaziS, Meon S, Vadamalai G, Ahmad K, Nejat N (2012). Morphological and molecular characterization of *Fusarium* spp. associated with yellowing disease of black pepper (*Piper nigrum* L.) in Malaysia. Journal of General Plant Pathology, 78:160–169.

Siddique SS, Bhuiyan MKA, Momotaz R, Bari GMM, Rahman MH. (2014). Cultural characteristics, virulence and *In-vitro* chemical control of *Fusarium oxysporum* f. sp. *phaseoli* of bush bean (*Phaseolus vulgaris* L.), The Agriculturists 12:103–110.

Singha MI, Kakoty Y, Unni GB, Das J, Kalita CM (2016). Identification and characterization of *Fusarium* sp. using ITS and RAPD causing Fusarium wilt of tomato isolated from Assam, North East India. Journal of Genetic Engineering and Biotechnology 14:99–105.

Sites JW, Marshall JC (2004). Operational criteria for delimiting species. Annual Review of Ecology Evolution and Systematics. 35:199–227.

Staden R, Beal KF, Bonfield JK (1998) The Staden package, 1998. In: Misener S, Krawetz SA (eds) Bioinformatics methods and protocols. Humana, New York, pp 115–130.

Steenkamp ET, Wingfield BD, Desjardins AE, Marasas WFO, Wingfield MJ (2002). Cryptic Speciation in *Fusarium subglutinans*. Mycologia. 94:1032-1043.

Steenkamp ET, Makhari OM, Coutinho TA, Wingfield BD, Wingfield MJ (2014). Evidence for a new introduction of the pitch canker fungus *Fusarium circinatum* in South Africa. Plant Pathology, 63(3), 530–538.

SteinkellnerS, Mammerler R, Vierheilig H (2005). Microconidia germination of the tomato pathogen *Fusarium oxysporum* in the presence of root exudates. Journal of Plant Interactions 1:23–30.

SweetCL, Gordon TR (2012). First report of grass species (Poaceae) as naturally occurring host of the pine pathogen *Gibberella circinata*, Plant Disease. 96:908.

Takken F, Rep M (2010). The arms race between tomato and *Fusarium oxysporum*. Molecular Plant Pathology 11:309–314.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary

distance, and maximum parsimony methods, Molecular Biology and Evolution 28:2731–2739.

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512-526.

Tanyolac B, Akkale C (2010). Screening of resistance genes to Fusarium root rot and Fusarium wilt diseases in F3 family lines of tomato (*Lycopersicon esculentum*) using RAPD and CAPs, African Journal of Biotechnology 9:2727-2730.

Tivoli B, Deltour A, Molet D, Bedin P, Jouan B. (1986). Mise en évidence de souches de *Fusarium roseum* var. *sambucinum* résistantes au thiabendazole, isolées à partir de tubercules de pomme de terre [Demonstration of *Fusarium roseum* var. *sambucinum* strains resistant to thiabendazole, isolated from potato tubers]. Agronomie, 6:219–224.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The ClustalX Windows interface: flexible strategies for multiple alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Trabelsi R, Sellami H, Gharbi Y, Krid S, Cheffi M, Kammoun S, Dammak M, Mseddi A, Gdoura R, Triki MA (2017). Morphological and molecular characterization of *Fusarium* spp. associated with olive trees dieback in Tunisia. Biotechnology, 7:1.

Van Hove F, Waalwijk C, Logrieco A, Munaut F, Moretti A (2011). *Gibberella musae* (*Fusarium musae*) sp. nov., a recently discovered species from banana is sister to F. verticillioides. Mycologia, 103:570–585.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, New York, USA: 315–322.

Wulff EG, Sorensen JL, Lubeck M, Nielson KF, Thrane U, Trop J (2010). *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity, Environ Microbiology 12: 649–57.

Zainuddin M, Ain Izzati N, Baharuddin S (2010). Variability of Fusarium species associated with bakanae disease of rice based on virulence, vegetative and biological compatibility. Sydowia. 62:89–104.

3. *In vitro* ANTAGONISTIC POTENTIAL OF *Trichoderma* SPECIES AGAINST *Fusarium oxysporum* ASSOCIATED WITH WILT DISEASE IN TOMATO

3.1. ABSTRACT

The biological control of *Fusarium* spp., causal agent of tomato wilt disease, is of great environmental relevance due to the wide distribution of this disease. The objective of the present study was to collect, characterize and identify morphologically and molecularly *Trichoderma* spp. and quantify thier potential as biological control agents for *Fusarium oxysporum* in tomato production. Forty soil samples were collected from the rhizosphere and stem of healthy tomato plants from 12 localities of three states, from which 15 Trichoderma isolates were identified by means of morphological characteristics and analysis by rDNA internal transcribed spacer regions (ITS) and EF-1α primers. Likewise, the antagonistic potential of the isolates against *Fusarium oxysporum* were evaluated by means of the dual culture technique in potato dextrose agar medium (PDA) at three temperatures (24, 28 and 30 °C), incubated for 8 days, under laboratory conditions. Both morphological and molecular analysis confirmed the identification of the *Trichoderma* spp. The species found were: *T. asperellum*, *T. asperelloides*, *Trichoderma sp., T. stromaticum, Trichoderma koningiopsis* and *T. atroviridis*. The first four showed the highest ($P \leq 0.05$) biocontrol against *F. oxysporum* manifesting mycelial growth inhibition by 69.30, 66.91, 66.46, and 48.79 %, respectively. Observations under light microscope revlealed that the hyphae of *T. asperellum* established aggressive contact with the hyphae of the pathogen; this indicates that mycoparasitism was the principal biocontrol mechanism identified for controlling *F. oxsyporum*. Thus, *T. asperellum* represents an alternative for the *in vitro* suppression of *F. oxysporum* by means of mycoparasitism, reason why it should be considered as an alternative for controloing the pathogen under field conditions.

Kewords: Biological control, phylogeny, *in vitro*, mycoparasitism, *Trichoderma asperellum*.

3.2. RESUMEN

El control biológico de *Fusarium* spp., agente causal de la enfermedad de marchitez del tomate, es de gran relevancia ambiental debido a la amplia distribución de esta enfermedad. El objetivo del presente estudio fue colectar, caracterizar e identificar morfológica y molecularmente especies de *Trichoderma* spp. y cuantificar su potencial para el control biológico de *Fusarium oxysporum* en el cultivo de tomate. Fueron colectadas 40 muestras de suelos de rizosfera y tallo de plantas de tomate sanas de 12 localidades de tres esatados de la República Mexicana, a partir de las cuales se aislaron 15 cepas de *Trichoderma* sp., las cuales se identificaron mediante claves morfológicas y análisis de regiones espaciadoras transcritas internas de rDNA ITS e iniciadores EF-1α. Asimismo, fueron evaluadas por su potencial antagonista contra *Fusarium oxysporum* mediante el método de cultivo duales en medio de agar de papa dextrosa (PDA) en tres temperaturas (24, 28 y 30 °C), incubados durante 8 días, en condiciones de laboratorio. Los análisis morfológicos y moleculares confirmaron la identificación de las especies de *Trichoderma*: *T. asperellum*, *T. asperelloides*, *Trichoderma sp., T. stromaticum, Trichoderma koningiopsis* y *T. atroviridis*. Las cuatro primeras mostraron el mayor control ($P \leq 0.05$) contra *F. oxysporum*, al inhibir su crecimiento micelial en 69.30, 66.91, 66.46 y 48.79 %, respectivamente. Observaciones en microscopio óptico mostraron que las hifas de *T. asperellum* establecieron contacto físico agresivo con las hifas del patógeno, lo que indentificó al micoparasitismo como el principal mecanismo de control biológico de *F. oxsyporum*. Así, *T. asperellum* representa una alternativa para la supresión *in vitro* de *F. oxysporum* por medio de micoparasitismo, por lo que debe considerarse como una posibilidad de empleo en campo.

Palabras clave: Control biológico, filogenia, *in vitro,* micoparasitismo, *Trichoderma asperellum*.

3.3. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is of agricultural and economic importance in most countries, since it occupies 16 % of the world's vegetable production. Tomato can be cultivated in either open field, protected agriculture or in hydroponics conditions. In 2015, an area of 8.5 million ha^{-1} was used for tomato production, with a total production of 161 million tons (FAO, 2016). In Mexico, this crop is one of the most important vegetable crop for food and industrial purposes. Plants are a major source of secondary metabolites such as terpenoids, alkaloids, glycosides, phenols and tannins, these play an important role in plant defense mechanisms against pest and diseases. The presence of phytopathogenic agents such as *Phytophthora infestans*, *Alternaria solani*, *Septoria lycopersici*, *Verticilium dahlae,* and *Fusarium oxysporum* the causal agent of tomato wilt disease has affected tomato yield over the years (Leyva-Mir, González-Solano, Rodríguez-Pérez, & Montalvo-Hernández, 2013).

Fusarium affects the plants by damaging the vascular vessels, mainly xylem in the roots (Chakrabarti, 2013), thus, reduces the absorption rate of water and minerals, which results in low crop yields and mayor economic losses for the tomato industry (Shankara, de Jeude, de Goffau, Hilmi, & van Dam, 2005). The control of this fungus is mainly by the use of treatments based on fungicides. However, the inappropriate and excessive use these products can be a hazard to human health and can create environmental consequences. Furthermore, new wilt resistant varieties are introduced in the market to reduce the effect of the disease but are often overcome by new races of the pathogen, thus making it very difficult to control the fungus (Boix-Ruíz *et al*., 2014). Therefore, alternative approaches to control *Fusarium* wilt is needed to decrease the excessive use of these fungicides.

In addition, the need to create a better understanding on the *in vitro* and *in vivo* interactions between pathogen and the plant that can help to improve the ways in which the fungus is controlled (Schmoll & Schuster, 2010; Vinale *et al*., 2008). The most common biological control agent is *Trichoderma* spp. which has been developed into several commercial biological control products and is used both in field crop and greenhouse system (Mokhtar & Dehimat, 2012; Rojo, Reynoso, Ferez, Chulze, &

Torres, 2007). The genus *Trichoderma* is a filamentous imperfect fungi and is the most common saprophyte found in the rhizosphere. It is highly interactive in root, soil and foliar environments, but further studies are need to determine its adaptation and effectiveness in tomato plants. Such information could be valuable in developing new and combined strategies to control *Fusarium* wilt in tomato.

3.4. MATERIALS AND METHODS

3.4.1. Morphology and growth characteristics of *Trichoderma* species and pathogen cultures

The isolates used in the present study were collected from the rhizosphere soils and stem of healthy tomato plants, obtained form 12 different locations of three states (Morelos, State of Mexico, and Tlaxcala), from which 15 Trichoderma isolates were identified. Soil dilution technique was used to dilute the samples collected from each state. Soil samples (10 g) were homogenized and weighed to carry out the dilution process (10^{-3}) determined by using a hemocytometer. A 100 µL of each diluted sample was pipetted onto Petri dishes with Potato Dextrose Agar (PDA) medium added with 0.5 $g \cdot L^{-1}$ streptomycin. The Petri dishes were then incubated for 7 days, at 24 °C under light and darkness, to observed colony growth.

The slides containing *Trichoderma* mycelium and spores were removed and placed on a slide; each slide was stained with a drop of cotton blue. Morphological identification was done based on cultural (colony and growth rate) characterization and light microscope observations (Bissett, 1991a) of the fungal colonies. The confirmation at the species-level was carried out according to an interactive key provided by (Samuels *et al*., 2002). The identified *Trichoderma* isolates were purified by single spore culture technique and preserved in refrigerator, at -20°C for further studies. The plant pathogenic fungus *F. oxysporum* was originally isolated on PDA medium from the stem and root of naturally infested tomato plants. The plants were collected from field plots located in the states of Morelos, Puebla, and Tlaxcala.

3.4.2. Growth rate of *Trichoderma* spp. in different concentration of culture medium

The growth rate of 15 *Trichoderma* species was examined on three different potato dextrose agar (PDA) media concentrations. These were as follows; Synthetic PDA medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5 g L^{-1} of streptomycin (Sigma-Aldrich, USA), incubated at 24 °C. Semi synthetic PDA medium supplemented with 0.5 g L^{-1} of streptomycin (Sigma-Aldrich, USA), 1 ml L^{-1} of lactic acid, incubated at 28 °C, and Semi synthetic PDA, supplemented with 0.5 g L^{-1} streptomycin (Sigma-Aldrich, USA), 1 ml L^{-1} of lactic acid, incubated at 30 °C. A 5 mm mycelia plug of each isolate were placed at the center of the 90 mm petri dish with respective medium and were incubated at the respected temperatures (Papavizas and Lumsden 1982). A completely randomized experimental design with three replicates was used. The diameter of each colony was measured every day for seven days after inoculation (DAI).

Table 5. *Trichoderma* isolates obtained from the rhizosphere and stem of healthy tomato plants in the states of Morelos, State of Mexico and Tlaxcala.

Isolata code	Location	State	Type of sample
UACH-242	Chapingo	State of Mexico	Stem
UACH-243	Yautepec	Morelos	Rhizosphere soil
UACH-244	Atlatlahucan	Morelos	Rhizosphere soil
UACH-245	Chapingo	State of Mexico	Stem
UACH-246	Chapingo	State of Mexico	Stem
UACH-247	Oaxtepec	Morelos	Rhizosphere soil
UACH-248	Oaxtepec	Morelos	Rhizosphere soil
UACH-249	Huamantla	Tlaxcala	Rhizosphere soil
UACH-250	Oaxtepec	Morelos	Rhizosphere soil
UACH-251	Oaxtepec	Morelos	Rhizosphere soil
UACH-252	Apanquetzalco	Morelos	Rhizosphere soil
UACH-253	Apanquetzalco	Morelos	Rhizosphere soil
UACH-254	Oaxtepec	Morelos	Rhizosphere soil
UACH-255	Santa. Catarina	Morelos	Rhizosphere soil
UACH-256	Atlatlahucan	Morelos	Rhizosphere soil

3.4.3. Dual culture technique of *Trichoderma* spp against *Fusarium oxysporum*, *in vitro*

Trichoderma isolates were tested against *Fusarium oxysporum* associated with tomato wilt using the dual culture technique (Morton and Stroube 1955; Katarzyna Nawrot-Chorabik, 2013). Each Petri-dish (9 cm) containing PDA was inoculated with two 5 mm diameter mycelial discs; one of the *Fusarium oxysporum* isolate and one of the *Trichoderma* isolate placed 7 cm apart from each other. The *Fusarium oxysporum* discs were placed in the Petri-dishes 48 hours before the *Trichoderma* discs. The inoculum discs were obtained from the margin of actively growing 7 days old fungal cultures. The experiment was repeated twice. A completely randomized experimental design was used with three Petri dishes for each antagonist. Each treatment was incubated at 24 °C until the PDA medium was completely covered with pathogen mycelia. Radial growth of the *Fusarium oxysporum* isolates was measured, and percent inhibition of average radial growth was calculated in relation to growth of the controls as follows: I = (C-T/C) × 100. Where I = percentage of inhibition: C = radial growth of pathogen (control); T = radial growth of *Fusarium oxysporum* (mm) in the presence of *Trichoderma* isolates (Edington *et al*., 1971). Microscopic examinations were made at 100X magnification for signs of mycoparasitism at the interaction zone between the two cultures to evaluate the mode of action of the *Trichoderma* isolates in the Petri dishes during the dual culture test.

3.4.4. Mycoparasitic activity of *Trichoderma* spp.

The hyphal interaction between the *Trichoderma* spp and the *Fusarium oxysporum* isolates were studied in PDA medium, incubated in dark and light conditions at 28 °C. Mycelium contact, intersection and subsequent overlapping of both fungus was registered for any morphological changes in growth of *Trichoderma* spp. Cover strips 20 x 40 mm strips were removed from the interaction zone, placed on sterilized microscope slides and observed under oil immersion at 100X magnification using the Olympus biological microscope model CX31. Mycoparasitic manifestations at different

stages of development were recorded and photographed using an Olympus camera compared with hyphae of the same age as the control.

3.4.5. Molecular characterization of antagonistic isolates

Seven days old, purified single spore mycelia colonies grown in Petri dishes with PDA medium were used for the extraction of DNA. The colonies were scrapped carefully and placed in a sterilized mortar. Then, using a pestle and with sufficient liquid nitrogen, the colonies were ground to a fine powder. The samples were transferred to a microcentrifuge tube where 500 µL of extraction buffer was added (0.1 M Tris pH 8, 10 mM EDTA, 2 % SDS, and 0.2 $mg \cdot mL^{-1}$ proteinase K). The tubes were vortex and placed in a warm bath at 38 °C for 30 min. Then, 30 µL of 10 % CTAB and 70 µL of 5 M NaCl was added and kept at 65 °C for 30 min. Then, 50 µL of 5 M potassium acetate was added and the mixture was incubated a -20°C for 5 min. 700 µL of chloroform: isoamyl alcohol (24:1) was added to all the tubes, followed by centrifugation at 13,000 x g for 10 min. The upper aqueous phase was pipetted out into a fresh 1.5 mL tube with 640 µL of ice-cold isopropanol and 60 µL of 3M pH 5.8 sodium acetate. Each sample was incubated at -20°C for 10 min and centrifuged again at 13,000 x g for 10 min. The pellet was washed with 500 mL of 70 % ethanol and was centrifuged again at 13,000 x g for 5 min. The ethanol was decanted and the pellet was dried at room temperature for 20 min and finally dissolved in distilled water of DNAasa and RNAasa (Promega ®).

3.4.6. PCR amplification

For PCR, the universal primers ITS 5 (5’ GGA AGT AAA AGT CGT AAC AAG G 3’), ITS 4 (5’ TCC TCC GCT TAT TGA TAT GC 3’), EF1-728F (5’ CAT CGA GAA GTT CGA GAA GG 3’), EF1-986R (5’ TAC TTG AAG GAA CCC TTA CC 3’) were used (White et al., 1990; O’Donnell et al., 1998). The components of the reaction were prepared to a final volume of 50 µL, by adding PCR buffer (1x), 2.5 µL $MgCl_2$, 0.2 mM of 1 µL dNTP, 0.8 µL of each primer, 1U of DNA polymerase Promega ® and 100 ng of DNA. The PCR was carried out in a C-1000TM Thermal Cycler BIORAD ® (EE. UU.).

The initial amplification conditions were 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 10 min. For the EF1-728/EF1-986R primers, the alignment temperature used was 54 °C. The amplified products were tested with electrophoresis on 1% agarose gels, with TAE 1 X buffer and 5 µL of the PCR product at 90 volts. The gel was analyzed in a Transilluminator Gel Doc-It TM 300 UVP (U. K.). The amplified products were sent to Marcogen Korea to be sequenced. The sequences obtained were compared with those in the database of NCBI with Blastn.

3.4.7. Phylogenetic analyses

The phylogeny was reconstructed by analyses from sequences of the ITS region and EF-1α gene. Forward and reverse sequences were assembled using the Staden Package (Staden *et al*., 1998). Sequences generated in this study were supplemented with additional sequences obtained from GenBank, based on BLASTn searches and the literature. Sequences of the isolates were aligned using ClustalX v. 1.81 (Thompson *et al*., 1997) and manual adjusted. Individual alignments of ITS and EF-1α dataset were analysed with Maximum Likelihood and Maximum Parsimony using MEGA5 (Tamura *et al*., 2011) with partial deletion of gaps, substitution models proposed by this program and 1000 bootstrap replicates. The obtained sequences were deposited in the NCBI database.

3.5. RESULTS AND DISCUSSION

3.5.1. Morphological characteristics and growth rate of the *Trichoderma* species

A total of 40 soil samples was obtained and processed in which 15 *Trichoderma* species were isolated from the rizosphere of healthy tomato plants collected from 12 loctaions of three states of Mexico. The identification of the *Trichoderma* isolates was carried out based on morphological and microscopic observation. All isolates, to some lesser degree presented the typical form of *Trichoderma* spp. colonies with the formation of

green concentric rings (Figure 7) at the sporulation sites (Bissett, 1984; Samuels *et al*., 2002; Samuels, 2006). The green color varied in tone in each of the isolates (Webster, 1964; Druzhinina *et al*., 2006). However, although the morphology characteristics serves to identify this fungi, it can become very difficult to distinguish among species hence the reason why it is necessary to confirm these findings with molecular methods (Hermosa *et al*., 2000; Shoresh and Harman, 2008a).

In this study, ten out of fifteen *Trichoderma* isolates were classified as *T. asperellum*. (UACH-243, UACH-245, UACH-247, UACH-249, UACH-250, UACH-251, UACH-253, UACH-254, UACCH-255 and UACH-256), one as *Trichoderma stromaticum* (UACH-242), one as *Trichoderma koningiopsis* (UACH-244), one as *Trichoderma* spp. (UACH-246), one as *Trichoderma atroviridis* (UACH-248), and one as *Trichoderma asperelloides* (UACH-252). All ten *Trichoderma asperellum* grow rapidly on PDA medium forming some fluffy white mycelia at first, which turned whitish green (isolates UACH243, UACH-253, UACH254, and UACH-255), and then turned dull green with the production of conidia (Hoyos-Carvajal *et al*., 2009a).

The *T asperellum* isolates presented the formation of branched conidiophore, smooth central axis from which secondary branches arise and tend to be paired. The phialides were flask shaped with a 2.4 µm L/W ratio. The conidial shaped was subglobose to ellipsoidal (Migheli *et al*., 2009). The average conidia measured was 1.8 µm with smooth walls. The colony appearance on the PDA medium were green, yellow green to dark green grown at 24 °C and 30 °C. All isolates presented Chlamydospore averaging from 6.5 to 15.3 × 4.1 to 12.6 µm (Samuels *et al*., 1999). The colony growth rate was from 62 mm at 24 °C. No pigmentation on medium was presented.

T. asperellum has been reported in Russia, Nepal, and North India (Kullning *et al*., 2000), in Southeast Asia (Kubicek *et al*., 2003), in China (Zhang *et al*., 2005), and in the rainforest soils and other specific habitats such as river sand, humus and wood in Peru, Mexico, Guatemala, and Colombia (Hoyos-Carvajal *et al*., 2009a). This shows evidence of the significant biodiversity of *Trichoderma* in both temperate and tropical regions around the world.

Isolates 2 (UACH-242) belongs to *Trichoderma stromaticum* that presented conidia dark green but often yellow, broadly ellipsoidal to ovoid measuring 5.3 µm to 2.8 µm, presenting both ends broadly rounded and the base narrowed in a single conidial mass. Colonies produced pustules in the center of the colony with of dark green pigmentation. Conidia were formed in compacted pustules. Conidial en masses on phialides are observed (Sanogo *et al*., 2002), with ampulliforme and sharp constricted phialides 2.2 to 4.8 × 3.3 to 10.7 µm. Fertile branches arising at the base of mycelium, typically one or a few cells in length, often densely clustered, producing unicellular lateral branches; phialides terminating all branches, densely clustered (Samuels *et al*., 2012). Chlamydospores measured 4.2 to 13.5 µm in diameter.

It has been reported that *Trichoderma stromaticum* is associated with “witches-broom” disease in cocoa (Pomella *et al*., 2007) and serves as a mycoparasite of this disease and is currently being applied in the fields to obtain adequate control. This diversity could be explored in the development of efficient biological control agents against the disease. Factors that may affect the application and performance of this specie the field, such as sporulation on rice substrate and on the brooms and growth at various temperatures, must be taken into consideration.

Isolate 3 (UACH-244) belongs to *Trichoderma koningiopsis* that presented colonies that were dense and of an abundant white to light green in color. The conidiophores presented abundant branches from the main axis, pair with longer or shorter internodes between branches. The phialides are short and crowded. Branches arise at an angle less than 90 °C. Phailides were straight, hooked, narrowly lageniform, and swollen in the middle (Samuels *et al*., 2006). Several phialides arises from the same point and were crowded. The conidia were deep to light green to yellow in coloration, ellipsoidal, lacking visible basal abscission scar and smooth (Samuels *et al*., 2006b). Chlamydospores were fertile to sparse, terminal to intercalary, globose to subglobose ranging from 8.8 to 10.1 µm. According to Samuels *et al*. (2006), *T. koningiopsis* was previously identified as *T. koningii*, common in tropical America, occurring also on natural substrata in East Africa, Europe and Canada, from ascospores in eastern North America, and as an endophyte in *Theobroma*. Tsurumi *et al*. (2010) also explored its biodiversity in Mongolia, Japan, Vietnam, and Indonesia.

Isolate 4 (UACH-246) *Trichoderma* spp. presented conidia that were subglobose to ellipsoidal, apex broadly rounded and more narrowly rounded at base. Conidiophores were highly branched, and entire structure was pyramidal and were spread to the top and smooth or rounded, wide near the base. Phialides were arisen mostly in crowded but had an angle with conidiophore and showed whorls (2 to 6) on the terminal branches. The phialides were bowling pin, lageniform and the length was 3.8 to 11.4 × 1.6 to 3.7 µm. Chlamydospores were not present.

Isolate (UACH-248) belongs to *Trichoderma atroviridis*. These isolates presented conidiophores comprising a distinct, 3.5 to 4.0 µm wide, smooth central axis from which secondary branches arise. They also present secondary branches in paired and unilateral, consisting of a single cell near the tip of the conidiophore, a single phialide or 2 to 4 divergent phialides. Phialides were flask-shaped, all branches arising at <90°, more or less swollen in the middle. Conidia were subglobose to ellipsoidal, finely warted, dark green in mass. Chlamydospores were abundant, subglobose measuring from 7.5 to 11.8 × 6.3 to 9.8 µm, terminal. *Trichoderma atroviridis* is known for promoting efficient plant growth and stress resistance and is used as a biological control agent in sustainable farming systems (Chet and Inbar 1994; Benítez *et al*., 2004; Stewart and Hill, 2014).

Isolates (UACH-252) were classified as *Trichoderma asperelloides* which developed ovoid conidia and phialides in pairs or groups of three all along the conidiophore that were wider at the center than at the base. The conidia shape was subglobose to ovoid and conidia ornamentation was finely warted. The lateral branches of conidiophores were branched. The conidial color was olive green or dark green. The conidia length ranged between 2.2 to 4.1 x 3.3 to 5.7 µm.

In a study comparing morphological characteristics (Samuels *et al*., 2010) augmented that *Trichoderma asperellum* and *Trichoderma asperelloides* are among the few common species of *Trichoderma* for which no teleomorph is known. They mentioned that it is clear that *T. asperelloides* is far less diverse than *T. asperellum* and a possibly is derived from it. Based on the results obtained from our study the diversity of *T. asperellum* suggests the relationship and adaptation of this specie to the diverse complexity of crops found in Mexico. Furthermore, the lack of diversity in *T.*

asperelloides it is possible that one of the mat genes has been lost in this species and this could have resulted in genetic isolation and possibly the sympatric speciation of these saprobes. Hence the importance of carrying out phylogenetic analysis to clarify all suspicions.

Trichoderma spp. are widespread in the soil as saprophytic fungi highly competitive to plant pathogens. *Trichoderma* species and are important candidates for biocontrol of plant pathogens (Lieckfeldt, *et al*., 1998) as the also produce high quantities of chlamyospores. This production increases their capacity to survive under extreme conditions (Lewis and Papavizas, 1984). These authors also mentioned the *Trichoderma* has the potential to form great numbers of chlamydospores in natural soil and in fragments of organic matter. This effect helps to prevent the introduction of the fungus to the soil as conidia by its aggressiveness to colonize and establish themselves in organic matter in natural environment.

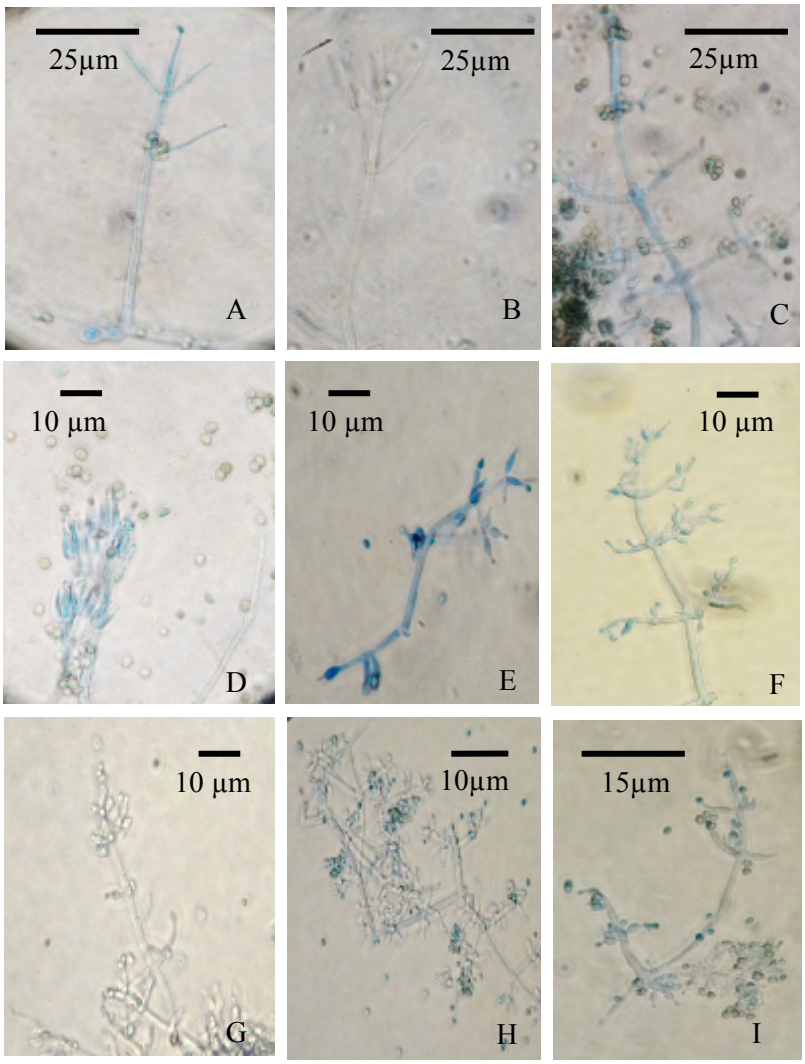


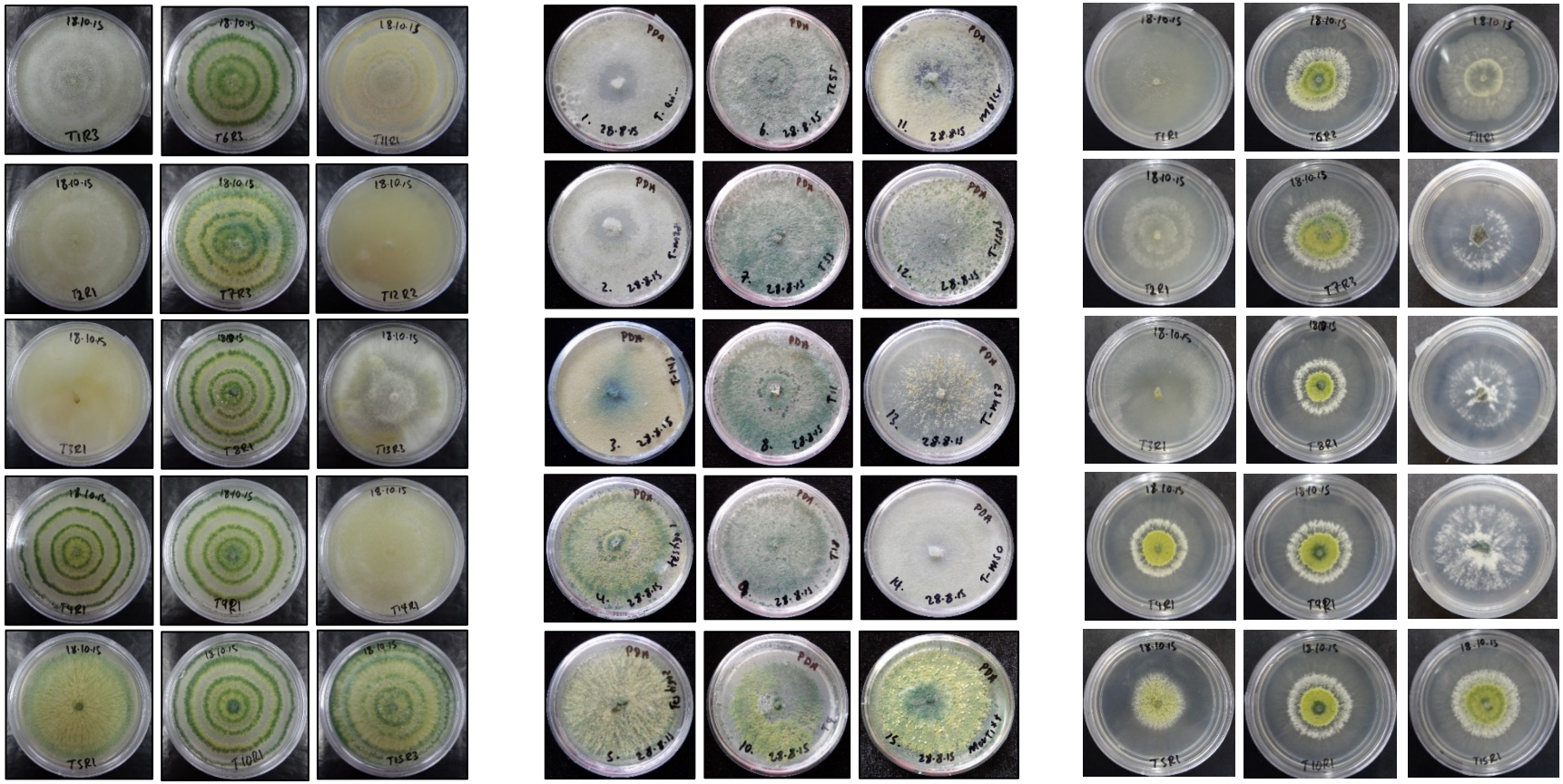
Figure 6 Morphological characteristics (conidiophores and phialides) of the Trichoderma isolates. (A)-(B) Fertile hair with long, straight, solitary and fertile apices, (C) Unpaired primary branches formed in nearly 90° to main axis, (D) Paired primary branches branching towards tips, with closely appressed phialides arised in whorls of two to three, (E)-(F) Paired primary branches, phialides held in whorls of two to three, (G)-(H) Unpaired primary branches branching towards tips, and (I) phialides solitary or held in whorls of two to three.

On the other hand, morphological characteristics are generally found to be highly variable (Kucuk and Kivanc 2003) *Trichoderma* isolates often can be readily identified to genus by a distinctive morphology that includes rapid growth, bright green or white conidial pigments, and a repetitively branched, but otherwise poorly defined

conidiophore structure. According to Druzhinina and Kubicek (2003) morphological analyses is highly prone to error and that more than half of the *Trichoderma* spp. that have been deposited in culture collection are given names that are morphologically wrong. Bissett (1991a) mentioned that a clear morphological definition for the genus *Trichoderma* would be problematic since the conidiophore branching structure is highly variable, and in many cases superficially resembles unrelated genera such as *Verticillium* and *Gliocladium*. In most of these cases, the *Trichoderma* anamorphs of Hypocrea can be differentiated in having a less regular pattern of branching at an indefinite number of levels, as opposed to the more regular verticillate or biverticillate branching in *Verticillium*; and the production of a terminal penicillate arrangement of branches and more regularly aculeate phialides on a relatively well-defined stipe in *Gliocladium*. Another example, is the convergent phialides of *Trichoderma virens* in other words *Trichoderma*-like conidiophores cannot be taken as an argument for excluding this species from *Trichoderma*. Thus, the use of gene sequence analyses is becoming more and more popular and can complete most of the phylogenetic analyses (Druzhinina and Kubicek, 2003; Liu *et al*., 2004; Szekeres, 2005; Jom-in and Akarapisan, 2009).

3.5.2. Growth rate of *Trichoderma* spp. in different PDA media concentration

This study was done to find out the best temperature and growing PDA medium for the *Trichoderma* isolates. After 7 days of incubation, the average mycelial growth rate was registered (Table 6). All the species of *Trichoderma* produced sporulation on the different PDA medium and at the temperature levels of 24, 28, and 30 °C (Figure 7)



A B C

Figure 7. Growth pattern of 15 *Trichoderma* isolates grown under three modified PDA medium, 5 days after incubation (A) Synthetic PDA media, at 24 °C, (B) Semi-synthetic PDA media, at 28 °C, and (C) Semi-synthetic PDA media, at 30 °C.

Elad *et al*., 1981 reported a *Trichoderma* selective medium as a tool for isolating *Trichoderma* species. In our study, PDA medium supplemented with streptomycin and lactic acid was chosen as the selective medium, and it turns out to be effective. In assessing the best suitable temperature for the growth of *Trichoderma* isolates, these were grown on synthetic and semi synthetic PDA medium at three temperatures: 24, 28, and 30 °C. The growth of the microorganism decreased at 30 °C, compared to the other two temperatures. The maximum colony growth of the mycelium was observed at 28 °C temperature. The statistical analysis showed that the growth of the *Trichoderma* species was significantly (P≤0.05) affected with an increase of the temperature (Table 6). The highest growth was obtained by *Trichoderma asperellum* (UACH-253 and UACH-243) with an average of 69.33 cm and 68.4, respectively, *Trichoderma koningiopsis* (UACH 248) with 66.91 cm and *Trichoderma asperelloides* (UACH 244) with 66.46 cm, in the semi synthetic PDA grown a temperature of 28 °C. A similar pattern was observed for the isolates grown in synthetic PDA medium, at 24 °C obtained lower values ranging from 64 to 66 cm. At the higher temperature of 30 °C growth of all *Trichoderma* isolates were slower compared to the 28°C and 24 °C. The average growth rate ranged from 46 to 64 mm of total growth 5 days after incubation (Table 6). *Trichoderma* spp (UACH 246) obtained the lowest value with 46.98. The optimum temperature for growth for the isolates was 28 °C and should not excide 30 °C. These results are in accordance with those of who reported that the most favorable temperature for growth and sporulation of *Trichoderma atroviridis* strain was between 25–30°C (Schwarze *et al*., 2012).

In a study related to optimal culture conditions needed for the growth of *Trichoderma* spp. *T. asperellum* obtained an optimum of 30 °C and a maximum of >35 °C, and *T. viride* has an optimum temperature of 22.5 °C and a maximum of 30°C. They mentioned that the temperature tolerance of the isolates might be an important factor among isolates since the fact that they act as promoters or inhibitors of plant growth (Lindsey and Baker, 1967; Menzies, 1993) or even as contaminants in mushroom cultures (Seaby, 1998).

Table 6. The mean average of the growth of Trichoderma isolates grown in PDA media with slight modifications.

***Trichoderma* isolates**	**Isolate code**	**Synthetic PDA media (24 °C)**	**Semi-synthetic PDA media (28 °C)**	**Semi-synthetic PDA media (30 °C)**
Trichoderma stromaticum	UACH-242	62.86 c	66.41 abc	59.60 cde
Trichoderma asperellum	UACH-243	66.67 a	68.41 ab	62.91 ab
Trichoderma asperelloides	UACH-244	65.94 ab	66.46 abc	61.78 abc
Trichoderma spp.	UACH-245	59.46 ef	66.99 ab	57.35 de
Trichoderma spp.	UACH-246	48.79 ef	59.80 e	46.98 f
Trichoderma spp.	UACH-247	62.14 cde	63.50 cd	62.03 abc
Trichoderma koningiopsis	UACH-248	65.78 ab	66.91 ab	64.59 a
Trichoderma asperellum	UACH-249	62.24 cd	65.56 bc	59.99 bcd
Trichoderma asperellum	UACH-250	61.39 cde	66.45 abc	59.61 cde
Trichoderma asperellum	UACH-251	61.36 cde	53.37 f	59.41cde
Trichoderma atroviridis	UACH-252	58.56 f	60.60 de	56.64 e
Trichoderma asperellum	UACH-253	64.01 abc	69.33 a	62.28 abc
Trichoderma asperellum	UACH-254	62.28 cd	63.41 dc	49.11 f
Trichoderma asperellum	UACH-255	60.11 def	54.57 f	60.69 bc
Trichoderma asperellum	UACH-256	63.57 bc	61.02 de	61.95 abc
HSD		45.65	50.25	44.27

Note: HSD: Honest significant difference
Different letters in each column indicates significant difference (Tukey, $P<0.05$).

In a study carried out by (Souza, 2006) on the mycelial growth and sporulation for all 91 *T. stromaticum* isolates grown in two different culture media (CMA and PDA), at 25, 30, and 35 °C indicated that none of the isolates were able to grow on both media at 35 °C. On CMA, there was a significant difference ($P \leq 0.001$) in growth rate at 25 and 30 °C. On PDA, no significant differences in growth rates were observed between groups of isolates for each temperature ($P \leq 0.1$), although a significant reduction ($P \leq 0.001$) in growth was observed at 30 °C. They also examined sporulation level by visual inspection after 14 days of incubation on both media and at both temperatures and found that at 25 °C, all isolates sporulated on both media, however, at 30 °C only about 55 % isolates sporulated on CMA and none were able to sporulate on PDA.

The growth of these organisms is a consequence of the nutritional sources used as also of the physical conditions (temperature and moisture levels) employed to cultivate them. In another study conducted by Bandopadhyay *et al*., 2003 they found that the optimum at temperature for the growth and sporulation of *Trichoderma* species were at 25-30 °C. In general, the optimum temperature for the growth of many *Trichoderma* spp. is between 25 and 30 °C (Klein and Eveleigh, 1998). There is considerable experimental evidence that *Trichoderma* germination and growth rates are proportional to water activity and temperature (Kredics *et al*., 2003), up to some limiting value that depends on the climatic origin of a given *Trichoderma* isolate (Ribera *et al*., 2016; Schwarze *et al*., 2012); but it was assumed that tolerance to a wide range of unfavorable temperature and moisture conditions is not critical for biological control by *Trichoderma*. Singh and Sudhir (2009) also reported the same findings and observed that temperature had a significant influence on the growth and sporulation of eight *Trichoderma* species in which the most favorable temperature was found between 25-30 °C. Kunming (2004) examined the potential application value of *Trichoderma harzianum* Th azad strains (Th-B) in culture media on different temperatures and observed that the strain grew best at 15-35 °C and optimum growth is seen at 25-30 °C. Sharma *et al*. (2005) reported that none fungi could grow at temperature 40 °C. Singh *et al*. (2011) reported that the most favorable temperature for growth and sporulation of *Trichoderma atroviride* was between 25-30 °C (240-260 mg dry wt.), followed by 40 °C (60 mg dry wt.).

3.5.3. Dual culture technique of *Trichoderma* spp against *Fusarium oxysporum*, *in vitro*

Fifteen *Trichoderma* isolates were screened *in vitro* for their antagonism against the *F Fusarium oxysporum* by using the dual cultural technique. Mycelial interaction is a basic method to assess antagonistic properties of microorganisms such as *Trichoderma* spp. The single cultures *Trichoderma* spp. grew actively and colonized the entire PDA medium surface within just five days, whereas the *Fusarium oxysporum* isolates took a least 8 days to fill the petri dishes. Growth inhibition *Fusarium oxsyporum* during the *in vitro* interaction with *Trichoderma* species at 4 and 6 DAI are shown in (Figure 8). Percent growth inhibition of pathogen was higher in *Trichoderma asperellum* (UACH-250) (66.73%) followed by *Trichoderma asperelloides* (UACH-248) (66.70%), and *Trichoderma asperellum* (UACH-243) (62.86%). While *Trichoderma koningiopsis* (UACH-244) and *Trichoderma stromaticum* (UACH-242) showed less reduction in growth of the pathogen obtaining 56.38 and 46.71% respectively at 4 DAI (Figure 8). The reduction of mycelial growth and spore production of the pathogen was higher in the dual culture Petri dishes compared with the pathogen control (UACH-251). Now, at 6 DAI the similar pattern of growth inhibition was observed with maximum increases 9.77% in (UACH-250) (76.5%) followed by 8.80% in T5 (69.81.6%), and by 8.79% in (UACH-248) (70.88). The isolate (UACH-242) recorded values below 53% of growth inhibition at both evaluation dates. Thus, in general, isolates *T. asperellum* and *T. asperelloides* showed better growth inhibition towards *Fusarium oxsyporum* compared to the other *Trichoderma* spp.

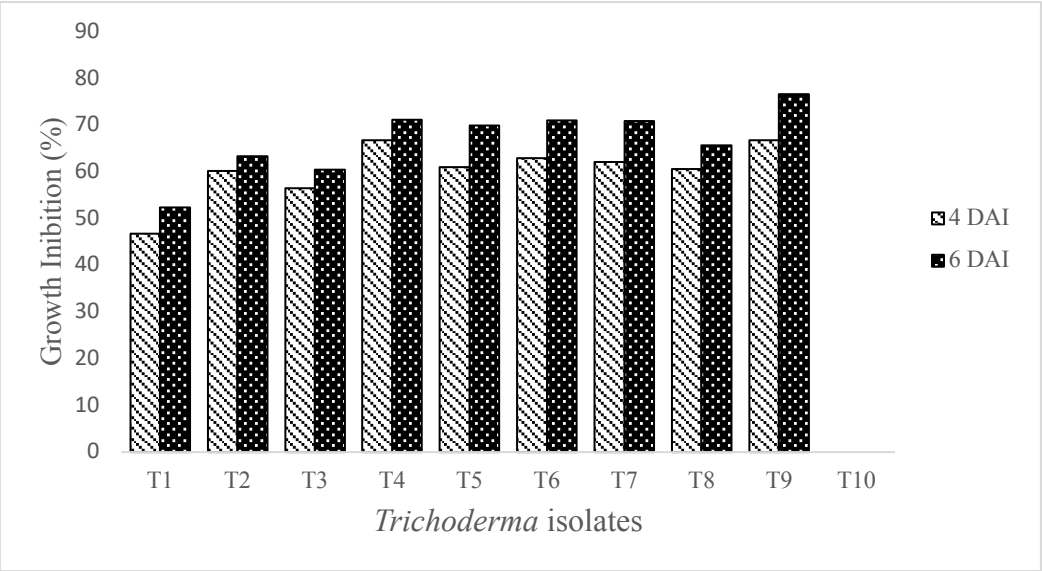


Figure 8. Percentage of growth inhibition of *Fusarium oxsyporum* during in vitro antagonism with *Trichoderma* spp. at 4 and 6 DAI. UACH-242 = *T stromaticum* × *Fusarium oxsyporum*, UACH-243 = *T asperellum* × *Fusarium oxsyporum*, UACH-244 = *T koningiopsis* × *Fusarium oxsyporum*, UACH-244 = *T asperelloides* × *Fusarium oxsyporum*, UACH-249 = *T asperellum* × *Fusarium oxsyporum*, UACH 250 = *T asperellum* × *Fusarium oxsyporum*, UACH-251 = *T asperellum* × *Fusarium oxsyporum*, UACH-252 = *T atroviridis* × *Fusarium oxsyporum*, UACH 253 = *T asperellum* × *Fusarium oxsyporum*, Control = *Fusarium oxsyporum* only.

This probably due to the limitation of available nutrients and space. The level of inhibition increased as the cultures developed in age (48 and 72 hours after incubation), when the pathogen had little space to grow, and when there was no clear zone of inhibition between the antagonist and pathogen (Figure. 9). The ability of *Trichoderma* spp. to produce metabolites in the growth inhibition of pathogens such as *Rhizoctonia solani*, *Curvularia lunata*, *Bipolaris sorokiniana*, and *Colletotrichum gloeosporioides* has been reported by several authors (Yang *et al*., 2006; Monteiro *et al*., 2010; Zivkovic *et al*., 2010). Its mechanisms vary from volatile antibiotics, i.e. 6-pentyl-a-pyrone (6PP) and most of the isocyanide derivates; water-soluble compounds; and peptaibols, which are linear oligopeptides of 12–22 amino acids rich in a-aminoisobutyric acid and N-acetylated. (Le Doan *et al*., 1986; Rebuffat *et al*., 1989; Harman *et al*., 2004; Vinale *et al*., 2008). These metabolites induce the production enzymes and trigger a cascade of physiological changes, stimulating rapid and directed growth of *Trichoderma* sp. (Zeilinger *et al*., 1999), thus reducing the growth and development of the pathogen.

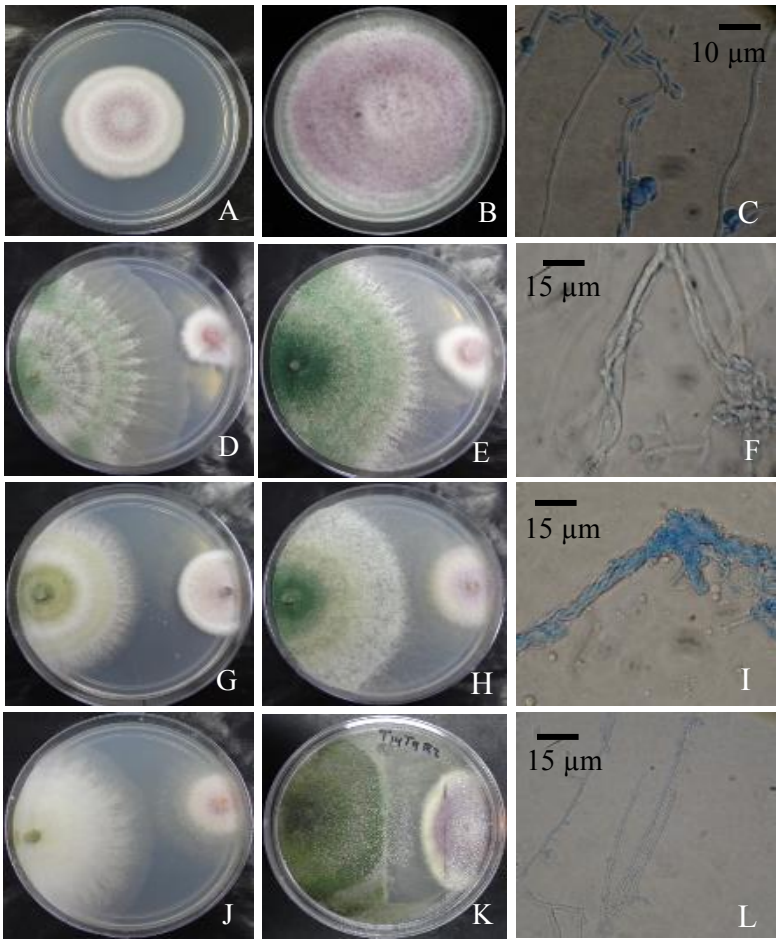


Figure 9. Dual culture technique the *Fusarium oxysporum* and *Trichoderma* isolates, 3-6 days after establishing the experiment. A)-B) *Fusarium oxysporum* (UACH-221) used as the control C) Macro y micro conidios of *Fusarium oxysporum*. D)-E) *Trichoderma asperellum* F) High level of coiling by *Trichoderma asperellum* G)-H) *Trichoderma asperelloides* I) High coiling level by *Trichoderma asperelloides* J)-K) *Trichoderma stromaticum* L) *Trichoderma stromaticum* penetrating the mycelium of *Fusarium.*

3.5.4. Mycoparasitic activity of *Trichoderma* spp.

The first apparent physical contact between the *Trichoderma* and *Fusarium* isolates occurred within 3-4 DAI, followed by growth inhibition at 6 DAI. *Trichoderma asperellum* (UACH-253) showed maximum pathogen growth inhibition. *Trichoderma asperellum* (UACH- 253) showed signs of mycoparasitism by destroying the host and stopping its sporulation (Figure 9).

Antibiosis and mycoparasitism are the well known mechanisms involved in biocontrol of pathogens by *Trichoderma*; competition for nutrition, space and dominance being equally important and mutually inclusive phenomenon. The complete course of interaction between *Trichoderma* spp. and *Fusarium oxysporum* as observed on the dual culture plates can be divided into three phases. The evaluation under microscope showed that *Trichoderma asperellum* was capable of overgrowing and degrading *Fusarium oxysporum* mycelia, coiling around the hyphae with apressoria and hook-like structures (Figure 9 and 10). The complex mechanisms of mycoparasitism, which include directed growth of *Trichoderma* and attachment and coiling of *Trichoderma* enabled the hyphae of *Trichoderma* spp. to firmly attach to the surface of its host mycelium. However, not all the isolates used the same mechanism, some antagonists used other mechanism against *Fusarium* just like non coiling effects with penetrating the hyphae. While, *T. asperellum* (UACH-253) showed the accumulation of conidia around the mycelium of the pathogen and not directly attaching itself to the hyphae (Figure 9). The above can be considered as phases in the process of mycoparasitism by the *Trichoderma* spp.

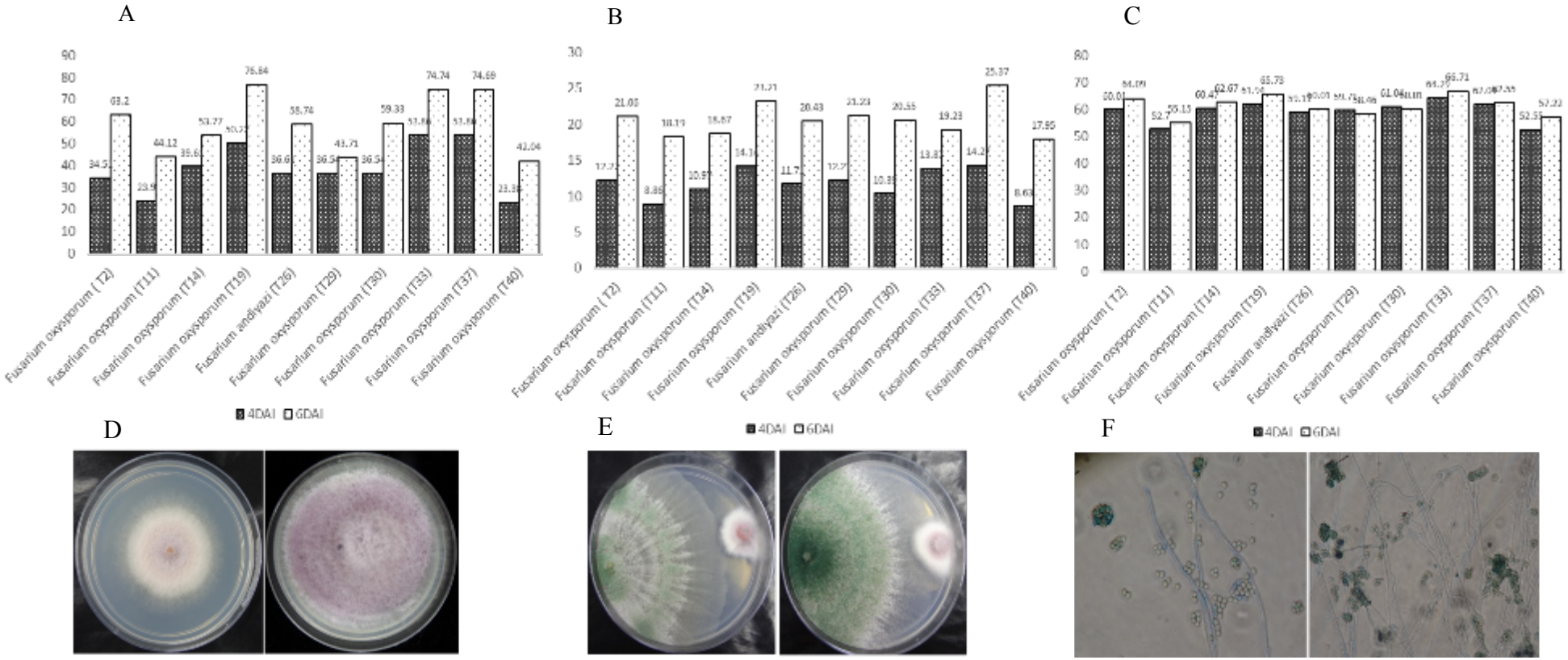


Figure 10. Percentage of inhibition and growth rate of the *Fusarium* isolates in relation to the *Trichoderma* isolates 3 and 6 days after the inoculation (DAI). A) Growth of the *Fusarium* isolates with *Trichoderma asperellum* B) Growth of the *Fusarium* isoaltes with the *Trichoderma asperelloides* C) Growth of the *Fusarium* isoaltes with the *Trichoderma stromaticum* D) Fusarium control isolates E) Dual culture technique F) Conidios of *Trichoderma* spp.

According to Vinale *et al*., (2008) the initial phase marked by interaction without mycelia contact in which diffusible metabolites from both the organisms decide the fate of interaction. The intermediate phase in which *Trichoderma* may or may not be able to overcome the inhibitory effect of *Fusarium*. In the intermediate phase, some chemo-attractive mechanisms may also effect the growth of the species by penetrating the hyphae. And, the final phase where *Trichoderma* parasitizes and kills the *Fusarium*. Moreover, the antagonistic activity of the *Trichoderma* strains is generally related to the hydrolytic enzyme activity especially chitinase (chitin) and β (1,3) glucanase (pachyman). These enzymes are closely related to mycoparasitism, and on the other hand, they are contributing to the host induced systemic resistance (ISR) to pathogen infection. Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host. Differential antagonistic activity has been observed for various *Trichoderma* spp., which demonstrates semi-specificity in the interaction of *Trichoderma* with *M. phaseolina*. Direct mycoparasitism is considered to be the main mechanism of action for *T. asperellum* s. lat. (Viterbo and Chet 2006, Nagayama *et al*., 2007, Tondje *et al*., 2007). Tondje *et al*. (2007) reported that culture filtrates of *T. asperellum* s. str. contain substantial laminarinase activity and lesser amounts of carboxymethylcellulase, which may function in degrading walls of *Phytophthora megakarya*. Dharmaputra et al. (1994) tested two isolates of *T. harzianum* and one isolate of *T. viride* against Ganoderma and found that all isolates inhibited the mycelial growth of the pathogen, but *T. harzianum* (isolate B10-1) showed the best performance. Etabarian (2006) reported that *T. viridie* (MO) reduced the colony area of *Macrophomina phaseoli* by 19.2 and 34.9 % using the dual culture and cellophane methods, respectively. Other than mycelial interaction and hyperparasitism by the *Trichoderma* species, scientists have also considered the action use of antibiotic metabolites as a contributing mechanism in the biocontrol of plant pathogens (Ghisalberti and Rowland, 1993). This study showed that secondary metabolites produced by *Trichoderma* strains were effective inhibitors of growth of *C. paradoxa*. The ability of *Trichoderma* species to produce inhibitory substances against microorganisms has been described by Dennis and Webster (1971) and Jinantara (1995).

Molecular characterization of the *Trichoderma* isolates

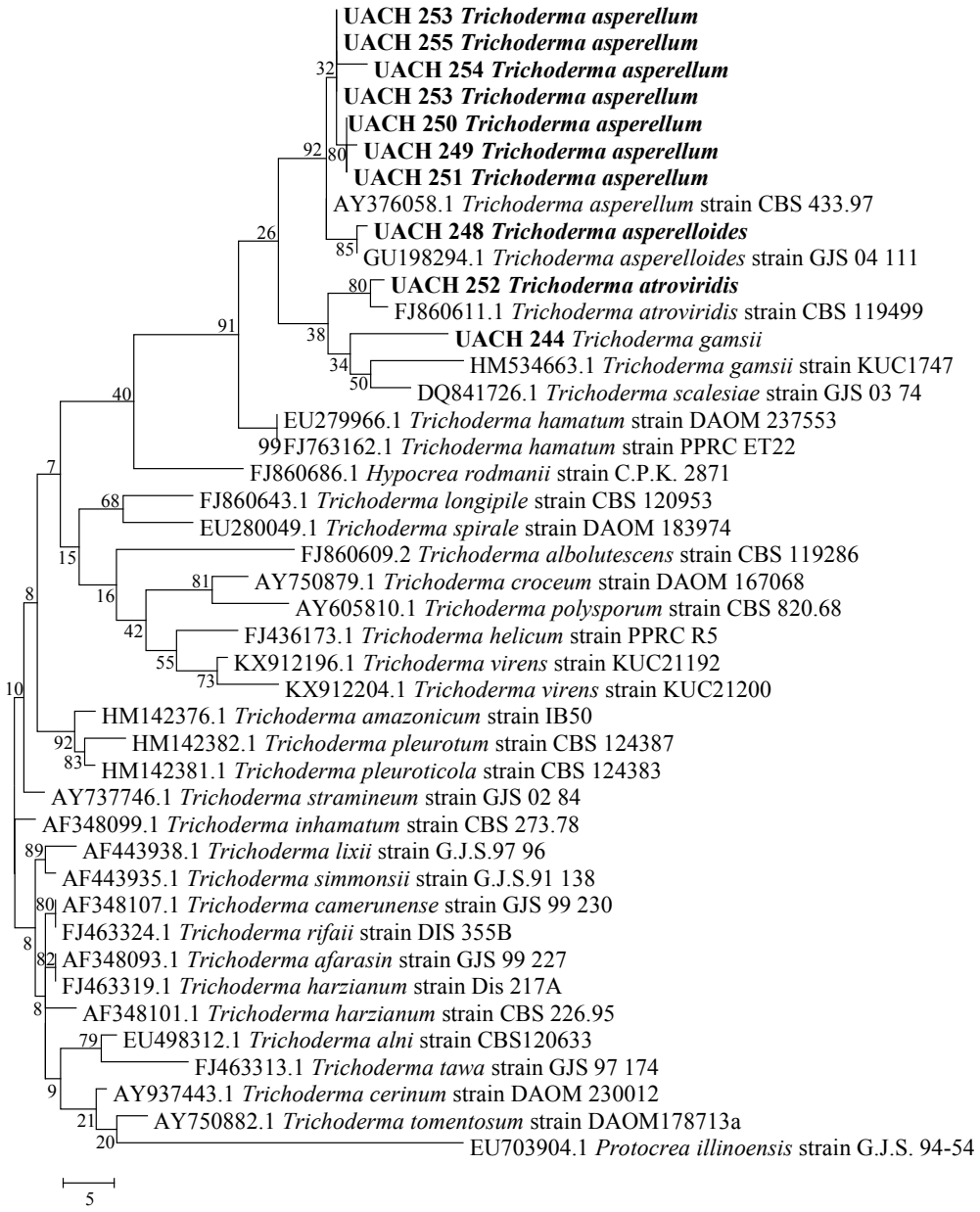


Figure 11. Figure. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 263 is shown. The consistency index is (0.438914), the retention index is (0.769944), and the composite index is 0.406929 (0.337939) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 152 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

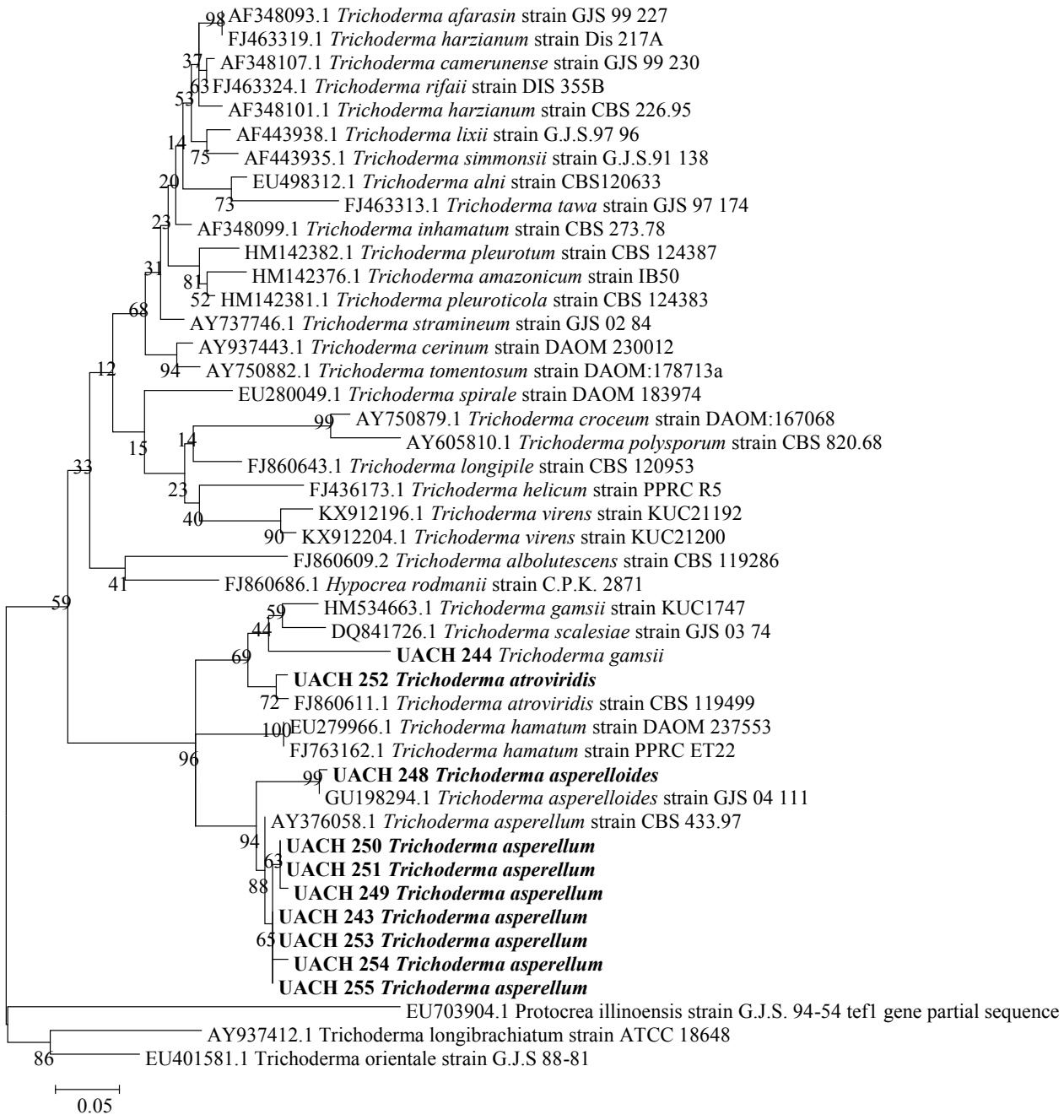


Figure 12. Molecular Phylogenetic anaylsis by Maximum Likelihood Method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2048.1709) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 168 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

3.6. CONCLUSIONS

In the present study five species of *Trichoderma* (*T. asperellum*, *T. stromaticum*, *T. atroviride, T. koningiopsis,* and *Trichoderma* spp.) were identified in association with wilting disease of tomato in Mexico, which will be very helpful information for developing new strategies for the adequate control of this pathogen.

Some of the isolates of *Trichoderma* spp. included in this study significantly inhibited the growth of the pathogen. All the Trichoderma isolates inhibited the growth ranging from 40 to 70 %. Thus, it is well known that all isolates collected from different samples of soil are not equally antagonistic to pathogen and searching of effective isolate to locally suit the purpose is important.

It was found that the isolates the different species of *Trichoderma* have mycoparasitic activity on *F. oxysporum*, observing coiling and penetration of the antagonist hyphae over those of the pathogen.

The molecular analysis confirman that *Trichoderma asperellum* is the most prodominat species amoug the isolates used in the work.

3.7. REFERENCES

Bandopadhyay, S., Subhendu, J.and Dutta, S. (2003). Effect of different pH and temperature levels on growth and sporulation of Trichoderma. Environment and Ecology 21: 770-773.

Barnett H L, Hunter BB (1998) Illustrated Genera of Imperfect Fungi. 4ª ed. Aps Press, USA

Benítez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7:249–260.

Bissett, J. (1991a): A revision of the genus Trichoderma. II. Infrageneric classification. Can. J. Bot. 69, 2357 – 2372.

Boix-Ruíz, A., Gálvez-Patón, L., de Cara-García, M., Palmero-Llamas, D., Camacho-Ferre, F., & Tello-Marquina, J. C. (2014). Comparison of analytical techniques used to identify tomato-pathogenic strains of *Fusarium oxysporum*. Phytoparasitica, 43(4), 471–483. https://doi.org/10.1007/s12600-014-0444-z

Chakrabarti, A. (2013). Genomics of Soil- and Plant-Associated Fungi (Vol. 36). https://doi.org/10.1007/978-3-642-39339-6

Chet I, Inbar J (1994) Biological control of fungal pathogens. Appl Biochem Biotechnol 48:37–43.

Druzhinina I. and Kubicek CP. 2003. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters. J. Zhejiang Univ. SCI. 6 B (2):110-112

Druzhinina IS, Kopchinskiy AG, kubicek CP (2006). The first 100 *Trichoderma* species characterized by molecular data. Mycoscience 47:55-64.

Elad, Y., Chet, I. & Henis, Y. (1981). A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica. 9, 59–67.

Edington LV, Khew KL, Barron GI (1971). Fungitoxic spectrum of benzimidazole compounds. Phytopathology 61: 42-44

FAO. (2016). FAO stat. Annual Crop Production Statistics. Food and Agriculture Organization of the United Nations. Statistics Division. Retrieved from http://faostat3.fao.org/download/Q/QC/E

Hermosa MR, Grondona I, Iturriaga EA (2000) Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Appl Environ Microbiol 66:1890-1898.

Hoyos-Carvajal, L., Orduz, S., & Bissett, J. (2009a). Genetic and metabolic biodiversity of Trichoderma from Colombia and adjacent neotropic regions. Fungal Genetics and Biology.Vol. 46. No. 9, (April 2009), pp. 615–631.

Jom-in S, Akarapisan A. 2009. Characterization of double-stranded RNA in *Trichoderma* spp. isolates in Chiang Mai province. J. Agricult. Techmol. 5(2):261-270.

Katarzyna Nawrot-Chorabik (2013). The Use of Interactions in Dual Cultures *in vitro* to Evaluate the Pathogenicity of Fungi and Susceptibility of Host Plant Genotypes, Environmental Biotechnology - New Approaches and Prospective Applications, Prof. Marian Petre (Ed.), In Tech.

Klein D, Eveleigh DE. Ecology of Trichoderma. In: Kubicek CP, Harman GE, editors. Trichoderma and Gliocladium. Vol. 1. Basic Biology, Taxonomy and Genetics. London: Taylor and Francis Ltd.; 1998. pp. 57–74.

Kredics L, Antal Z, Doczi I, Manczinger L, Kevei F, Nagy E. Clinical importance of the genus Trichoderma. A review. Acta Microbiol Immunol Hung. 2003; 50:105–117.

Kubicek, C., Bissett, J., Druzhinina, I., Kulling-Grandiger, C., Szakacs, G. (2003). Genetic and metabolic diversity of Trichoderma: a case study on South East Asian isolates. Fungal genetics and biology. Vol. 38, No. 3, pp. 310-319.

Kucuk C, Kivanc M. 2003. Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical, and physiological features. Turk J. Biol. 27:247-253.

Kulling, C., Szakacs, G., & Kubicek, C. (2000). Molecular identification of Trichoderma species from Russia, Siberia and the Himalaya. Mycological Research. Vol. 104, No. 9, (December 1999), pp. 1117-1125.

Le Doan, T., El-Hajii, M., Rebuffat, S., Rajeswari, M.R., Bodo, B., 1986. Fluorescein studies on the interaction of trichorzianine A IIIc with model membranes. Biochimica et Biophysica Acta 858, 1–5.

Lewis, J.A. and Papavizas, G.C. 1984. Chlamydospore formation by *Trichoderma* spp.in natural substrates. Can. J. Microbiol., 30: 1-7.

Leyva-Mir, S. G., González-Solano, C. M., Rodríguez-Pérez, J. E., & Montalvo-Hernández, D. (2013). Behavior of advanced lines of tomato (*Solanum lycopersicum* L.) to phytopathogens at Chapingo, Mexico. Revista Chapingo Serie Horticultura, XIX (3), 301–313. https://doi.org/10.5154/r.rchsh.2012.12.070

Lieckfeldt E, Kuhls K, Muthumeenakshi S. 1998. Molecular taxonomy of *Trichoderma* and *Gliocladium* and their teleomorphs. In: Kubicek C P, Harman G E, editors. *Trichoderma* and *Gliocladium*. London, United Kingdom: Taylor & Francis Ltd. pp. 35–56.

Liu X, Zhuang J-H, Gao Z-G, Yang C-C, Chen J. 2004. Construction of engineering *Trichoderma* strains and their characteristics against tomato gray mold. J. Zhejiang Univ. (Agric. & Life Sci.) 30(4):419-420.

Lindsey D L, Baker R. 1967. Effect of certain fungi on dwarf tomatoes grown under gnotobiotic conditions. Phytopathology.;57:1262–1263.

Menzies J G.1993 A strain of *Trichoderma viride* pathogenic to germinating seedlings of cucumber, pepper and tomato. Plant Pathol. 1993; 42:784–791

Migheli Q, Balmas V, Komon˜-Zelazowska M, Scherm B, Fiori S, Kopchinskiy A, Kubicek CP, Druzhinina S. 2009. Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of Hypocrea/Trichoderma. Environ Microbiol 11:35–46.

Mokhtar, H., & Dehimat, A. (2012). Antagonism capability *in vitro* of *Trichoderma harzianum* against some pathogenic fungi. Agriculture and Biology Journal of North America, 3(11), 452–460. https://doi.org/10.5251/abjna.2012.3.11.452.460

Monteiro VN, Silva RN, Steindorff AS, Costa FT, Noronha EF, (2010) New insights in *Trichoderma harzianum* antagonism of fungal plant pathogens by secreted protein analysis. Curr Microbiol 61: 298–305.

Papavizas, G. C., and Lumsden, R. D. 1982. Improved medium for isolation of *Trichoderma* spp. from soil. Plant Dis. 66:1019-1020.

Pomella, A.W.V., De Souza, J.T., Niella, G.R., Bateman, R.P., Hebbar, K.P., Loguercio, L.L., Lumsden, R.D., 2007.*Trichoderma stromaticum* for management of witches' broom in Brazil. In: Vincent, C., Goettel, M., Lazarovits, G. (Eds.), Biological Control: A Global Perspective, Case Studies from Around the World. CABI Publishing, Wallingford, UK, pp. 210-217.

Ribera, J., Tang, A.M.C., Schubert, M., Lam, R.Y.C., Chu, L.M., Leung, M.W.K., 2016. *In-vitro* evaluation of antagonistic *Trichoderma* strains for eradicating *Phellinus noxius* in colonised wood. J Trop For Sci. 28, 457-468.

Rebuffat, S., El Hajji, M., Hennig, P., Davoust, D., Bodo, B., 1989. Isolation, sequence and conformation of seven trichorzianines B from *Trichoderma harzianum*. International Journal of Peptide and Protein Research 34, 200–210.

Rojo, F. G., Reynoso, M. M., Ferez, M., Chulze, S. N., & Torres, A. M. (2007). Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. Crop Protection, 26(4), 549–555. https://doi.org/10.1016/j.cropro.2006.05.006

Sanogo, S., Pomella, A. W. V., Hebbar, P. K., Bailey, B., Costa, J. C. B., Samuels, G. J., and Lumsden, R. D. 2002. Production and germination of conidia of

Trichoderma stromaticum, a mycoparasite of *Crinipellis perniciosa* on cacao. Phytopathology 92:1032-1037.

Samuels GJ (2006) *Trichoderma*: Systematics, the sexual state, and ecology. Phytopathology 96:195-206.

Samuels, G., Suarez, C., Solis, K., Holmes, K., Thomas, S., Ismaiel, A., & Evans, H. (2006b). *Trichoderma theobromicola* and *T. paucisporum*: two new species isolated from cacao in South America. Mycological Research.Vol. 110, No. 4, pp. 381–392

Samuels, G, Ismaiel, A. (2009). *Trichoderma evansii* and *T. lieckfeldtiae* two new *T. hamaturn*-like species. Mycologia. Vol. 101, No. 1, pp. 142-156.

Samuels G, Ismaiel A, Bon M De Respinis S Petrini O (2010). *Trichoderma asperellum* sensu lato consists of two cryptic species. Mycologia. 102. 944-66. 10.3852/09-243.

Samuels, G. J., Ismaiel, A., de Souza, J. & Chaverri, P. (2012) *Trichoderma stromaticum* and its overseas relatives. Mycological Progress. 11, 215–254

Schmoll, M., & Schuster, A. (2010). Biology and biotechnology of *Trichoderma*. Applied Microbiology and Biotechnology, 87(3), 787–799.

Schwarze, F.W.M.R., Jauss, F., Spencer, C., Hallam, C., Schubert, M., 2012. Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. Biol Control. 61, 160-168.

Seaby D. 1998.*Trichoderma* as a weed mould or pathogen in mushroom cultivation. In: Kubicek C P, Harman G E, editors. *Trichoderma* and *Gliocladium.* London, United Kingdom: Taylor & Francis Ltd; pp. 267–288.

Sharma, R.L., Singh, B.P., Thakur, M.P. and Thapak, S.K. (2005). Effect of media, temperature, pH and light on the growth and sporulation of *Fusarium oxysporum* f.sp. *lini*, Ann. Pl. Protec. Sci. 13: 172 -174.

Shoresh M, Harman GE (2008a). The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: A proteomic approach. Plant Physiol147:2147-2163.

Singh, O.P. and Kumar, S. (2009) *Trichoderma* spp. Growth as influenced by Temperatures. Ann. Pl. Prot. Sci. 17: 225-274.

Singh, A., Shahid, M., Pandey, N.K., Kumar, S., Srivastava, M. and Biswas, S.K. (2011). Influence of Temperature, pH and media for growth and sporulation of *Trichoderma atroviride* and its shelf life study in different carrier based formulation. J. Pl. Dis. Sci. 6: 32-34.

Shankara, N., de Jeude, J. V. L., de Goffau, M., Hilmi, M., & van Dam, B. (2005). Cultivation of tomato. Agromisa Fondation and CTA Wageningen.

Steyaert, J.M., R.J. Weld, A. Stewart, 2010. Isolate-specific conidiation in Trichoderma in response to different nitrogen sources. Fungal Biology 114: 179-188

Stewart A, Hill R (2014) Applications of Trichoderma in plant growth promotion. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG (eds) Biotechnology and biology of *Trichoderma.* Elsevier, London, pp 415–428.

Szekeres A. 2005. Echophysiological and molecular investigation of *Trichoderma* strains isolated from winter wheat rhizosphere. Dissertation Summary. Acta Biologica Szegediensis 49(3-4):61.

Tsurumi, Y., Inaba, S., Susuki, S., Kamijo, S., Widyastuti, Y., Hop, D., Balijinova, T., Sukarno, N., Nakagiri, A., Susuki, K., & Ando, K. (2010). Distribution of Trichoderma species in four countries of Asia. 9th International Mycological Congress. Edimburg, Scotland.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., & Lorito, M. (2008). Trichoderma-plant-pathogen interactions. Soil Biology and Biochemistry, 40(1), 1–10.

Webster J. 1964. Culture studies on Hypocrea and Trichoderma. I. Comparison of perfect and imperfect states of H. gelatinosa, H. rufa, and Hypocrea sp. 1. Trans Br Mycol Soc.;47: 75–96.

Yang, D.M., Y. Bi, X.R. Chen, Y.H. Ge and J. Zhao, 2006. Biological control of postharvest diseases with *Bacillus subtilis* (B1 strain) on muskmelons (*Cucumis melo* L. cv. Yindi). Acta Horticulturae, 712: 735-740.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M., Kubicek, C.P., 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics and Biology 26, 131–140.

Zivkovic, S., S. Stojanovic, Z. Ivanovic, V. Gavrilovic, T. Popovic and J. Balaz, 2010. Screening of antagonistic activity of microorganisms against *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*. Arch. Biol. Sci., 62: 611-623.

4. ANTAGONISTIC POTENTIAL OF *Trichoderma* SPECIES AGAINST *Fusarium oxysporum* IN TOMATO PLANTS GROWN UNDER GREENHOUSE CONDITIONS

4.1. ABSTRACT

The efficacy of *Trichoderma* species may differ due to variations in ecosystems. This study was conducted to assess the biocontrol efficacy of *Trichoderma* isolates against The objective of the present investigation was to evaluate the efficacy of the biological control of *Fusarium oxysporum*, by means of six Trichoderma isolates in two substrates under greenhouse conditions. For this, in 30-day-old tomato plants were established in soil substrate and coconut fiber, where two procedures were performed to evaluate the biological control of the following isolates *T. stromaticum* UACH-242, *T. asperellum* UACH-243, *Trichoderma* spp UACH-246, *Trichoderma asperelloides* UACH-248, *T. atroviridis* UACH-252, and *T. asperellum* UACH-253. The first method consisted in the simultaneous inoculation of the corresponding Trichoderma isolates and a pathogenic strain of *Fusarium oxysporum*. In the second inoculation method the tomato plants were inoculated first with *Fusairum oxysporum* and later with *Trichoderma* spp. Likewise, control plants inoculated only with *Fusarium oxysporum* were used for the experiment. They showed a lower incidence compared to the plants infested with *Fusarium* spp. ($P \leq 0.05$). The plants inoculated with the two isolates: *Trichoderma asperellum* (UACH-253) and *Trichoderma atroviridis* (UACH-252) obtained adequate yields inspite the fact that tomato plants were inoculated with the pathogen under greenhouse conditions showing proper performance against Tomato wilt pathogen under greenhouse conditions. For this we recommened that these isolates should be evaluated under field condition, and should therefore be considered for further studies.

4.2. RESUMEN

La eficacia del control biológico de *Fusarium oxysporum,* agente causal de la marcitez del tomate, mediante *Trichoderma*, es modificada por las condiciones de los sistemas de producción. El objetivo de la presente investigación fue evaluar la eficacia del

control biológico de *Fusarium oxysporum*, mediante seis aislados de *Trichoderma* en dos sustratos bajo condiciones de invernadero. Para ello, en plantas de tomate de 30 días de edad establecidas en sustrato de suelo y firba de coco, utilizando los siguientes asildos, *T. stromaticum* UACH-242, *T. asperellum* UACH-243, *Trichoderma* spp UACH-246, *Trichoderma asperelloides* UACH-248, *T. atroviridis* UACH-252, y *T. asperellum* UACH-253, se realizaron dos procedimientos para evaluar el control biológico. El primero consistió en la inoculación simultánea del aislado correpondiente de *Trichoderma* y una cepa patógénica de *Fusarium oxysporum.* El segundo método de inoculación se realizado fue la inoculación del *Fusairum oxysporum* y posteriormente los asilados de Trichoderma. Así mismo, se realizó un testigo con inoculación únicamente de *Fusarium oxysporum*. La mostraron una menor incidencia en comparación con el control infestado con *Fusarium* spp. ($P \leq 0.05$). Dos aislamientos: *Trichoderma asperellum* (UACH-253) y *Trichoderma atroviridis* (UACH-252) mostraron un rendimiento adecuado contra el patógeno de marchitez del tomate en condiciones de invernadero, aunque estos aislados deben evaluarse en condiciones de campo, sin embargo, parecen ser prometedores aislados para más estudios.

4.3. INTRODUCTION

Fusarium oxysporum is an abundant saprophyte (Karim *et al*., 2016) in soil and organic matter and is found colonizing the rhizosphere of many plant species (Moe, 2013; Szoboszlay *et al*., 2016), and is becoming an increasing problem globally (Pinaria *et al*., 2010; Macedo *et al*., 2017). *Fusarium oxysporum* is a fungal pathogen that infects plant roots, rapidly colonizes xylem tissue, restricting water and nutrient uptake, causing wilting, and eventually kills the plant (Gawehns *et al*., 2014). *Fusarium* produces chlamydospores (Leslie and Summerell, 2006) within infected plant and root tissues and can persist in the soil for up to 20 years or more (Alabouvette, 1986; Peng, *et al*., 1999), preventing the optimal development of the host plant (Recorbet, & Alabouvette, 1997).

The genus Fusarium has numerous specialized forms known as formae speciales (f.sp.) (Van Dam *et al*., 2016) that infects a range of host plants causing diseases such

as Vascular wilt (Lanubile *et al*., 2016), Fusarium wilt (Ajilogba and Babalola, 2013), Crown-rot (Vitale *et al*., 2014), Root-rot (Huang *et al*., 2011), and Damping-off (Maciel *et al*., 2017). Soil-borne pathogens are considered as some of the most problematic in crop protection there are difficult to observe, control and avoiding their occurrence is almost impossible (Latiffah *et al*., 2007). *Fusarium* spp. can damage up to 100 different species and can produce a series of toxic secondary metabolites that are a threat to the agriculture (Berges *et al*., 2013).

Greenhouse technology has the advantage of precise manipulation of the environmental conditions that can directly determine high yields and quality of the crops (Chen *et al*., 2011), and prevents of fungal diseases such as *Alternaria solani*, *Phytophthora infestans*, *Botrytis cinerea*, and *Leveillula taurica*. Pest and disease control is probably the greenhouse practice with the greatest impact on the environment, however as the use of protected cultivation develops, plants become more susceptible to pests for several reasons, including monoculture cultivation and the use of selected, high-yielding varieties which sometimes stimulate pest and disease development (Raviv and Antignus, 2004). Biological control is gaining higher importance among farmers and researchers because of its efficiency and low negative impact on the environment (Mandeel, 2006; Carvalho, 2014). Furthermore, this alternative is perceived to be safer and to have a minimal environmental impact (Brimmer and Boland 2003). It also, helps to increase the activity of the secondary metabolism and root systems of the plants (Ding *et al*., 2014; Bahraminejad *et al*., 2015).

One of the most popular microorganisms for biocontrol of *Fusarium oxysporum* is *Trichoderma* spp., (Bissett 1991a) that can be found in different types of soil (agricultural land, grasslands, forests, and dessert soil), over a wide range of climatic zones (Zhang *et al*., 2005). This fungus has the ability to colonize under distinctive environmental conditions due to its high reproductive capacity (Bissett 1991b, Harman et al. 2004), low nutritional requirements and its growth capacity which is favored by organic matter, moisture content, and optimal temperature conditions of 25-30 °C (Srobar, 1978; Bhai & Dhanesh, 2008). *Trichoderma* spp., also has the capacity to survive under extreme conditions such as low and high temperatures, pH and salty

environments (Lewis and Papavizas, 1984; Asha *et al*., 2013). *Trichoderma* spp. have a symbiotical relation with the roots of the plants, helping the nutrient flux, the development of the plants (Althaf and Srinivas, 2013), and in the synthesis and liberation of enzymes such as polysaccharoses, cellulose, xylanases and quintanses (Verma *et al*., 2007). Some studies have also proven that the biological control can stimulate the secondary metabolism resulting in higher concentrations of bioactive substances (Mukherjee *et al*., 2012b; Abdelrahman *et al*., 2016). Many species of *Trichoderma*, if given optimal conditions, establish stable and long-lasting colonisations of root surfaces and even penetrate into the epidermis and a few cells below this level (Harmen *et al*., 2004).

The method of inoculation is very important for the success of the biological control strategy and can differ in regard with different substrates (El Komy *et al*., 2015). The inoculation of the substrates with *Trichoderma* spp. has shown numerous positive results under hydroponics conditions (Mwangi *et al*., 2011). However, there is still considerable interest in finding more efficient mycoparasitic fungi of the *Trichoderma* spp., which can adapt to different soil and substrate conditions under greenhouse conditions. Most research is focused on the application of *Trichoderma* spp. to the conventional open field and the results in higher plant biomass, controlling the pathogen or improving the rooting systems of the plants (Ming *et al*., 2013). The secondary metabolism is not vital for the survival of the plants but it has a defensive role against pathogens that are responding to improper growing conditions (Hartmann, 2007; Atkinson and Urwin, 2012; Rejeb *et al*., 2014).

4.4. MATERIALS AND METHODS

4.4.1. Trial location and plant material

This study was conducted in a greenhouse in the growing season of 2016 – 2017, at the Horticulture Institute, Department of Plant Science, Universidad Autonoma Chapingo, located in Chapingo, State of Mexico, Mexico, at 19° 29’ N and 98° 53’ W, at an elevation of 2,245 m. A moderate susceptible tomato cultivar “Rio Grande”

(Commercial variety) and the “L3” (Experimental line) of the tomato plant breeding program of the Universidad Autónoma Chapingo was used for the experiment. The plants were sown in polyseterene trays and were covered for the germination process. After germination, the trays were uncovered and kept relative humidity and temperature conditions for the first stages of seedling development. After 30 days, the seedlings were transplanting in sterilized soil substrate and coconut fiber (Inoculation method 1) and peat-moss (Inoculation method 2). The environmental parameters of the greenhouse were set as following: temperature 30 ± 4 °C, relative air humidity 75 ± 15 %, automated irrigation correlated with the solar radiation applied daily, 1-2 hr. intervals. The nutrient solution was made by mixing Stiener solution (marco and micronutrients) concentrate with water, reaching an electrical conductivity (EC) of 1.6 mS.

4.4.2. Isolates and inoculum preparation

Six isolates of *Trichoderma*, originally isolated from Tomato (*Solanum lycopersicum* L.) grown soil by researchers of the Department of Agricultural Parasitology and the Forestry Department the Universidad Autónoma Chapingo, were identified as *T. asperellum*, *T. atroviridis*, *T. stromaticum*, and *Trichoderma* spp. The isolates were kept at the Institute of Horticulture at the Department of Plant Science, Chapingo, Mexico, with the code *T. stromaticum* UACH-242, *T. asperellum* UACH-243, *Trichoderma* spp UACH-246, *Trichoderma asperelloides* UACH-248, *T. atroviridis* UACH-252, y *T. asperellum* UACH-253 (Table 7). The isolates were grown in potato dextrose agar (PDA) and incubated for seven days at 30 °C ± 2 °C. After incubation, the *Trichoderma* spores were harvested from Petri dish by adding 10 ml of sterile water, and the spores were transferred immediately to plastic bags containing 200 grs of sterilized rice grains and stored at room temperature for 25 days. Spore concentration was adjusted to 10^7 $CFUg^{-1}$ (Durman *et al*., 1999) and was determined using a haemocytometer for each isolate.

Table 7. List of *Trichoderma* isolates used in the greenhouse trials

Isolate code	State	*Trichoderma* spp.	Inoculation method (1)	Inoculation method (2)
UACH-242	State of Mexico	*Trichoderma stromaticum*	x	x
UACH-243	Morelos	*Trichoderma asperellum*	x	
UACH-246	State of Mexico	*Trichoderma* spp.	x	
UACH-248	Morelos	*Trichoderma asperelloides*	x	
UACH-252	Morelos	*Trichoderma atroviridis*	x	x
UACH-253	Morelos	*Trichoderma asperellum*	x	x

Two isolates of *Fusarium oxysporum* UACH-221 and UACH-235, originally isolated from naturally infected tomato field and identified by Isaac, *et al*. (2018) exhibiting a high levels of pathogencity were used in the experiment. The isolates were reactivated from 15 % glycerol kept at -80 °C. The conidial suspensions were prepared in distilled water from a 7-day-old culture on carnation leaf agar (CLA) for *Fusarium* spp. (Nelson *et al*., 1983). The cultures were uniformly inoculated with conidial suspensions and incubated at 28 °C under a 12 h light/dark cycle.

4.4.3. Inoculation methods and substrate preparation

Tomato plants grown in 200-cavity polystyrene trays filled with peat moss were inoculated with *Fusarium* spp. by two methods: 1) Root Dip and soil infestation Method (Punja and Parker, 2000), and 2) Direct Inoculation Method using syringe (Keeling, 1982).

4.4.3.1. Method 1. Root Dip and soil infestation

The plants were extracted from the seedling trays and the roots were washed to remove the excess of peat moss. The root tips were cut at approx. 1 cm and wounded plants were dipped in 35 ml of suspension of *Fusarium oxsyporum* (10^7 spores·ml^{-1}) for 1 hr. The concentrated solution was mixed with water 1:50 to have the same concentration as the *Trichoderma* spp. solution. Using the steam sterilization technique soil substrate

and coco fiber were sterilized for 3 hours and were placed in 25 x 25 cm-diameter plastic bags. The amount of subtrate used in this experiment was 6-8 kg by each plastic bags. One tomato plant of the Rio Grande variety was transplanted per bag. The ratio of soil medium to fungal cultures was 600:1 v/v (6 kg of substrate medium: 10 g of fungal preparation).

The experiment was carried out in a completely randomized block design. Experimental unit consisted of four tomato plants and each treatment consisted of five replicates. Therefore, 20 tomato plants (4 plants per unit x 5 replications) were evaluated for each treatment combination with a total of 19 treatments for each substrate type (2 *Fusarium* isolates x 8 *Trichoderma* isolates + 2 controls [1 control without *Trichoderma*] + 1 total control [without *Fusarium* and without *Trichoderma*]). There was a total of 760 plants tested in this experiment. The plastic bags were maintained in a greenhouse at 30 ± 4 °C and watered 5 times per day for 3 months. During this period, the plants showing tomato wilting symptoms, severity, disease incidence, total number of fruits, fruit and commercial yields 60 days after inoculation (DAI) was recorded. Data were analyzed statistically by analysis of variance and means of treatments were separated with Tukey's test using SAS v. 9.3 (2012).

Table 8. Treatments and isolates used in the expermeint where the tomato plants were inoculated using the method 1 in soil and coco-fiber substrate

Treament	Tomato variety	Type of substrate	*Fusarium* isolate	Antagonist and Control method
T1	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma stromaticum*
T2	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma asperellum*
T3	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma* spp
T4	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma asperelloides*
T5	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma atroviridis*
T6	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma asperellum*
T7	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	Sportak 45 CE (procloraz)
T8	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	*Trichoderma harzianum* (PHC T-22) commercial strain
T9	Riogrande	Soil substrate	UACH-221 (*Fusarium oxyporum*)	Negative control
T10	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma stromaticum*
T11	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma asperellum*
T12	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma* spp.
T13	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma asperelloides*
T14	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma atroviridis*
T15	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma asperellum*
T16	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	Sportak 45 CE (Procloraz)
T17	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	*Trichoderma harzianum* (PHC T-22) commercial strain
T18	Riogrande	Soil substrate	UACH-235 (*Fusarium oxyporum*)	Negative control
T19	Riogrande	Soil substrate	Without Pathogen	Positive control

Note: The same treatments were used in the coco fiber substrate

4.4.3.2. Method 2. Direct Inoculation using syringe

The plants of the L3 experimental line were extracted from the seedling trays and were transplanted immediately in 8 oz Styrofoam cups. Seedlings were inoculated 7 days after planting by inserting a syringe with 15 ml *Fusarium* conidia in the neck of the tomato plant. A second application ok the *Fusarium* inoculum was carried out 7 days after the first application. Seedlings were inoculated three times during the experiment at 7-day intervals. Sterilized peat moss (Steam sterilization technique for 1.5 hrs) was used as the substrate and was watered to saturation after planting and maintained at near water-holding capacity during the experiment.

A total of 300 plants was used for this part of the experiment (100 hundred per each *Fusarium* isolate, UACH-221, UACH-235 and Race 3) tested in this experiment and were maintained in a greenhouse at 30 ± 4 °C. During this period, the plants showing symptoms of tomato wilting, severity, disease incidence, plant height, degree of root rot, fresh shoot wieght, dry shoot wieght, fresh fruit weight and dry fruit weight were recorded 30 days after inoculation (DAI). A completely randomized design with five replications was used to carry out the experiment. The experimental unit consisted of five tomato plants and each treatment consisted of four replicates. Data were analyzed statistically by ANOVA and means of treatments were separated with Tukey's test using SAS v. 9.3 (2012). A total of 15 treatments and are presented in the Table 9.

Table 9. Treatmeant and isolates used as the expermeint where the tomato plants were inoculated using the method 2

Treament	Tomato variety	Substrate type	*Fusarium* isolate	Antagonist
T1	Experimental Line 3	Peatmoss	UACH-221	*Trichoderma stromaticum*
T2	Experimental Line 3	Peatmoss	UACH-221	*Trichoderma atroviridis*
T3	Experimental Line 3	Peatmoss	UACH-221	*Trichoderma asperellum*
T4	Experimental Line 3	Peatmoss	UACH-221	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*
T5	Experimental Line 3	Peatmoss	UACH-221	Negative control 1
T6	Experimental Line 3	Peatmoss	UACH-235	*Trichoderma stromaticum*
T7	Experimental Line 3	Peatmoss	UACH-235	*Trichoderma atroviridis*
T8	Experimental Line 3	Peatmoss	UACH-235	*Trichoderma asperellum*
T9	Experimental Line 3	Peatmoss	UACH-235	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*
T10	Experimental Line 3	Peatmoss	UACH-235	Negative control 2
T11	Experimental Line 3	Peatmoss	Race 3	*Trichoderma stromaticum*
T12	Experimental Line 3	Peatmoss	Race 3	*Trichoderma atroviridis*
T13	Experimental Line 3	Peatmoss	Race 3	*Trichoderma asperellum*
T14	Experimental Line 3	Peatmoss	Race 3	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*
T15	Experimental Line 3	Peatmoss	Race 3	Negative control 3

4.4.4. Variables used in this study

Severity (SEV): Foliar disease severity was rated 30 days after sowing. Plant assessments were made using a foliar disease severity scale, where 1 = no symptoms (0 % foliage affected); 2 = slight symptom of mosaic development on leaves (1-20 % foliage affected); 3 = moderate symptom development with chlorosis and necrosis on foliage (21-50 % foliage affected); 4 = heavy symptom development with chlorosis and necrosis (51-80 % foliage affected); and 5 = severe chlorosis and necrosis (81-100 % foliage affected) (Huang & Hartman, 1998).

Disease incidence (INCI): Disease intensity (DI) based on the wilting or the necrotic symptoms was calculated using the following equation:

$DI = [(ni \times si) / (N \times S)] \times 100\%$

Where, ni: number of tomato plants with ith score of symptoms, si: the value of the ith score of symptoms, N: total number of tested tomato plants, and S: the highest value of score of symptoms (Cachinero et al. 2002).

Plant Height: For each of the experiments, the height of the plants was measuered at three stages of development the plant using a measuring tape.

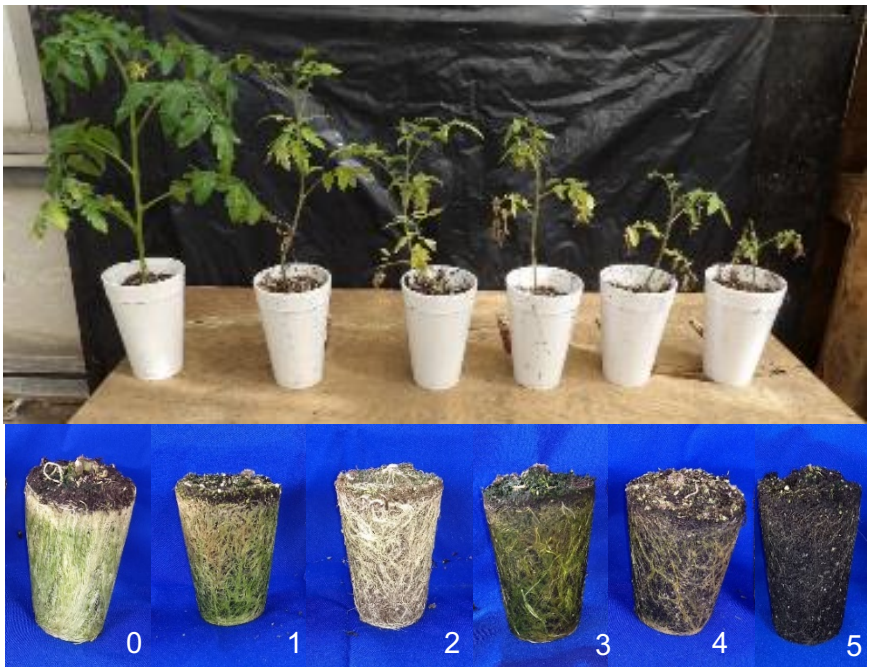
Shoot Fresh Weight (SFW): At the end of the experiment, shoots were separated from roots and weighed.

Fruit Fresh Weight (FFW): At the end of the experiment, fruit were harvested and weighed.

Shoot Dry Weight (SDW): At the end of the experiment, shoot was separated from roots, dried at 70 ^{o}C for 48 hours and weighed.

Fruit Dry Weight (FDW): At the end of the experiment, harvested fruits were dried at 70 ^{o}C for 48 hours and weighed.

Root damage incidence (RDI): using a visual scale, the root development after being inoculated with *Fusarium oxysporum* and *Trichoderma* isolates, the treatment was classified as follows:



Severity scale

0=100% Root development

1=80 % Root development

2=60 % Root development

3=40 % Root development

4=20 % Root development

5=Very little Root development and plant death

(Mak et al. 2004b).

Figure 13 Severity scale of root damage during experiment on tomato roots caused by *Fusarium* spp.

Total Fruit Number: the number of fruits including marketable and non-marketable fruits) were counted before the weighing process.

Total Yield: was measured g· plant^{-}1 at two ocassion during the experiment in including fruits with blossom end rot (usaually below 80 g) and misshaped fruits (usually over 150 g).

Marketable Fruit Number: the fruits were classified into comerical size (above 80 g) and these were counted before weighing them.

Marketable Yield: This was measured in g· plant^{-1}, including fruits with fresh weight between 88 and 150 g.

4.5. RESULTS AND DISCUSSION

4.5.1. Effect of *Trichoderma* spp. in the growth and development of tomato plants after *Fusarium* inoculation method 1 in soil substrate.

In the present study, four out of the six isolates, showed promising ability after their inoculation in soil substrate against *F. oxysporum* under greenhouse conditions (Table 10), in comparison to the control.

The best disease control was achieved in treatments UACH-221 (*Fusarium oxyporum*) + Sportak 45 CE (Procloraz), UACH-221 (*Fusarium oxyporum*) + *Trichoderma harzianum* (PHC T-22) commercial strain and UACH-235 (*Fusarium oxyporum*) + *Trichoderma harzianum* (PHC T-22) comercial strain, all demonstrating only 0 % of disease incidence, respectively (Table 10), compared to the control which registered 80 % disease incidence. The *T. asperellum* and *T. asperelloides* were also quite competitive against the pathogen. For example, the UACH-253 *Trichoderma asperellum* isolate enhanced plant height, total fruit, total yield and number of marketable fruits significantly ($P < 0.05$) (Table 10).

The highest number of fruits plant^{-1} was obtained from UACH-235 (*Fusarium oxyporum*) + Sportak 45 CE (Procloraz) with 61.22 followed by UACH-221 (*Fusarium oxyporum*) + UACH-242 (*Trichoderma stromaticum*) the and UACH-221 (*Fusarium oxyporum*) + UACH-253 (*Trichoderma asperellum*) with 57.81 and 57.21 fruits respectively. The positve and negative control plants produced fair amounts of fruits while the lowest number of fruits were produced by UACH-221 (*Fusarium oxyporum*) + UACH-246 (*Trichoderma* spp.) with 38.81 fruits.

Yield averages obtained from the *Trichoderma* application were statistically at par, but significantly higher then the negative control. Application UACH-221 (*Fusarium oxyporum*) + UACH-253 (*Trichoderma asperellum*) resulted in the highest yield of 3426.61 g plant^{-1}, which was followed by UACH-235 (*Fusarium oxyporum*) + UACH-253 (*Trichoderma asperellum*) with 2712.61 g plant^{-1} and UACH-221 (*Fusarium oxyporum*) + *Trichoderma stromaticum* with 2704.82 g plant^{-1}. The lowest yield,1670.41 and 1610. 41 g plant^{-1}, was obtained from the control plants (Table 10).

With regards to marketable yield, the positive control resulted in the highest marketable yield of 2091.83 g plant^{-1} which was followed by UACH-221 (*Fusarium oxyporum*) + commercial strain *Trichoderma harzianum* (PHC T-22) with 1937.62 g plant^{-1} and UACH-235 (*Fusarium oxyporum*) + Sportak 45 CE (Procloraz) with 1631.24 g plant^{-1}.

Table 10. Growth and development of tomato plants after *Fusarium* inoculation in soil substrate

Treat	*Fusarium oxyporum* Isolates	Microorganisms	Plant height (10 DAI) (cm)	Plant height (17 DAI) (cm)	Plant height (24 DAI) (cm)	Sev (1-5 scale) (%)	Inci (%)	Total Fruit	Total Yield (g)	Marketable Fruit	Marketable Yield (g)
1	UACH-221	UACH-242 (*Trichoderma stromaticum*)	19.3 bc	33.2 b-e	45.3 cde	1.15 c	20 abc	57.8 a	2704.8 abc	16.4 ab	1495.2 ab
2	UACH-221	UACH-243 (*Trichoderma asperellum*)	20.4 bc	31.4 b-g	47.0 c	1.21 c	32 abc	42.6 a	1899.8 bc	11.8 ab	1033.2 ab
3	UACH-221	UACH-246 (*Trichoderma* spp)	18.4 bc	30.9 c-h	43.5 c-f	1.51 c	40 a	38.8 a	1656.8 c	8.8 ab	806.2 ab
4	UACH-221	UACH-248 (*Trichoderma asperelloides*)	21.1 b	34.7 bcd	59.7 b	1.32 c	4 bc	47.2 a	1876.6 bc	11.0 ab	992.0 ab
5	UACH-221	UACH-252 (*Trichoderma atroviridis*)	20.5 bc	31.9 b-f	46.1 cd	1.05 c	4 bc	47.0 a	2530.0 abc	16.8 ab	1464.2 ab
6	UACH-221	UACH-253 *(Trichoderma asperellum)*	26.6 a	47.7 a	70.1 a	1.25 c	4 bc	57.2 a	3426.6 a	18.0 ab	1428.0 ab
7	UACH-221	Sportak 45 CE (Procloraz)	20.4 bc	30.4 d-h	46.2 cd	1.42 c	0 abc	46.8 a	2522.2 abc	14.6 ab	1631.2 ab
8	UACH-221	Commercial strain *Trichoderma harzianum* (PHC T-22)	20.8 bc	30.6 c-h	46.2 cd	1.41 c	0 c	53.2 a	2558.0 abc	13.0 ab	1937.6 ab
9	UACH-221	Negative control	13.9 de	25.5 h	38.2 ef	2.93 a	40 a	54.8 a	1610.4 c	13.0 ab	1075.2 ab
10	UACH-235	UACH-242 (*Trichoderma stromaticum*)	18.4 bc	28.7 e-h	43.3 c-f	1.15 c	8 abc	40.6 a	2217.0 abc	11.8 ab	1042.8 ab
11	UACH-235	UACH-243 (*Trichoderma asperellum*)	18.5 bc	30.3 d-h	46.7 c	1.25 c	20 abc	48.4 a	2545.6 abc	14.4 ab	1318.2 ab
12	UACH-235	UACH-246 (*Trichoderma* spp)	17.6 bcd	27.3 fgh	39.0 def	1.45 c	24 abc	43.8 a	1787.0 bc	8.8 ab	750.2 b
13	UACH-235	UACH-248 (*Trichoderma asperelloides*)	20.2 bc	35.9 bc	58.1 b	1.22 c	8 abc	48.6 a	2676.0 abc	15.8 ab	1619.4 ab
14	UACH-235	UACH-252 (*Trichoderma atroviridis*)	20.8 bc	33.3 b-e	45.0 c-f	1.51 c	8 abc	45.8 a	2345.6 abc	15.4 ab	1441.0 ab
15	UACH-235	UACH-253 *(Trichoderma asperellum)*	21.4 b	36.5 b	60.2 b	1.14 c	4 bc	47.0 a	2712.6 abc	13.6 ab	1274.4 ab
16	UACH-235	Sportak 45 CE (Procloraz)	17.9 bcd	32.7	46.0 cd	2.21 b	8 abc	61.2 a	2422.6 abc	6.8 b	941.6 ab
17	UACH-235	Commercial strain *Trichoderma harzianum* (PHC T-22)	16.5 cde	31.0 b-h	42.8 c-f	1.05 c	0 c	39.2 a	1864.0 bc	11.4 ab	1019.6 ab
18	UACH-235	Negative control	12.9 e	26.1 gh	37.8 f	2.3 ab	36 ab	43.8 a	1670.4 c	16.8 ab	1294.6 ab
19	Without Pathogen	Positive control	20.7 bc	31.5 b-g	47.2 c	1.1 c	12 abc	49.8 a	3052.2 ab	20.8 a	2091.8 a
		HSD	**4.45**	**5.50**	**7.42**	**0.60**	**4.87**	**31.12**	**1333.7**	**13.27**	**1295.6**

Each value represents the mean of 5 replicates. For each experiment, values within a column followed by a same letter are not significantly different according to Tukey test (P≤0.05).

[a]Plants were drenched with sterile distilled water. [b]Plants were grown without pathogen and antoganist

4.5.2. Effect of *Trichoderma* spp. in the growth and development of tomato plants after *Fusarium* inoculation in coco fiber substrate.

The isolates, *Trichoderma asperellum* UACH-253 and *Trichoderma atroviridis* UACH-248 showed an adequate biocontrol of *Fusarium oxysporum* in coconut fiber substrate, obtaining a lower wilt incidence of 4 %, respectively, when compared with control whoch showed higher percentages of incidence (55%). *Trichoderma asperellum* UACH-253 also showed higher fruit yields by presenting (3958 and 3588.2 g/planta), as well as the total fruits (80 and 70 per plant during the experiment and commercial fruit weight (1802 and 1248) (table 11.). When using soil as a substrate, *Trichoderma asperellum* (UACH-243) had the best biocontrol effect, since the incidence of enferemdad was 8%; In addition, it allowed to express a greater yield, number and weight of commercial fruit were 2545 $g \cdot plant^{-1}$, 80.8 fruits and 1318g, respectively).

The isolates of UACH-242 *Trichoderma stromaticum* and UACH-253 *Trichoderma asperellum* was followed, with the number of commercial fruits of 57.2 and 47, yield of 3426.6 respectively. It is important to mention that with fiber coconut substrate; the control was more effective than that observed in soil.

The lowest yield,1965 and 1935 $g \cdot plant^{-1}$, was obtained from the negative control plants and UACH-221 (*Fusarium oxyporum*) + UACH-246 (*Trichoderma* spp.) (Table 11). On the other hand, for the marketable yield, the UACH-221 (*Fusarium oxyporum*) + UACH-243 (*Trichoderma asperellum*) obtaining 1976 g $plant^{-1}$ which was followed by UACH-235 (*Fusarium oxyporum*) + Sportak 45 CE (Procloraz) with 1924 g $plant^{-1}$. and UACH-235 (*Fusarium oxyporum*) + UACH-253 (*Trichoderma asperellum*) with 1802 g $plant^{-1}$. These values were slighty under the values obtained in the soil substrate.

Table 11. Growth and development of tomato plants after *Fusarium* inoculation in coco fiber substrate.

Treat	Fusarium isolates	Microorganisms	Plant height (10 DAI) (cm)	Plant height (17 DAI) (cm)	Plant height (24 DAI) (cm)	Sev (1-5 scale) (%)	Inci (%)	Total Fruit	Total Yield (g)	Marketable Fruit	Marketable Yield (g)
1	UACH-221	UACH-242 (*Trichoderma stromaticum*)	19.8 ab	29.9 bc	48.5 abc	1.2 c	12 c	77.4 a	3043 ab	18.2 a	1573.8 a
2	UACH-221	UACH-243 (*Trichoderma asperellum*)	20.2 ab	31.1 bc	47.3 abc	1.6 bc	32 abc	73.4 a	3420ab	21.8 a	1976.0 a
3	UACH-221	UACH-246 (*Trichoderma* spp)	20.8 ab	31.1 bc	47.5 abc	1.3 bc	16 bc	57.4 a	1935 b	12.0 a	939.2 a
4	UACH-221	UACH-248 (*Trichoderma asperelloides*)	20.0 ab	28.1 bc	48.3 abc	1.6 bc	16 bc	60.4 a	2922 ab	21.8 a	1787.6 a
5	UACH-221	UACH-252 (*Trichoderma atroviridis*)	20.1 ab	31.4 bc	47.7 abc	1.1 c	4 c	55.2 a	2201 ab	14.6 a	1189.2 a
6	UACH-221	UACH-253 *(Trichoderma asperellum)*	25.6 ab	41.1 a	70.4 ab	1.6 bc	4 c	80.8 a	3958 a	12.6 a	1248.4 a
7	UACH-221	Sportak 45 CE (procloraz)	20.7 ab	32.4 bc	49.6 abc	1.8 bc	20 abc	67.2 a	3051 ab	19.4 a	1601.4 a
8	UACH-221	Commercial strain *Trichoderma harzianum* (PHC T-22)	20.6 ab	33.1 b	46.6 abc	1.2 c	8 c	56.2 a	2800.8 ab	16.6 a	1529.6 a
9	UACH-221	Negative control	14.9 b	26.9 c	57.6 abc	2.7 a	60 a	61.0 a	2331. 1 ab	19.4 a	1496.8 a
10	UACH-235	UACH-242 (*Trichoderma stromaticum*)	20.1 ab	28.8 bc	44.6 c	1.3 bc	8 c	60.6 a	2421.4 ab	15.8 a	1296.4 a
11	UACH-235	UACH-243 (*Trichoderma asperellum*)	20.9 ab	29.8 bc	46.8 abc	1.8 bc	20 abc	73.6 a	2840.6 ab	15.8 a	1206.8 a
12	UACH-235	UACH-246 (*Trichoderma* spp)	19. 6 ab	29.3 bc	44.4 c	1.5 bc	16 bc	59.2 a	2402.9 ab	14.8 a	1025.4 a
13	UACH-235	UACH-248 (*Trichoderma asperelloides*)	21.9 ab	28.1 bc	62.6 abc	1.5 bc	8 c	57.6 a	2194.0 ab	13.2 a	1074.2 a
14	UACH-235	UACH-252 (*Trichoderma atroviridis*)	31.9 a	30.7 bc	48.6 abc	1.3 c	20 abc	62.0 a	2361.4 ab	14.2 a	1122.4 a
15	UACH-235	UACH-253 *(Trichoderma asperellum)*	23.5 ab	42.3 a	71.9 a	1.2 c	20 abc	69.0 a	3588.2 ab	19.0 a	1802.0 a
16	UACH-235	Sportak 45 CE (Procloraz)	19.7 ab	31.2 bc	45.1 b	1.5 bc	24 abc	71.6 a	3491.0 ab	22.8 a	1924.4 a
17	UACH-235	Commercial strain *Trichoderma harzianum* (PHC T-22)	19.8 ab	29.0 bc	44.8 b	1.4 bc	28 abc	62.2 a	2939.6 ab	20.4 a	1684.0 a
18	UACH-235	Negative control	16.3 b	30.5 bc	45.3 b	2.1 ab	56 ab	72.4 a	1964.8 b	13.0 a	1066.4 a
19	Without Pathogen	Positive control	22 ab	31.6 bc	47.7 abc	1.6 c	8 c	62.2 a	2645.7 ab	18.0 a	1668.4 a
		HSD	**12.696**	**5.81**	**19.4**	**0.84**	**40.48**	**38.2**	**1825**	**20.4**	**1703**

Each value represents the mean of 5 replicates.

For each experiment, values within a column followed by a same letter are not significantly different according to Tukey test ($P<0.05$).

[a]Plants were drenched with sterile distilled water.

[b]Plants were grown without pathogen and antoganist

These results confirm the usefulness of evaluating different isolates in different substrate type, for their mycoparasitic properties which could lead to preparation of biopesticides. Isolates of some species namely *T. harzianum, T. viride* and *T. virens* are well known and their formulations are used by many growers but there might be more effective species of *Trichoderma*, therefore evaluating strains in various substrate type would be beneficial. We found that *T. asperellum* and *T. atroviridis* other alternatives in controlling *F. oxysporum*, the while Ashrafizadeh *et al.,* (2002); Ozbay *et al.,* (2004); Hajieghrari *et al.,* (2008) and Otadoh *et al.,* (2011) in their screening experiments mentioned other species of *Trichoderma* as most efficient isolates against this particular pathogen. There are reports that *Trichoderma harzianum* increases the solubility of phosphates and micronutrients such as zinc, copper, iron and manganese all plant nutrients with low solubility and this enhances growth of the roots and the above ground parts of the plant.

Our results of greenhouse experiment agree with some reports and in contrast with some others. Akrami *et al*. (2011) reported that 44 % disease reduction was observed when a combination of *T. harzianum* and *T. asperellum* were used against the pathogen while Boureghda and Bouznad (2009) observed that *T. atroviride* isolate (Ta. 13), with 83.92% disease reduction was the most effective species under greenhouse condition. Also, Tsai *et al*. (2008) reported that among five tested isolates, *T. asperellum* TA strain was the best for promoting the growth of *Anoectochilus* plants and reduction of the disease, but others such as Ashrafizadeh *et al*. (2002); Ozbay *et al*. (2004); Soltani *et al*. (2005) and Otadoh *et al*. (2011) mentioned other *Trichoderma* species as effective antagonists against Tomato wilt of several crops under greenhouse condition.

In a study comparing the effect of four of the microorganisms (*P. brevicompactum*, *T. atroviride*, *P. marginalis*, and *P. putida*) significantly increased the marketable fruit yield compared to the control in rockwool (Wang *et al*., 2000; Safronova *et al*., 2006). The total yield was also increased by the microorganisms as compared to the control, however not significantly. In the organic medium, the inoculation of the plants with *T. atroviride* lead to a significant increase in the marketable and total yields as compared to the control. The shoot wieght was not markedly affected by the inoculation of the

microorganisms and ranged from 482 to 509 cm (rockwool) and 447 to 471 cm (organic medium).

In another study carried out by Poldma *et al*. (2002) who determined that *T. viride* treated cucumber plants produced significantly higher total yield. Also, in cabbage, while total yield determined after 28 days was significantly higher, the effect after 42 days was not significant in plants treated with *T. longipile* and *T. tomentosum* (Rabeendran *et al*., 2000). In a similar study canied out in *Cucumis sativus* grown in soil in an unheated glasshouse, using *T. harzianum*, increased the total and early yield. While the increase in total yield was statistically significant, the effect was not significant in early yield (Altintas & Bal, 2005; Vitale *et al*., 2012).

Its important to mention that *Trichoderma* spp. did not increase yield in all the crops studied. In onion, Poldma *et al*. (2001) found that yield from *T. viride* treated plots were inferior to the control. In tomato grown in soil, in the same unheated glasshouse as the cucumber experiment mentioned above, *T. harzianum* did not induce significant increase in total and marketable yield. Whereas in the same study, the early yield from *Trichoderma* treated plants compared to the control was higher but the effect of *T. harzianum* was not statistically significant (Bal & Altintas, 2006). Rabeendran *et al*., (2000) showed that the application of *Trichoderma* was significantly effective only in early yield but not in total yield. For the cases in which *T. harzianum* is effective only in early yield but not in total yield, it may be speculated that under certain conditions adverse to *Trichoderma*, populations may decline rapidly being effective only transiently at the beginning.

The mechanisms of plant-growth-promoting ability, in addition to the biological control capability, of *Trichoderma harzianum* was studied *in vitro* using the T-22 strain and it was determined that *T. harzianum* is able to solubilize minerals from their solid states, i.e. from rock phosphate, $Mn0_2$, Fe_20_3 and metallic zinc, making them available for plant use and subsequently promoting plant growth (Altomare *et al*., 1999). In suppmt of the above work, Yedidia *et al*. (2001) determined in cucumber plants grown axenically, that it is only in the presence of *T. harzianum* that concentrations of important minerals such as P, Fe, Mn and Zn increased in the plant tissues, which displayed increased shoot length, dry weight and leaf area. Also in the explanation of the positive effects, it was

pointed out that *T. harzianum* was able to colonize roots establishing a symbiotic relationship in which the defence system of the hosts was induced (Brimner & Goland, 2003; Dubey *et al*., 2011). Alternatively, in certain cases, success of the antagonistic fungi, e.g. *Trichoderma*, may be limited due to disadvantages they face in their own establishment in the root zone in the soil. The disadvantage may arise from competitive ability of the species employed. The pathogenic fungi may be more aggressive and take advantage of the available nutrients in the root zone much faster than the antagonistic fungi. Celar (2003) found that *Fusarium* spp. can be more competitive than *Trichoderma* in the fast and selective consumption of available nutrients and under certain conditions addition of some substrates is helpful in conferring effectiveness to the antagonistic fungi. Therefore, it may be speculated that under such circumstances establishment of *Trichoderma* populations takes time, not being effective in any way immediately after the application. Results showed, in general, that fruit quality was positively affected by the application of *T. harzianum*.

4.5.3. Effect of *Trichoderma* spp. in the growth and development of tomato plants after *Fusarium* Inoculation in peatmoss.

The selected antagonists (*Trichoderma stromaticum*, *Trichoderma atroviridis*, *Trichoderma asperellum*) were tested for their ability to reduce the incidence and yield parameters of tomato under greenhouse conditions. The control plants did not show wilting symptoms, while inoculated ones showed various degrees of wilting at 40 DAI. The colonies of *Fusarium oxysporum* were successfully re-isolated from inoculated tomato plants showing the symptoms, indicating association between the symptoms and *Fusarium oxsyporum* (UACH-221, UACH 235 and Race 3) infection. All trichoderma species had influence on plant height. The mixture of the *Trichoderma* spieces (*Trichoderma stromaticum* UACH-242 + *Trichoderma atroviridis* UACH-252 + *Trichoderma asperellum* UACH-253) significantly increased the Plant Height, Fruit Fresh Weight (FFW), and Root Damage Incidence (RDI) compared to the control (Table 12).

Table 12.- Growth and development of tomato plant using the Direct Inoculation Syringe Method 2 in peatmoss.

No.	*Trichoderma*	*Fusarium*	Plant Height (cm)			Inci	Sev	RDI	SFW	SDW	FFW	FDW
			15 (DAI)	40 (DAI)	60 (DAI)	(%)	(%)	(%)	(g)	(g)	(g)	(g)
T1	*Trichoderma stromaticum*	UACH-221	15.8 ab	41.5 ab	67.5 ab	40 ab	61 a	55 b	246.3 ab	71.9 ab	236.3 a	31.2 a
T2	*Trichoderma atroviridis*	UACH-221	15.7 ab	41.7 ab	69.2 ab	25 b	54 a	65 ab	348.8 ab	86.5 ab	341.3 a	45.8 a
T3	*Trichoderma asperellum*	UACH-221	15.8 ab	43.7 ab	65.0 ab	5 b	46 a	77 ab	453.8 a	95.5a	458.8 a	54.5 a
T4	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*	UACH-221	16.6 a	44.3 a	70.6 ab	15 b	51 a	68 ab	375.3 ab	86.6ab	585.0 a	60.9a
T5	Negative control 1	UACH-221	14.6 ab	41.3 ab	63.6 ab	65 a	45 a	56 ab	272.5 ab	69.9 ab	327.5 a	43.4 a
T6	*Trichoderma stromaticum*	UACH-235	15.0 ab	39.1 ab	66.9 ab	15 b	51 a	66 ab	243.8 ab	74.7 ab	302.5 a	46.3 a
T7	*Trichoderma atroviridis*	UACH-235	15.4 ab	41.8 ab	72.8 a	25 b	46 a	68 ab	345 ab	87.3 ab	552.8 a	63.0 a
T8	*Trichoderma asperellum*	UACH-235	16.4 a	41.8 ab	72.2 a	5 b	52 a	69 ab	452.5 ab	84.7 ab	578.8 a	64.0 a
T9	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*	UACH-235	15.4 ab	42.4 ab	66.4 ab	5 b	45 a	74 ab	338.8 ab	87.3 ab	386.3 a	46.4 a
T10	Negative control 2	UACH-235	14.3 ab	40.7 ab	60.6 ab	65 a	61 a	59 ab	271.3 ab	77.6 ab	355.0 a	46.2 a
T11	*Trichoderma stromaticum*	Race 3	14.6 ab	42.2 ab	61.2 ab	10 b	53 a	62 ab	281.3 ab	84.6 ab	288.8 a	41.1 a
T12	*Trichoderma atroviridis*	Race 3	14.8 ab	39.7 ab	64.8 ab	20 b	51 a	71 ab	285 ab	85.4 ab	283.8 a	38.8 a
T13	*Trichoderma asperellum*	Race 3	13.6 b	40.1 ab	70.1 ab	5 b	46 a	80 ab	323.8 ab	96.9 a	330.0 a	43.4 a
T14	*Trichoderma stromaticum* + *Trichoderma atroviridis* + *Trichoderma asperellum*	Race 3	15.8 ab	43.4 ab	67.2 ab	5 b	45 a	77 ab	302.5 ab	88 ab	408.8 a	49.9 a
T15	Negative control 3	Race 3	14.6 ab	38.31 b	56.19 ab	30 ab	52 a	72 ab	178.6 b	56.6 b	223.8 a	33.3 a
HSD			**2.7**	**5.85**	**18.8**	**39.1**	**21.26**	**24.6**	**258.9**	**35.5**	**468.5**	**44.5**

Each value represents the mean of 5 replicates.

For each experiment, values within a column followed by a same letter are not significantly different according to Tukey test ($P<0.05$).

[a]Plants were drenched with sterile distilled water.

[b]Plants were grown without pathogen and antoganist

Several *Trichoderma* spp. could be effectively used in biocontrol of soil borne plant pathogens and identifying efficient species adapted to different agroecosystems seem to be useful for their further evaluation. Several reports have indicated that biocontrol efficiency of *Trichoderma* spp. against Tomato wilts may differ in different regions of the world ie, a highly antagonistic species against a particular pathogen in a given region may react poorly against the same pathogen in another region (Ashrafizadeh *et al*., 2002; Hajieghrari *et al.,* 2008; Otadoh *et al*., 2011) which could be due to differences in various agroclimatic conditions.

T. harzianum is capable of invading roots, but is typically restricted to the outer layers of the cortex (Yedidia *et al*., 1999). Infection is accompanied by the production of several classes of signal compounds from the fungus that activate plant resistance responses (Harman *et al*., 2004). Observed significant ($P < 0.05$) growth due to *Trichoderma asperellum* inoculation in tomatoes confirms other reports that *Trichoderma* spp with host plant has an improved growth effect (Nemec *et al*., 1996; Hermosa *et al*., 2012). Increased growth of the tomato plants due to *Trichoderma* inoculation is mainly attributed to improved phosphorous and micronutrient uptake in the host plant, although in the prescense of the pathogen *Fusarium oxysporum*. The tomato plants have the abilty to emited secondary root tips, this helped to improved the absortion process.

4.6. CONCLUSIONS

In our evaluations, four out of the six isolates belonging to the four *Trichoderma* species, two isolates: *Trichoderma asperellum* (UACH-253) and *Trichoderma atroviridis* (UACH-252) showed proper performance against Tomato wilt pathogen under greenhouse conditions, although these isolates need to be evaluated under field condition they, however, seem to be promising isolates for further studies.

4.7. REFRENCES

Abdelrahman M, Abdel-Motaal F, El-Saved M, Jogaiah S, Shigyo M, Ito S, Tran LS (2016). Dissection of Trichoderma longibrachiatum-induced defense in onion

(Allium cepa L.) against Fusarium oxysporum f. sp. cepa by target metabolite profiling. Plant Science 245:128-138.

Ajilogba C F, Babalola O O. (2013). Integrated management strategies for tomato Fusarium wilt. Biocontrol Sci. 2013;18(3):117-27.

Akrami M, Golzary H, Ahmadzadeh M (2011) Evaluation of different combinations of Trichoderma species for controlling Fusarium rot of lentil. Afr J Biotechnol 10(14): 2653–2658

Alabouvette C.1986. Fusarium-wilt suppressive soils from the Châteaurenard region: review of a 10-year study. Agronomie, EDP Sciences, 1986, 6 (3), pp.273-284.

Althaf HSK, Srinivas P (2013). Association of arbuscular mycorrhizal fungi and other rhizosphere microbes with different medicinal plants. Research Journal of Biotechnology 8(6):24-28.

Altintas, S., & Bal, U. (2005). Application of *Trichoderma harzianum* increases yield in cucumber (Cucumis sativus) grown in an unheated glasshouse, Journal of Applied Horticulture, 7, 25-28.

Altomare, C., Norvell, W.A., Bjokrnan, T. & Harman, G.E. (1999). Solubilization of phosphates and micronutlients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Applied and Envimnmenta/. Micmbiology, 65, 2926-2933.

Asha N.N. Raghunandan B.L. and. Shivaprakash M.K (2013). Survival and population studies of *Trichoderma* spp. Madras Agric. J., 100 (4-6): 554-558.

Ashrafizadeh, A., Etebarian, H. and Zamanizadeh, H. (2002). Evaluation of Trichoderma isolates in controlling Fusarium wilt of melon. Iranian journal of plant pathology, 41: 39-57.

Atkinson NJ, Urwin PE (2012). The interaction of plant biotic and abiotic stresses: from genes to field. Research Journal of Biotechnology 8(6):24-28.

Bahraminejad S, Abbasi S, Amiri R (2015). The effect of some medicinal and ornamental plant extracts against *Fusarium oxysporum*. Journal of Crop Protection 4(2):189-197.

Bal, U. & Altintas, S. (2006). Effects of *Trichoderma harzianum* on yield and fruit quality characteristics of tomato (*Lycopersicon esculentum* Mill.) grown in an unheated greenhouse, Australian Joumal t~f' Experimental Awiculture, 46, 131-136.

Berges MSL, Hera C, Sulyok M, Schäfer K, Capilla J, Guarro J, Di Pietro A (2013). The velvet complex governs mycotoxin production and virulence of *Fusarium oxysporum* on plant and mammalian hosts. Molecular Microbiology 86(1):49-65.

Bhai RS, Dhanesh J, (2008). Occurrence of fungal diseases in vanilla (Vanilla planifolia Andrews) in Kerala. Journal of Spices and Aromatic Crops 17, 140–8.

Bissett, J. (1991a). The revision of the genus Trichoderma II. infrageneric classification. Canadian. Journal of Botany, 69: 2357-2372.

Bissett, J. (1991b). The revision of the genus Trichoderma III. Section Pachybasidium. Canadian. Journal of Botany, 69: 2357-237.

Boureghda, H and Bouznad, Z. (2009). Biological control of Fusarium wilt of chickpea using isolates of *Trichoderma atroviride*, *T. harzianum* and *T. longibrachiatum*. Acta Phytopathol Entomol Hung, 44(1): 25–38.

Brimmer, T. and G.J. Boland, (2003). A review of the non-target effects of fungi used to biologically control plant diseases. Agric. Ecosyst. Environ., 100: 3-16.

Brimner, T.A. & Goland, G.J. (2003). A review of the non-target effects of fungi used to biologically control plant diseases. Agriculture, t'cosystems and Environment, 100, 3-16.

Cachinero JM, A Hervas, RM Jimenez-Diaz, M Tena (2002). Plant defence reactions against Fusarium wilt in chickpea induced by incompatible race 0 of *Fusarium oxysporum* f.sp. ciceris and non-host isolates of *F. oxysporum* Plant Pathol, pp. 765-776

Carvalho, Daniel D. C., Lobo Junior, Murillo, Martins, Irene, Inglis, Peter W., & Mello, Sueli C. M. (2014). Biological control of *Fusarium oxysporum* f. sp. *phaseoli* by *Trichoderma harzianum* and its use for common bean seed treatment. Tropical Plant Pathology, 39(5), 384-391

Celar, F. (2003). Competition for ammonium and nitrate forms of nitrogen between some phytopathogenic and antagonistic soil fungi. Biological Control, 28, 19-24.

Chen, L. L., Yang, X., Raza, W., Li, J., Liu, Y., Qiu, M., Zhang, F. & Shen, Q. (2011). *Trichoderma harzianum* SQR-T037 rapidly degrades allelochemicals in rhizospheres of continuously cropped cucumbers. Appl Microbiol Biotechnol 89, 1653–1663.

Ding J, Zhang Y, Zhang H, Li X, Sun Z, LiaoY, Xia X, Zhao Y, Shi K, Yu J (2014). Effects of *Fusarium oxysporum* on rhizosphere microbial communities of two cucumber genotypes with contrasting Fusarium wilt resistance under hydroponic conditions. European Journal of Plant Pathology 32(2):125-135.

Dubey SC, Bhavani R, Singh B (2011) Integration of soil application and seed treatment formulations of Trichoderma species for management of wet root rot of mungbean caused by Rhizoctonia solani. Pest Manag Sci 67: 1163-1168.

Durman, S., Menendez, A., Godeas, A. (1999). Evaluación de *Trichoderma* spp. como antagonista de *Rhizoctonia solani in vitro* y como biocontrolador del damping-off de plantas de tomate en invernadero. Revista Argentina de Microbiología, 31: 13-18

EL Komy, M. H.; Saleh, A. A.; Eranthodi, A.; Molan, Y. Y. (2015). Characterization of novel *Trichoderma asperellum* isolates to select effective biocontrol agents against tomato fusarium wilt. The Plant Pathology Journal, v. 31, n. 1, p. 50-60,

Gawehns F., Houterman P.M., Ait Ichou F., Michielse C.B., Hijdra M., Cornelissen B.J.C., Rep M., and Takken F.L.W. (2014). The *Fusarium oxysporum* Effector Six6 Contributes to Virulence and Suppresses I2-Mediated Cell Death. MPMI 27: 336–348.

Hajieghrari, B., Torabi - giglou, M., Mohamadi, M. R. and Davari, M. (2008). Biological potential of some Iranian *Trichoderma* isolates for control of soil born plant pathogenic fungi. African Journal of Biotechnology, 7 (8): 967-972

Harman G E, Howell C R, Viterbo A, Chet I and Lorito M., (2004). Trichoderma species – opportunistic, avirulent plant symbionts. Nature Reviews Microbiology Vol.2 pp43-56.

Harman, G. E., Lorito, M. and Lynch, J. M. (2004). Uses of *Trichoderma* spp. to alleviate or remediate soil and water pollution. In: Advances in Applied Microbiology.

Volume 56. (Laskin, A. I., Bennett, J. W. and Gadd, G. M., Eds.). Elsevier Academic Press, San Diego, CA, USA. 313–330.

Hartmann T (2007). From waste products to ecochemicals: fifty years' research of plant secondary metabolism. Phytochemistry 68(22):2831-2846

Hermosa R, Viterbo A, Chet I, Monte E (2012) Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology-Sgm 158:17–25

Huang, Y.H.; Hartman, G.L. (1998) Reaction of selected soybean genotypes to isolates of *Fusarium solani* f. sp. *glycines* and their culture filtrates. Plant Disease, v.82, p.999-1002.

Huang C H, Roberts P D, and Datnoff L E. (2011). Silicon Suppresses Fusarium Crown and Root Rot of Tomato Journal of Phytopatholgy 159:546–554

Karim, N. F. A., Mohd, M., Nor, N. M. I. M., & Zakaria, L. (2016). Saprophytic and Potentially Pathogenic Fusarium Species from Peat Soil in Perak and Pahang. Tropical Life Sciences Research, 27:1–20.

Keeling BL (1982) A seedling test for resistance to soybean stem canker caused by Diaporthe phaseolorum var. caulivora. Phytopathology 72:807-809

Lanubile A, Ellis ML, Marocco A, Munkvold GP.(2016). Association of Effector Six6 with Vascular Wilt Symptoms Caused by *Fusarium oxysporum* on Soybean. Phytopathology.106:1404-1412.

Latiffah Z, Mohd Zariman M, Baharuddin S. (2007) Diversity of Fusarium species in cultivated soils in Penang. Malaysian Journal of Microbiology.3(1):27–30

Leslie, J.F., y Summerell, B.A. 2006. The Fusarium Laboratory Manual. Blacwell. Iowa USA. 388 p

Lewis, J.A. and Papavizas, G.C. (1984). Chlamydospore formation by *Trichoderma* spp.in natural substrates. Can. J. Microbiol., 30: 1-7.

Macedo R, Sales LP, Yoshida F, Silva-Abud LL, Lobo M Junior. (2017), Potential worldwide distribution of Fusarium dry root rot in common beans based on the optimal environment for disease occurrence. PLoS One;12(11)

Maciel, CG, Santos W, C, Muniz R F, Brião M F, and Lemos B, D. (2017). *Fusarium oxysporum* and *F. verticillioides* associated with damping-off in *Pinus* spp. Revista Ciência Agronômica, 48: 134-141

Mandeel Q.A. (2006). Biodiversity of the genus Fusarium in saline soil habitats. Journal of Basic Microbiology.;46:480–494.

Ming Q, Su C, Zheng C, Jia M, Zhang Q, Zhang H, Qin L (2013). Elicitors from the endophytic fungus *Trichoderma atroviride* promote Salvia miltiorrhiza hairy root growth and tanshinone biosynthesis. Journal of Experimental Botany 64(18):5687-5694.

Moe L A., (2013). Amino acids in the rhizosphere: From plants to microbes. American Journal of Botany 100 (9): 1692–1705.

Mukherjee et al., (2012b) P.K. Mukherjee, B.A. Horwitz, C.M. Kenerley Secondary metabolism in *Trichoderma*–a genomic perspective Microbiology (Reading, England), 158; 35-45

Mwangi, Margaret W., Monda, Ethel O., Okoth, Sheila A., & Jefwa, Joyce M. (2011). Inoculation of tomato seedlings with *Trichoderma harzianum* and Arbuscular Mycorrhizal Fungi and their effect on growth and control of wilt in tomato seedlings. Brazilian Journal of Microbiology, 42(2), 508-513.

Nelson, P.E.; Toussoun, T. A. y Marasas, W.F.O. (1983). Fusarium species, an illustrated manual for identification. The Pensnsylvania Styate University Press. University Park and London. 193 pp.

Nemec S, Datnoff LE, Strandbery T. (1996). Efficacy of bio control agents in planting mixes to colonize plant roots and control root diseases of vegetable and citrus. Crop Protection. 15:735–743.

Otadoh, J. A., Okoth, S. A., Ochanda, J. and Kahindi, J. P. (2011). Assessment of *Trichoderma* isolates for virulence efficacy on *Fusarium oxysporum* f. sp. *phaseoli*. Tropical and Subtropical Agroecosystems, 13: 99-107.

Ozbay, N., Newman, S. E. and Brown, W. M. (2004). The effect of the *Trichoderma* strains on the growth of tomato seedlings. Acta Horticulture, 635: 131-135.

Peng, H.X., Sivasithamparam, K. & Turner, D.W. (1999) Chlamydospore germination and Fusarium wilt of banana plantlets in suppressive and conducive soils are affected by physical and chemical factors. Soil Biology and Biochemistry 31:1363-1374.

Pinaria AG, Liew E, Burgess LW, (2010). Fusarium species associated with vanilla stem rot in Indonesia. Australasian Plant Pathology 39, 176– 83.

Poldma P., Merivee A., Johansson P., Ascard J., Alsanius B., (2001). Influence of biological control of fungal diseases with *Trichoderma* spp. on yield and quality of onion., in: 'New Sights in Vegetable Production'. Nordic Association of Agricultural Scientists, NJF Seminarium nr. 329. Segadi, Estonia, 05-08.09.2001, ISSN 0333-1350: 48-52

Poldma, P., Albrecht, A. & Merivee, A. (2002). Influence of fungus Trichoderma viride on the yield of cucumber in greenhouse conditions. In Proceedinxs of' the Conf'erence on Scientific Aspects of' Orxanic Fanning. Jelgava, Latvia 21-22 March 2002, pp. 176-180.

Punja Z. K., Parker M. (2000). Development of Fusarium root and stem rot, a new disease on greenhouse cucumbers in British Columbia caused by *Fusarium oxysporum* f.sp. *radices-cucumerinum*. Can. J. Plant Pathol. 22: 349-363.

Rabeendran N., Moot D.J., Jones E.E., Stewart A., (2000) Inconsistent growth promotion of cabbage and lettuce from *Trichoderma* isolates. N.Z. Plant Prot53: 143-146.

Raviv M, Antignus Y. (2004). UV radiation effects on pathogens and insect pests of greenhouse-grown crops. Photochem Photobiol;79(3):219-26.

Recorbet, G. & Alabouvette, C. (1997) Adhesion of *Fusarium oxysporum* conidia to tomato roots. Letters in Applied Microbiology 25: 375-379

Rejeb IB, Pastor V, Mauch-Mani B (2014). Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. Plants 3(4):458-475.

Safronova, V.I., Stepanok, V.V., Engqvist, G.L., Alekseyev, Y.V., Belimov, A.A., 2006. Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biology and Fertility of Soils 42, 267–272

SAS Institute Inc (2012). Base SAS® 9.3 Procedures Guide. Cary, NC: SAS Institute Inc.

Soltani, H., Zafari, D. and Rohani, H. (2005). A study on biological control of crown, root and tuber fungal diseases of potato by *Trichoderma harzianum* under *in-vivo*

and fieldcondition in Hamadan. Agricultural Research (water, soil & plant in agriculture). 5: 13-25.

Srobar, S. (1978) The influence of temperature and pH on the growth of mycelium of the causative agents of Fusarioses in wheat in Slovakia Czechoslovakia. UVTI (Ustav Vedeckotechnickych Informaci) Ochrana Rostlin 14: 269-274

Szoboszlay M, White-Monsant A, Moe LA (2016) The Effect of Root Exudate 7,4′-Dihydroxyflavone and Naringenin on Soil Bacterial Community Structure. PLOS ONE 11(1): e0146555.

Takken, F. L. W. (2015). The effector repertoire of *Fusarium oxysporum* determines the tomato xylem proteome composition following infection. Frontiers in Plant Science, 6, 967.

Tsai, C. C., Tzeng, D. S. and Hsieh, S. P. Y. (2008). Biological control of Fusarium stem rot of *Anoectochilus formosanus* Hayata by *Trichoderma asperellum* TA strain. Plant Pathol. Bull. 17: 243-254.

Van Dam P, Fokkens L, Schmidt SM, Linmans JH, Kistler HC, Ma LJ, Rep M. (2016) Effector profiles distinguish formae speciales of *Fusarium oxysporum*. Environ Microbiol;18(11):4087-4102.

Verma M., Brar S. K., Tyag, R.D., Surampalli, R.Y., Valero, J.R. (2007). Review: Antagonistic fungi, Trichoderma spp.: Panoply of biological control. Biochemical Engineering Journal 37: 1-20

Vitale A, Cirvilleri G, Castello I, Aiello D, Polizzi G (2012) Evaluation of *Trichoderma harzianum* strain T22 as biological control agent of *Calonectria pauciramosa*. BioControl 57(5):687–696

Vitale A, Rocco M, Arena S, Giuffrida F, Cassaniti C, Scaloni A, Lomaglio T, Guarnaccia V, Polizzi G, Marra M, Leonardi C. (2014). Tomato susceptibility to Fusarium crown and root rot: effect of grafting combination and proteomic analysis of tolerance expression in the rootstock. Plant Physiol Biochem.;83:207-16.

Wang, C., Knill, E., Glick, B.R., De´fago, G., (2000). Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its gacA derivative CHA96 on their

growth-promoting and disease-suppressive capacities. Canadian Journal of Microbiology 46, 898–907.

Yedidia, 1., Srivastva, A.K., Kapulnik, Y. & Chet, I. (2001). Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant and Soil, 235, 235-242.

Yedidia, I., Benhamou, N. and Chet, I. (1999). Induction of defense responses in cucumber plants (Cucumis sativus L.) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology, 65, 1061–1070

Zhang, X., G. Hegerl, F. W. Zwiers, and J. Kenyon (2005), Avoiding inhomogeneity in percentile based indices of temperature extremes, J. Clim., 18, 1641 – 1651.

5. GENERAL DISCUSSION

Tomato wilt disease plants is a serious threat to agricultural production. Its nature and mode of survival in the agricultural ecosystem makes it very difficult to control by a single plant protection approach (Porta-Puglia and Mifsud, 2005; Baysal *et al*., 2009; Edel-Hermann *et al*., 2012; Taghdi *et al*., 2015; Nirmaladevi *et al*., 2016). This disease is widely distributed in tomato growing areas of Mexico and must not be ignored because it causes severe damages under suitable environmental conditions (Arie *et al*., 2007; Panthee and Chen, 2010, Inami *et al*., 2014). In this study, the most prevalent Fusarium species identified by morphological and molecular characteristics, associated with tomato wilt were *Fusarium oxysporum*, *Fusarium circinatum*, and *Fusarium andiyazi*. Among them *Fusarium oxysporum* was the most prevalent suggesting that this may be major Fusarium species found in the three states (Puebla, Morelos, and Tlaxcala) where the samples were obtained.

The results showed that most *Fusarium oxysporum* isolates showed significantly higher pathogenicity on the tomato cultivars than any other Fusarium species, inducing severe wilt symptoms. This suggest that the other Fusarium isolates in this study might not have been derived from the soils and plant tissues with authentic vascular wilt symptoms, but superficial wilt symptoms derived from root and/or stem rots which affect adversely on the absorption and translocation of water and nutritional substances (Agrios, 2004; Lopez-Berges *et al*., 2013).

Some authors Waalwijk *et al*., (1996) and O'Donnel and Cigelnik, (1997) indicated that the *Fusarium oxysporum* taxon is actually a genetically heterogeneous polytypic morphospecies whose strains represent some of the most abundant and widespread

microbes of the global soil microflora and also found in wide range of aquatic ecosystems (Gordon and Martyn, 1997; Palmero *et al*., 2009; Swathi *et al*., 2013). *F. oxysporum* also has the ability to exist as saprophytes, and to degrade lignin and complex carbohydrates associated with soil debris. Furthermore, they are also pervasive plant endophytes that can colonize plant roots and may even protect plants or be the basis of disease suppression. The *F. oxysporum* isolates distributed in the tomato fields in the present study showed the high morphological variations and high diversifications in the phylogenetic analysis based on EF-1α gene sequences. These results suggest morphological, genetic and pathological variations in *F. oxysporum* complex might have been derived hardly from common components of selection pressure that drives variations of pathogen's characteristics (Wachter and Hill, 2016). These variations may be driven by the relationships of the pathogens with host plants for the changes of pathological characteristics and by environmental and soil factors influencing their survival, growth and reproduction for the changes of morphological and genetic characteristics (Singha, 2016).

On the other hand, the Trichoderma species are known as biological control agents and are widely used in agriculture as an alternative to synthetic chemical products (Chet, 1987, Althaf and Srinivas, 2013). Isolates of this fungus have been employed against a wide spectrum of phytopathogenic fungi, including *F. oxysporum*, (Bernal-Vicente *et al*., 2009). Developments in the control achieved with this antagonist have been based on the use of isolates possessing greater efficacy, characterized by greater adaptability and persistence in the medium to which it is applied and/or greater aggressiveness against a particular phytopathogen (Rincón *et al*., 2008). Such isolates are characterized mainly by the secretion of hydrolytic enzymes such as chitinases,

glucanases and proteases – capable of hydrolyzing the cell wall of the phytopathogenic fungi (Markovich and Kononova, 2003; Seidl *et al*., 2006; Verma *et al*., 2007, Rincón *et al*., 2008).

Phylogenetic analysis of the different isolates of the genus Trichoderma and their subsequent sequencing allowed us to identify various species of Trichoderma (*T. asperellum*, *T. stromaticum*, *T. atroviride, T. koningiopsis,* and *Trichoderma* spp.). This analysis also revealed the wide genetic variation among the *T. asperellum* (the most predominant specie obtained among all isolates of this study); this could be explained due to the processes of recombination that occured during the sexual phase of reproduction (Sharma *et al*., 2009). When confronted in dual culture with the pathogenic fungus *F. oxysporum*, the growth of the selected isolates of *T. asperellum* was considerably more rapid than that of the pathogen, an important point for the survival of the antagonist in the medium (Samuels, 1996). In the dual culture, the greatest mycoparasitic effect was observed by the isolates UACH-253 (*T. asperellum*) and UACH-248; this was in accord with mycoparasitic activities that were greater than compared to the other isolates tested suggesting the production of inhibitory substance(s) by the antagonist which diffuses through the media causing growth inhibition of the pathogen inoculums though other processes such as mycoparasitism and competition may be involved. Similar reports by Elad *et al*. (1982), Ahmand and Baker (1986), and Tondje *et al*., (2007) indicated that direct parasitism of Trichoderma on hyphae of other fungi, production of extracellular lytic enzymes for cell wall degradation and competition may play a major role in the control of soil borne plant pathogens by Trichoderma species. Other research has indicated the role of these genes in the activation of other chitinases, acting as signals to indicate that the process

of cell wall degradation has commenced (Ramot *et al*., 2004; Vinale *et al*., 2008). The oligosaccharides liberated by the breakdown of the glucans of the cell wall (much more accessible than chitin) would activate the expression of the NAGases (Seidl *et al*., 2006), allowing the fungus to detect the presence of a host cell wall containing chitin.

Finally, the potential of Trichoderma species as biocontrol agents against various plant diseases has been reported by several authors (Sharon *et al*., 2001, Otadoh *et al*., 2011). In the present investigation, fungal antagonist UACH-253 isolate caused significant reduction in tomato wilt incidence under greenhouse conditions. The inhibitory effect of these bio-agents against tested pathogen was probably due to mycoparatic activity as mentioned above Hermosa *et al*., 2012.

The increased virulence of the *F. oxysporum* isolates on the tomato may be derived from the pathogen adaptation to continuous cropping system of the tomato production, as microbial populations pathogenic to the crop in cropping cycle increase, accompanying the decrease of beneficial microorganisms (Chen *et al*., 2011). This suggests tomato plants growing in greenhouses are exposed to the increased disease pressure due to the increased pathogen populations, resulting in the severe disease development especially when the soil substrate.

In the present investigation, the plant height (PH), fruit fresh weight (FFW), and root damage incidence (RDI) were also increased in *T. asperellum* treated plants. Similar results on increased plant growth due to application of *Trichoderma gamsii* in cereals and legume crops (Rinu et al., 2013). The increase in plant growth might be associated with secretion of auxins, gibberellins and cytokinins.

The increase in production may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake and by producing siderophore or

antibiotics to protect plants from deleterious rhizosphere organisms (Sundaramoorthy and Balabaskar 2013). Therefore, the antagonist *T. asperellum* UACH-253 is chosen to be the most promising bio-control agent for *F. oxysporum*. On the bases of the above mentioned, this bio-agent can be considered for sustainable disease management programs as an option in controlling Fusarium wilt. All of the above menetioned is very helpful information for developing new strategies for the adequate control of this pathogen.

6. GENERAL CONCLUSIONS

In the present study three *Fusarium* spp. (*Fusarium oxysporum*, *Fusarium andiyazi*, and *Fusarium circinatum*) associated with wilting disease of tomato in Mexico were identified. It was not find any relationship between the identified Fusarium species with the sampled areas and the production system; this shows the high capacity of this pathogen to affect tomato established under any condition due to the genetic variability of Fusarium, which affected the high variability of the infection period and growth rate as observed in this study.

The *Trichoderma* isolates included in this study significantly inhibited the growth of the pathogen. All the Trichoderma isolates inhibited the growth ranging from 40 to 70 %. Thus, it is well known that all isolates collected from different samples of soil are not equally antagonistic to pathogen and searching of effective isolate to locally suit the purpose is important.

Trichoderma asperellum (UACH-253) and *Trichoderma atroviridis* (UACH-252) showed proper performance against Fusarium wilt under greenhouse conditions, although

these isolates need to be evaluated under field condition they, however, seem to be promising isolates for further studies. Thus, it is of significant important to continue monitoring and evaluating crop diseases development to avoid high losses in tomato production and to serach for new and promising alternatives.

7. GENERAL REFERENCES

Althaf HS, Srinivas P (2013). Evaluation of Plant Growth Promoting Traits by *Pseudomonas* and *Azotobacter* Isolated from Rhizotic Soils of Two Selected Agro forestry Tree Species of Godavari Belt Region, India. Asian J Exp Biol Sci 4 (3): 431–436

Arie T., Takahashi H., Kodama M., Teraoka T. (2007): Tomato as a model plant for plant-pathogen interactions. Plant Biotechnology, 24: 135–147.

Baysal, T., Ersus, S., & Apaydın, E. (2009). The effect of corn zein edible film coating on quality of intermediate moisture tomatoes. Gida, 34(6), 359–366

Bernal-Vicente A, Ros M, Tittarelli F, Intrigliolo F, Pascual JA (2009) Citrus compost and its water extract for cultivation of melon plants in greenhouse nurseries. Evaluation of nutriactive and biocontrol effects. Bioresour Technol 99: 8722-8728

Chen L, Yang X, Raza W, Li J, Liu Y, Qiu M, Shen Q (2011). *Trichoderma harzianum* SQR-T037 rapidly degrades allelochemicals in rhizospheres of continuously cropped cucumbers. Appl Microbiol Biotechnol.89:1653–1663

CHET, I. (1987). Trichoderrna - application, mode of action, and potential as a biocontrol agent of soilborne plant pathogens. In Innoruri. approaches to Plant Diseuse Control, pp. 137-1 60. Edited by I. Chet. New York: John Wiley.

Edel-Hermann V., Gautheron N., Steinberg C. (2012). Genetic diversity *of Fusarium oxysporum* and related species pathogenic on tomato in Algeria and other mediterranean countries. Plant Pathol. 61, 787–800.

Elad, Y., Y. Hadar, I. Chet and Y. Henis. (1982). Prevention with *Trichoderma harzianum* Rifai aggr, of reinfestation by *Sclerotium rolfsii* Sacc., and *Rhizoctonia*

solani Kuhn of soil fumigated with methyl bromide and improvement in disease control in tomato and peanuts. Crop Prot., 1: 199–211.

Gordon, T.R., Martyn, R.D. (1997): The evolutionary biology of *Fusarium oxysporum*. Ann Rev Pytopathol 35: 111-128.

Hermosa R., Viterbo A., Chet I., Monte E. (2012). Plant-beneficial effects of Trichoderma and of its genes. Microbiol 158, 17–25.

Gordon, T.R., Martyn, R.D. (1997). The evolutionary biology of *Fusarium oxysporum*. Ann Rev Pytopathol 35: 111–128.

Lopez-Berges MS, Hera C, Sulyok M, Schafer K, Capilla J, Guarro J, Di Pietro A. The velvet complex governs mycotoxin production and virulence of Fusarium oxysporum on plant and mammalian hosts. Mol Microbiol. 2013;87: 49–65.

Markovich NA, Kononova GL. (2003). Lytic enzymes of Trichoderma and their role in plant defense from fungal diseases: a review. Appl Biochem Microbiol.39:341–351

Nirmaladevi D, Venkataramana M, Srivastava RK, Uppalapati SR, Gupta VK, Yli-Mattila T, Clement Tsui KM, Srinivas C, Niranjana SR and Chandra NS (2016) Molecular phylogeny, pathogenicity and toxigenicity of *Fusarium oxysporum* f. sp. *lycopersici*. Scientific Reports

O'Donnell, K. and Cigelnik, E. (1997) Two Divergent Intragenomic rDNA ITS2 Types within a Monophyletic Lineage of the Fungus Fusarium Are Nonorthologous. Molecular Phylogenetics and Evolution, 7, 103.

Otadoh, J. A., Okoth, S. A., Ochanda, J. and Kahindi, J. P. (2011). Assessment of Trichoderma isolates for virulence efficacy on *Fusarium oxysporum* f. sp. *phaseoli*. Tropical and Subtropical Agroecosystems, 13: 99–107.

Palmer, J. M., Perrin, R. M., Dagenais, T. R. T., and Keller, N. P. (2009). H3K9 methylation regulates growth and development in *Aspergillus fumigatus*. Eukaryot. Cell 7:2052–2060.

Panthee DR, Chen F (2010). Genomics of fungal disease resistance in tomato. Currentgenomics, 11:30–39.

Porta-Puglia A., Mifsud D. (2005). First record of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in Malta. J. Plant Pathol.87, 150

Ramot, O., Viterbo, A., Friesem, D., Oppenheim, A. & Chet, I. (2004). Regulation of two homodimer hexosaminidases in the mycoparasitic fungus *Trichoderma asperellum* by glucosamine. Curr Genet 45, 205–213.

Rincón, A. M.; A. C. Codón; T. Benítez (2008). Hidrolasas y genes fúngicos de interés en biocontrol, Pallás, V.; C. Escobar; P. Rodríguez; J. F. Marcos (Editores). Herramientas biotecnológicas en Fitopatología, Ediciones Mundi Prensa, Madrid.

Rinu K., Sati P., Pandey A., (2013). *Trichoderma gamsii* (NFCCI 2177): a newly isolated endophytic, psychrotolerant, plant growth promoting, and antagonistic fungal strain. J Basic Microbiol, 1-10.

Samuels GJ (1996) Trichoderma: a review of biology and systematics of the genus. Mycol Res 100:923–935.

Seidl, V., Marchetti, M., Schandl, R., Allmaier, G. & Kubicek, C. P. (2006). Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. FEBS J 273, 4346–4359.

Sharma, M., Varshney, R.K., Rao, J.N., Kannan, S., Hoisington, D., Pande, S., (2009). Genetic diversity in Indian isolates of *Fusarium oxysporum* f. sp. *ciceris*, chickpea wilt pathogen. Afr. J. Biotech. 8, 1016–1023

Sharon E, Bar-Elad M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001). Biological control of root knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology, vol 91, (7), pp 687–693.

Singha MI, Kakoty Y, Unni GB, Das J, Kalita CM (2016). Identification and characterization of *Fusarium* sp. using ITS and RAPD causing Fusarium wilt of tomato isolated from Assam, North East India. Journal of Genetic Engineering and Biotechnology 14:99–105.

S. Sundaramoorthy and P. Balabaskar (2013). Biocontrol efficacy of Trichoderma spp. against wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici.* J App Biol Biotech; 1 (03): 36–40.

Swathi J, Sowjanya KM, Narendra K, Krishna Satya A (2013). Bioactivity assay of an isolated marine *Fusarium* sps. Int. J. Biosci. Biotechnol. 5(5):179–186

Taghdi Y., Hermosa R., Domínguez S., Rubio M. B., Essalmani H., Nicolas C., *et al*. (2015). Effectiveness of composts and *Trichoderma* strains for control of Fusarium wilt of tomato. Phytopathol. Mediterr. 54, 232–240.

Tondje PR, Roberts DP, Bon MC, Widmer T, Samuels GJ, Ismaiel A, Begoude AD, Tchana T, Nyemb-Tshomb E, Mdoumbe-Nkeng M, Bateman R, Fontem D, Hebbar KP. (2007). Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. Biol Control 43:202–212

Verma, M., Brar, S.K., Tyagi, R.D., Sahai, V., Prévost, D., Valéro, J.R., Surampalli, R.Y., (2007). Bench-scale fermentation of *Trichoderma viride* on wastewater sludge: rheology, lytic enzymes and biocontrol activity. Enzyme Microb. Technol. 41, 764–771.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., & Lorito, M. (2008). Trichoderma-plant-pathogen interactions. Soil Biology and Biochemistry, 40(1), 1–10

Waalwijk C, De Koning JRA, Baayen RP, Gams W (1996) Discordant groupings of *Fusarium* spp from sections Elegans, Liseola and Dlaminia based on ribosomal ITS1 and ITS2 sequences. Mycologia 88: 316–328

Wachter J, Hill S. (2016) Positive selection pressure drives variation on the surface-exposed variable proteins of the pathogenic Neisseria. PLoS One.11