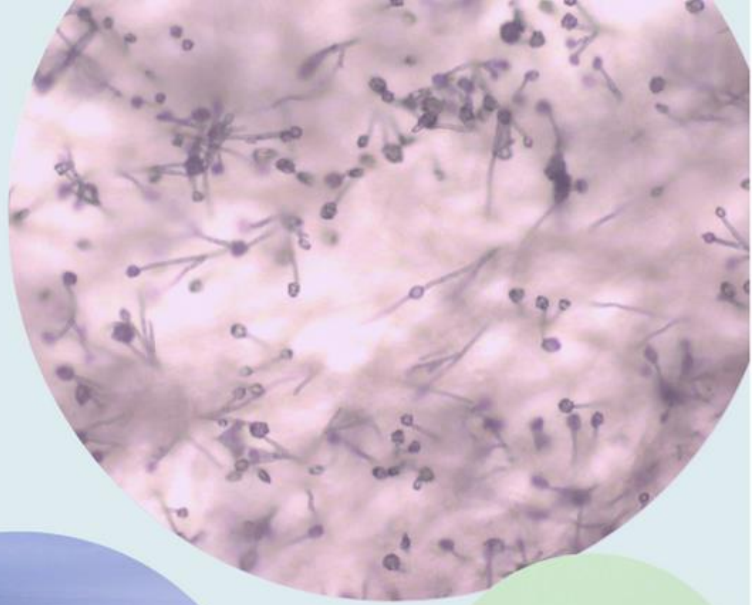

PH.D. THESIS

Etiology, epidemiology and control of Fusarium crown and foot rot of zucchini caused by *Fusarium solani* f. sp. *cucurbitae*

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Etiology, epidemiology and control of Fusarium crown and foot rot of zucchini caused by *Fusarium solani* f. sp. *cucurbitae*

Etiología, epidemiología y control de la podredumbre de la base del tallo del calabacín causada por *Fusarium solani* f. sp. *cucurbitae*

Memoria presentada por **Ana Pérez Hernández** para optar al grado de Doctora

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El calabacín (*Cucurbita pepo L.*) es una de las hortalizas más importantes a nivel mundial. Esta especie es, en general, poco susceptible a enfermedades causadas por hongos de suelo con síntomas en las raíces y base del tallo, sin embargo, en la primavera 2007 se detectó por primera vez en la provincia de Almería una enfermedad telúrica que causaba marchitez, clorosis, necrosis en la base del tallo, y finalmente la muerte de las plantas. El agente causal fue identificado como *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* (Snyder y Hans). Este hongo ascomiceto tiene una especificad parasitaria sobre las cucurbitáceas, y es capaz de infectar en condiciones de campo a melón, sandía, pepino y calabaza. Puede transmitirse a través de las semillas y producir clamidosporas para conservarse en el suelo.

La presente tesis doctoral se centra en el estudio de esta forma especializada de *Fusarium solani,* así como de diversos métodos para su control. De esta forma, en el Capítulo II se comienza con la realización de una prospección fitopatológica en invernaderos de la provincia de Almería, principalmente dedicados al cultivo de calabacín, aunque también se visitaron invernaderos donde se cultivaban pepino, melón o sandía. Una vez confirmada la extensión de la enfermedad a lo largo de la provincia de Almería, se obtuvo una colección de aislados, y se realizó un estudio detallado de una selección de éstos. Para ello en primer lugar se estudió el poder patógeno sobre plantas y frutos de calabacín. Todos los aislados de *F. solani* estudiados, excepto uno, fueron altamente patógenos causando enfermedad en todas las plantas inoculadas, y una elevada tasa de mortandad. Además, todos los aislados fueron capaces de producir podredumbre en los frutos. A partir de estos resultados se seleccionó un aislado moderadamente agresivo y un aislado muy agresivo, y se estudió su patogenicidad sobre varias especies de cucurbitáceas, así como varias especies pertenecientes a otras familias botánicas ampliamente cultivadas en la provincia de Almería. Los resultados confirmaron la especificidad parasitaria de esta forma especializada sobre las cucurbitáceas, que resultaron todas susceptibles frente a la enfermedad, mientras que el resto de las especies ensayadas no resultaron ser hospedadoras. Se observaron diferencias entre las distintas especies, resultando el

pepino significativamente menos susceptible que el resto de cucurbitáceas. Posteriormente, se describieron las principales características morfológicas de *F. solani* f. sp. *cucurbitae*, entre las que destacan la producción de fiálidas largas y verticiladas, la formación de macroconidias relativamente largas y finas, así como la producción de largas cadenas de clamidosporas. Por último, a partir de las secuencias del factor de elongación traslación 1-α (*EF 1-α*) y de la segunda mayor subunidad de la RNA polimerasa II (*RPB2*) se realizó un estudio filogénico y se confirmó el encuadre de estos aislados dentro del complejo de especies de *Fusarium solani* (FSSC), mostrando que los aislados forman un grupo monofilético situado en el clado 3 del FSSC.

En el Capítulo III se describe la primera detección de la podredumbre de la base del tallo del melón en España; y en el Capítulo IV se describe la primera detección de *Phytophthora capsici* causando podredumbre de la base del tallo del calabacín en España, una enfermedad de síntomas similares a los causados por *F. solani* f. sp. *cucurbitae*.

Por último, en el Capítulo V se estudian diversos métodos para el control de la podredumbre de la base del tallo del calabacín. En primer lugar, se investigó la conservación del hongo durante 18 meses en sacos de perlita infestados con el patógeno. Una vez se verificó la capacidad del mismo para sobrevivir en los sacos, se evaluaron varios métodos para el control de la enfermedad. En primer lugar se estudió el control genético mediante la caracterización de la susceptibilidad de una serie de cultivares comerciales de calabacín frente a le enfermedad, quedando patente que todos ellos fueron altamente susceptibles. Se ensayaron diversos productos químicos y formulados microbiológicos, entre los que se detectaron tres sustancias, procloraz, metil-tiofanato y carbendazima como altamente eficaces para su control, aunque su uso no está actualmente autorizado en calabacín en la Unión Europea. Por último, se probó la solarización y biosolarización de invernaderos con sistema de enarenado para la eliminación del inóculo del suelo durante periodos de seis semanas, demostrándose que ambos métodos son eficaces para la eliminación del inóculo del suelo y por tanto son adecuados para prevenir la enfermedad en posteriores cultivos.

Zucchini (*Cucurbita pepo* L.) is one of the most important crops produced worldwide. This species is generally not very susceptible to fungal soilborne diseases, however, in the spring of 2007, a telluric disease causing wilt, chlorosis, crown necrosis, and finally the death of the plants was detected for the first time in the province of Almería. The causal agent was identified as *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* (Snyder and Hans). This ascomycete fungus has host specificity on cucurbits, being able to infect melons, watermelons, cucumbers and squashes in field conditions. The pathogen may be seed-transmitted and it produces chlamydospores which can survive in the soil after the incidence of the disease.

This doctoral thesis has focused on the study of this *forma specialis* of *F. solani*, as well as in the assessment of several methods for disease control. Chapter II starts conducting a disease survey in greenhouses within the province of Almería, most of them dedicated to the cultivation of zucchini, although we also visited greenhouses dedicated to the cultivation of cucumber, melon and watermelon. Once the spread of the disease throughout the province of Almería was confirmed, a collection of isolates was obtained, and a detailed study of a selection of them was carried out. For this purpose, their pathogenicity on plants and fruits of zucchini was first studied. All isolates of *F. solani* studied except one were highly pathogenic to zucchini plants, causing disease in all inoculated plants, and a high mortality rate. In addition, all the isolates were able to produce fruit rot. From these results, a moderately aggressive isolate and a very aggressive one were selected, and their pathogenicity was studied on the main species belonging to the cucurbit family, as well as several species belonging to other botanical families that are widely cultivated in the province of Almeria. The results confirmed the host specificity of this special form on cucurbits, which were all susceptible to the disease, while the rest of the species tested were not hosts of the disease. Differences in susceptibility among the different cucurbits were observed, being cucumber significantly less susceptible to the disease. In addition, the main morphological characters of *F. solani* f. sp. *cucurbitae* were described, among which the production of long and verticillate phialides, the formation of relatively long and

slender macroconidia, as well as the production of long chlamydospore chains were the most remarkable characters. Finally, from the sequences of the translation elongation factor 1-α (*EF 1-α*) and the second largest subunit of RNA polymerase II (*RPB2*), a phylogenetic study was conducted which confirmed the classification of these isolates within the *Fusarium solani* species complex (FSSC), showing that the isolates form a monophyletic group within clade 3 of the FSSC.

Chapter III describes the first detection of the Fusarium crown and foot rot of meloncaused by *F. solani* f. sp. *cucurbitae* in Spain; and Chapter IV describes the first detection of *Phytophthora capsici* causing Crown and root rot in zucchini in Spain, a disease with similar symptoms to those caused by *F. solani* f. sp. *cucurbitae*.

Finally, Chapter V discusses various methods for controlling the Fusarium crown and foot rot of zucchini. First, the survival of the fungus in infested bags of perlite was assessed for 18 months. Once its ability to survive in the bags was verified, several methods for disease control were studied. First, genetic control was investigated by characterizing the susceptibility of several commercial zucchini cultivars to the disease, confirming that all of them were highly susceptible. Also, various chemical products and microbiological formulations were tested, among which three substances, prochloraz, thiophanate- methyl and carbendazim were highly effective in the control of the disease, although its use is not currently authorized in zucchini in the European Union. Finally, six-week periods of solarization and biosolarization of greenhouses with the so-called *enarenado* system for elimination of the inoculum in the soil were tested, and their efficacy for reducing the inoculum from the soil was demonstrated, and therefore they can be considered effective tools for preventing a disease outbreak in subsequent crops

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION...1

1. THE CULTIVATION OF ZUCCHINI AND MELON 1

1.1 Origin and taxonomy 1

1.1.1 Origin and taxonomy of zucchini...1

1.1.2 Origin and taxonomy of melon..2

1.2 Botanical characteristics and cultural requirements 4

1.2.1 Zucchini...4

1.2.2 Melon 6

1.3 Economic importance of zucchini and melon production 9

1.3.1 Global importance 9

1.3.2 National importance...11

1.4 Main soilborne diseases of zucchini and melon..13

1.4.1 Soilborne diseases of zucchini 13

1.4.2 Soilborne fungal diseases of melon...15

2. THE GENUS *Fusarium* 21

2.1 History of the *Fusarium* taxonomy..21

2.2 The species concepts in *Fusarium* 24

2.2.1 The morphological species concept 25

2.2.2 The biological species concept 26

2.2.3 The phylogenetic species concept..27

2.3. The special forms in *Fusarium*...28

2.4 Fusaium crown and foot rot of zucchini caused by *F. solani* f. sp. *cucurbitae* 30

OBJECTIVES 35

CHAPTER II

Pathogenic, Morphological, and Phylogenetic Characterization of *Fusarium solani* f. sp. *cucurbitae* Isolates from Cucurbits in Almería Province, Spain 37

ABSTRACT ....

INTRODUCTION 39

MATERIALS AND METHODS....42
Disease survey....42
Collection of isolates....43
Production of pathogen inoculum....43
Plant growth and maintenance....44
Pathogenicity trials on zucchini plants and fruit....44
Pathogenicity on other horticultural crops....46
Determination of mycelial growth rates and morphological characteristics....47
Genomic DNA extraction, PCR amplification, and sequencing....48
Phylogenetic analyses....48
Statistical analyses....50
RESULTS....51
Disease survey....51
Pathogenicity on zucchini plants and fruit....54
Pathogenicity on other horticultural crops....56
Morphology....59
Phylogeny....61
DISCUSSION....66
ACKNOWLEDGEMENTS....71

CHAPTER III

***Fusarium solani* f. sp. *cucurbitae*, Affecting Melon in Almería Province, Spain....73**
ABSTRACT....75
MAIN TEXT....75
ACKNOWLEDGMENTS....78

CHAPTER IV

Phytophthora Crown and Root Rot of Zucchini Squash in Almería, Spain....79
ABSTRACT....81

CHAPTER V

Survival of *Fusarium solani* f. sp. *cucurbitae* and Fungicide Application, Soil Solarization, and Biosolarization for Control of Crown and Foot Rot of Zucchini Squash 83

ABSTRACT 85

INTRODUCTION 85

MATERIALS AND METHODS 89

Isolate identification 89

Production of pathogen inoculum 89

Methods for growing plants 90

Temperature data acquisition 90

Soil-borne inocula survival assessment 91

Fungicide efficacy greenhouse evaluation 92

Cultivar susceptibility evaluation 95

Soil solarization and biosolarization 96

Statistical analyses 98

RESULTS 98

Pathogen survival assessment in bags of perlite 98

Fungicide efficacy greenhouse evaluation. 101

Cultivar experiments 102

Solarization and biosolarization 104

DISCUSSION 105

AKNOWLEDGEMENTS 109

CHAPTER VI

GENERAL DISCUSSION 111

CHAPTER VII

CONCLUSIONS 123

LITERATURE CITED 127

CHAPTER I

INTRODUCTION

1. THE CULTIVATION OF ZUCCHINI AND MELON

1.1 Origin and taxonomy

1.1.1 Origin and taxonomy of zucchini

Zucchini (*Cucurbita pepo* L.) is an annual and herbaceous plant belonging to the Cucurbitaceae family, to the genus *Cucurbita*. This is composed of 22 wild and five cultivated species: *C. pepo* L., *C. moschata* (Duchesne ex Lam.) Duchesne ex Poiret, *C. maxima* Duchesne ex Poiret, *C. ficifolia* Bouché and *C. argirosperma* Huber.

The species *C. pepo* was one of the first to be domesticated according to archaeological records, being its first record in Mexico, specifically in the Oaxaca Valley in the year 8.750 BC. There is evidence of domesticated varieties that were found in northern Mexico and the United States more than 4,000 years ago (Paris 2008). It is therefore considered that there were two independent domestication events of this species, one in southern Mexico and the other in the eastern United States of America (Decker 1988). It was introduced in Europe in the sixteenth century, after the conquest of the New World, from where it was distributed to the rest of Europe (Lira and Montes 1994, Lust and Paris 2016).

C. pepo is divided into three subspecies based on polymorphisms in its allozymes (Paris et al. 2003): *C. pepo* subsp. *fraterna* (Bailey) Andres, composed of wild varieties from the Northeast of Mexico (Andres 1987, Nee 1990), *C. pepo* subsp. *ovifera* (L.) Decker (= *C. pepo* subsp. *texana* (Scheele) Filov) which includes wild varieties of the United States, and *C. pepo* subsp. *pepo*, from which no wild varieties have been found (Decker 1988). The domesticated varieties for consumption are included within the *ovifera* and p*epo* subspecies, and these have been further classified according to the characteristics of their fruits: Cocozelle, Vegetable Marrow, Pumpkin and Zucchini, belonging to the subsp. *pepo*; and Crookneck, Straightneck, Scallop and Acorn belonging to the subsp. *ovifera*. As shown in Figure 1, there is great diversity in the shape of the fruits of *C.*

pepo, considered as possibly the most polymorphic species in their fruits (Duchesne 1786, Naudin 1856).

Figure 1. Different morphotypes of *C. pepo*. From left to right top row subsp. *ovifera*: *Acorn, Straightneck, Crookneck, Scallop*. Lower row subsp. *pepo*: *Pumpkin, Vegetable Marrow, Cocozelle* and *Zucchini*. Retrieved from Lust and Paris, 2016

The Zucchini morphotype is the most cultivated and of greater economic importance in the world within this species. Its first description dates from the beginning of the twentieth century in Italy (Tamaro, 1901), from where it was introduced to the United States. In the year 1937, zucchini was first described in the United States as a new introduction from Italy (Tapley et al. 1937). Currently, the cultivated varieties come from hybrids that were bred in the United States obtained from the Italian varieties (Lust and Paris, 2016).

1.1.2 Origin and taxonomy of melon

Melon (*Cucumis melo* L.) also belongs to the Cucurbitaceae family, to the genus *Cucumis*. This genus has a wide distribution of wild species from southern and central Africa, Asia, to the north of the Australian continent. For several years it has been considered by most authors that the melon was originated in Africa, because the

number of chromosomes in melon coincides with that of several African wild species, while the cucumber, of Asian origin, has a different chromosome number (Burger et al. 2010, Chen et al. 1998, Kirkbride 1993). However, recent studies suggest that *C. melo* originated in Asia (Renner et al. 2007, Schaefer et al. 2008), being its wild ancestor originating in India (Sebastian et al. 2010). The oldest reference to this crop has been found in Egyptian tombs from around 2,400 BC (Kirkbride 1993, Pangalo 1929). It is estimated that melons were present in Central Asia in the ninth century, from where they were introduced to the Iberian Peninsula probably through the Islamic conquest in the eleventh century (Paris et al. 2012). From there they were introduced in the rest of Europe, and later they were introduced by Christopher Columbus in the American continent in 1516 (Mallick and Masui 1986, Whitaker and Davis 1962).

One of the most accepted melon classifications is the one proposed by Pitrat et al. (2000), which divides the species into 16 groups or varieties, five of which are grouped in the subsp. *agrestis* (*conomon, makuwa, chinensis, acidulus* and *momordica)* and 11 in the subsp. *melo* (*cantalupensis, reticulatus, adana, chandalak, ameri, inodorus, flexuosus, chate, tibish, dudaim* and *chito*). Most melons grown in our country belong to the *cantalupensis* and *inodorus* groups.

Another classification (independent of the botanical classification) is the commercial classification of melons by types, which can be established in the following way (Torres, 1997):

• Yellow melon: of Spanish origin, yellow skin and creamy white flesh. It is divided into rough yellow and smooth round yellow.

• Spanish green melons: green color more or less dark, elongated shape and high size (1.5 to 3 kg). They are divided into three groups: Rochet, Piel de sapo and Tendral.

• Charentais melon: of French origin, which include both smooth and reticulated skin varieties.

• Melon Galia: of Israeli origin, rounded shape, yellow skin with a fine reticulate, greenish-white pulp and buttery texture.

• Cantaloup melon: of American origin, spherical shape, coarse reticulated throughout its surface, the flesh is salmon-colored and aromatic.

• Honeydew melon: smooth skin, faint green or almost white pulp.

• Other minor types

1.2 Botanical characteristics and cultural requirements

1.2.1 Zucchini

Zucchini, also known as summer squash or courgette, is an annual, non-vining bushy plant, provided with a main stem that normally shows a determinate growth, although it depends on the cultivar (López-Marín, 2017). The stem is thick, almost cylindrical in shape, with a pilose surface, and the rest of the plant organs such as leaves, flowers and tendrils emerge directly from it. Tendrils are formed from axillary meristems at leaf nodes and can reach 20 cm long, however, most of the commercial cultivars lack tendrils. The leaves are big, generally deeply lobed almost palmatifid, dark green colored with silver-gray splotches and streaks, up to 50 cm wide, with a pilose surface in the abaxial side. The petiole is very long and also pilose, stalk, arising from the main stem. The leaves are helicoidally alternate (Reche 2000, Serrano 1996).

Zucchini has extensive and superficial rooting habits, with a shallow distribution, developing in the first meter depth and showing a lesser development of the more deeply penetrating portions (Weaver and Bruner 1927). The root system is composed by a strong tap root and several lateral roots (Serrano 1996). Secondary roots may form from the internodes if the stem is in contact with the soil. The root system normally shows a profuse and rapid development (López-Marín 2017).

C. pepo is a monoecious species with large, bright, yellow-orange conspicuous flowers. They are comprised of a campanulate, 5-lobed corolla together with a 5-lobed calyx, forming a basal perianth tube (Hayward 1938). Staminate flowers are born on long, slender pedicels and produced near the crown of the plant, in the main stem. Pistillate flowers are larger with shorter and broader pedicels and are produced distally to staminate flowers on the main stem and lateral branches. Staminate flowering often

occurs few days before pistillate flowering (López-Marín 2017, Serrano 1996). Pollination is entomophillic, being some of the most important pollinators honey bees (*Apis melifera*), and bumblebees (*Bombus* spp.), among others (Shuler et al. 2005).

The fruit is a berry, fleshy, without a central cavity, unilocular, normally elongated and cylindrical, and somewhat stubby at its apical end, with a smooth and very delicate epidermis. Its color can be green, white or yellow, in different shades, marbled, reticulated, etc. Some cultivars may offer different forms (round, flattened and warty), such as the Pattypan squash or the Round zucchini. It is commercialized and consumed on an immature stage, in order to avoid the hardening of its epicarp, and the presence of big, mature seeds (López-Marín 2017). Optimal fruits weight between 100 to 300 g, normally 3 to 7 days after pollination (FAO 1990, Maynard and Hochmuth 2007). The seeds are oval and elongated, over 1 cm-long, smooth and with a rib along the edge, ivory white (López-Marín 2017).

Zucchini is widespread in protected cultivation in the Mediterranean countries where the young fruits and sometimes the staminate flowers before anthesis are largely consumed. It is cultivated in plastic houses and under low tunnels (FAO 1990).

Regarding temperature, zucchini, like other cucurbits, requires warm conditions for good growth, and it’s not likely to resist frosts. Consequently, its cultivation is limited to those areas where the risk of frost damage is absent. In the other hand, *C. pepo* requires lower temperatures than other cucurbits. For the optimal development of the plant, temperatures should be comprised between 20 to 25°C during the daytime and around 16 to 18°C during the night. It doesn’t tolerate very high or very low temperatures, nor very low humidity. The conditions that follow winter sowing in the Mediterranean regions determine a cycle of about 90-120 days, whilst autumn cultivation allows harvesting to start 60-90 days after sowing (FAO, 1990).

Zucchini plants develop well with relative humidity ranging 65-80%, and they have high water requirements, especially after the development and ripening of the first fruits. Zucchini can develop independently of the number of daylight hours a day; however, long days and high temperatures induce a greater appearance of male flowers and on

the contrary short days and low temperatures, the greater presence of female flowers (López-Marín 2017).

Although it doesn't have very specific requirement in terms of soil quality, adapting well to all types of soils including the sandy ones, it develops better in soils with a loam texture, with deep horizons. It does have huge requirements in organic matter, with a high level of nutrients in order to achieve a high fruit quality (López-Marín 2017).

1.2.2 Melon

Melon is an annual, trailing herbaceous plant with an often-angled stem, very hairy, provided with simple and spiral tendrils. The plant is composed by the main stem, and the secondary branches which arise from leaf axil meristems, being the first three or four the most developed ones (Alarcón and Fuentes 2016, Serrano 1996). The leaves are simple, alternate on long petioles, cordate with three to seven lobes, smooth in the adaxial surface and hairy on the abaxial surface, acute, deep green, with serrate margins and about 7 to 15 cm in diameter (Galera-García et al. 2003, Maroto 1995).

The root system is extensive but shallow, with a strong tap-root readily giving rise to numerous secondary and lateral roots. No adventitious roots are formed (FAO 1990, Serrano 1996). Most of the root system is found at around 30-40 cm depth, although some roots may reach 1.2 m (Alarcón and Fuentes 2017).

According to the type of flowers, muskmelon cultivars can be divided into monoecious, bearing separate staminate and pistillate flowers on the same plant, and andromonoecious, bearing staminate and hermaphrodite flowers (FAO 1990). The flowers are borne in the leaf axils and are pedunculated. Male flowers are formed in the main stem and appear before female and hermaphrodite flowers, which are formed in secondary and tertiary branches. The flowers have green calices consisting in five sepals that fuse at the base, and corollas consisting of five petals, bright to pale yellow, and small, approximately 3 cm in diameter. Female and hermaphrodite flowers have an inferior ovary. Pollination is entomophilic (Galera-García et al. 2003, Maroto 1995, Torres 1997).

The fruit is an infructescence called pepo that is composed by the skin (it can show different colors and be netted or reticulated), the placenta (where the seeds are located, and which is divided into 3-4 double lobes) and the pulp. The shapes, colors and dimensions of the fruits are very variable, as explained above. The seeds occupy the central cavity of the fruit, are fusiform, flat and white or yellowish in color, being able to form between 200 and 600 per fruit (Maroto 1995). Normally, the fruits reach maturity at around 30-45 days after pollination, presenting variable size depending on the variety. The sugar content in the fruits a key factor for the fruit quality and recollecting the fruit at its optimal maturation stage is essential (Maroto 1995).

In protected crops, melon is one of the crops with highest heat and light requirements, which makes it difficult to grow it in wintertime, even in heated greenhouses. Also, light is very important, not only for plant growth but also particularly for fruit quality. Plants exposed to low light intensity usually produce small and unsweet fruits (FAO 1990). For the best development of the plant, temperatures should be comprised between 24 to 30°C during the daytime and around 13 to 15°C during the night (Alarcón and Fuentes 2016). Low temperatures reduce growth and hasten female flower formation, while excessively high temperatures (above 30°C) increase respiration and accelerate fruit ripening, so that the fruits become yellow too early, have a low sugar content and are therefore of poor quality (FAO 1990).

Melon plants need plenty of water during the growth period and especially in the development and ripening of the fruits. The scarcity of water may lead to lower yields, both in quantity and quality. The number of hours of light is also very important, requiring at least 15 hours a day (Zapata et al. 1989). It prefers rich, deep, well-aired and drained soils, preferably with a pH between 6 and 7. Tolerates calcareous soils but is very sensitive to flooding that may produce root and fruit rots (Zapata et al. 1989).

In protected cultivation the muskmelon is usually a spring crop because its climatic requirements do not allow it to be grown in wintertime and competition with late open-air crops reduces the prices. The planting time depends on the region, however, in Mediterranean conditions, even in the most favorable areas, conditions are not

suitable for planting before mid-January without heating. Average plant density is about 1.8 plants/m^2 for flat and 2.5 plants/m^2 for climbing crops. It depends on plant vigor, on light and on the time of year. Higher densities are used for less vigorous plants and good light conditions. The higher the plant density, the higher the yield per m^2 but the lower the number of fruits per plant and their size (FAO 1990).

Planting is performed when the plants have two or three true leaves. In staked cultivation, the plants are hold with threads and develop vertically. Plants may be maintained with one or two main stems. Although the creeping cultivation system is the most used due to the reduction in labor, the staking cultivation system is used to achieve higher fruit qualities (Gómez-Guillamón et al. 1997).

Fruit collection depends on a number of factors such as the variety, cultivation area, planting season, and sugar content, among others. The most common is collecting fruits between April and June. The time that collection lasts is also very variable, not exceeding 30-40 days (Reche 2008).

1.3 Economic importance of zucchini and melon production

1.3.1 Global importance

Zucchini and melon are two of the most important crops spread throughout the world, being produced from the tropics to the temperate zones (Maroto 1995). According to the statistics of the FAO, in the year 2017 more than 27 million tons of different types of pumpkins, squash and gourds; and almost 32 tons of melons were produced in the world. The main producer for both crops was China, with almost 8 million tons of pumpkins, squash and gourds, and more than 17 million tons of melons. Spain was in seventh place in the production of pumpkins, squash and gourds, with 702,278 tons; and in the eighth place in the world in melon production with 655,677 tons (Figure 2, FAOSTAT 2017).

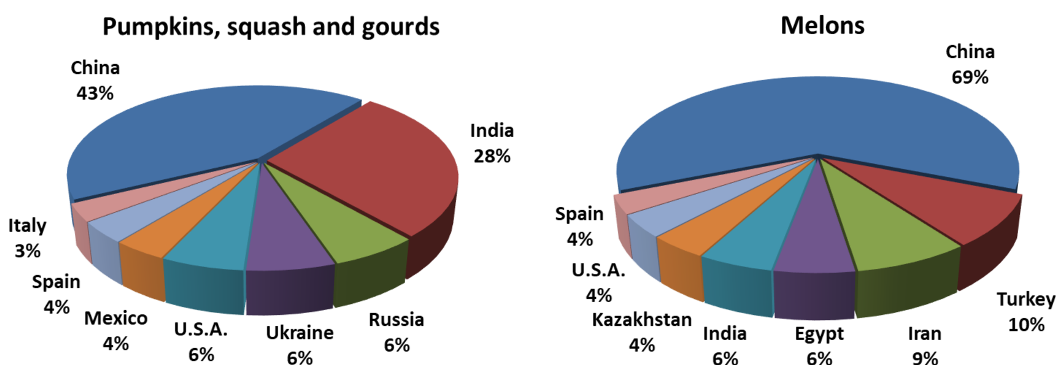

Figure 2. Main producing countries of zucchini and melon in the world. Own elaboration from FAOSTAT 2017.

The world production of pumpkins, squash and gourds has not stopped growing in recent years. Specifically, from 2005 to 2017, it increased by 36% as a result of the increase in the area dedicated to its cultivation, which was 33%. The melon, however, has suffered several fluctuations in the last decade, although it also follows a growing trend, having increased its world production by 16% between those years. It is important to remark, however, that the world surface dedicated to melon production has suffered a 4% decrease (Figure 3), so the increase in this case is due to the increase in yield of 20% compared to 2005, up to exceed 2,600 t/ha in 2017.

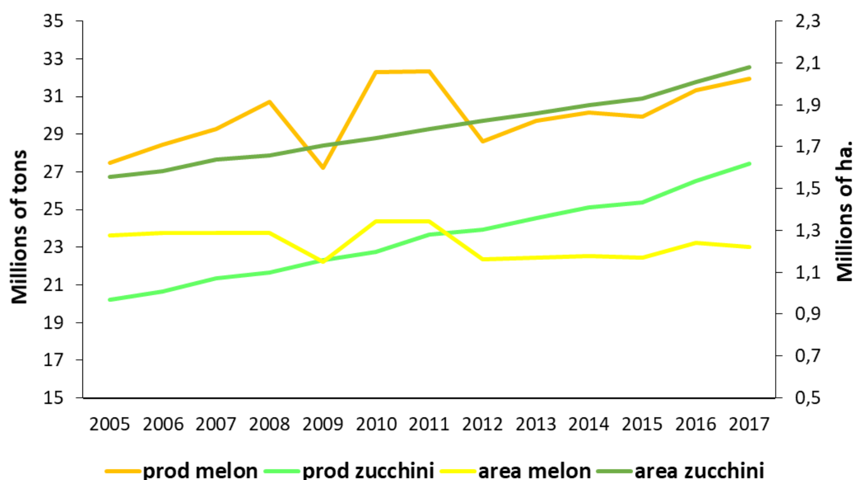

Figure 3. Evolution of the surface and world production of melons and pumpkins, squash and gourds since 2005. Own elaboration from FAOSTAT 2017.

Spain is the main melon exporting country worldwide, with 443,395 tons of melons exported in 2016, and the second exporter of pumpkins, squash and gourds after Mexico with 362,046 tons in the same year (Figure 4). The main importer is the United States, which in 2016 imported a total of 694,110 tons of melons and 474,412 tons of pumpkins, squash and gourds (FAOSTAT 2017).

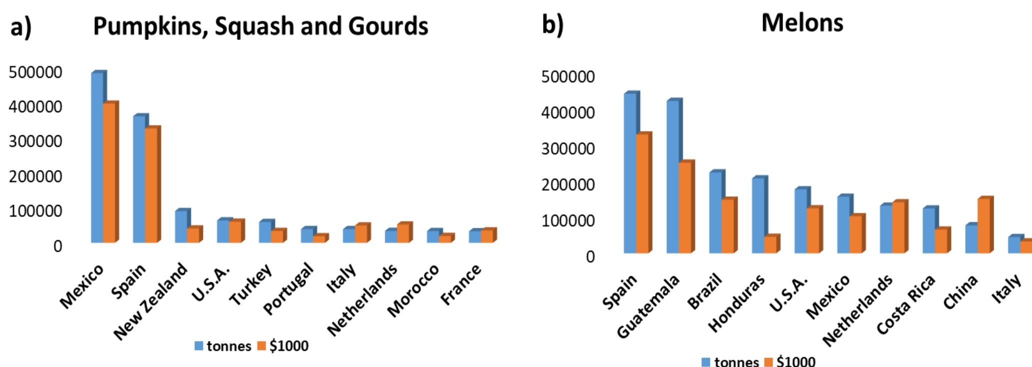

Figure 4. Main countries exporting a) pumpkins, squash and gourds and b) melons, worldwide in 2017. Values expressed in tons and thousands of dollars. Own elaboration from FAOSTAT 2017.

1.3.2 National importance

At a national level, both vegetables stand out for the surface that is dedicated to their cultivation. According to data from the Ministry of Agriculture, Fisheries and Food, in 2016 11,081 ha were devoted to the cultivation of zucchini in Spain, producing a total of 581,503 tons, and 20,686 hectares to the melon crop that produced 649,767 tons (MAPA 2017). However, the production trends of these crops have been opposite. In the case of zucchini, the total area dedicated to its cultivation in Spain has continued to grow, increasing its total area by more than 50% since 2006. In the same way, production has followed an upward trend in recent years. However, as shown in Figure 5, in 2014 there was a reduction in the production of zucchini, but not in its surface, probably due to the introduction in late 2012 of *Tomato leaf curl New Delhi virus* (ToLCNDV, Juárez et al. 2014) that severely affected zucchini cultivation in the province of Almería, especially during the first year after its introduction until the prevention and control measures of the disease were improved (Figàs et al. 2017, Juárez et al. 2014, Ruiz et al. 2017, Simón García 2016). In the case of melon, the area devoted to this crop has been drastically reduced, going from more than 40 thousand hectares in 2006, to slightly more than 20 thousand hectares in 2016. In the same way, its production has followed a similar evolution in Spain (Figure 5).

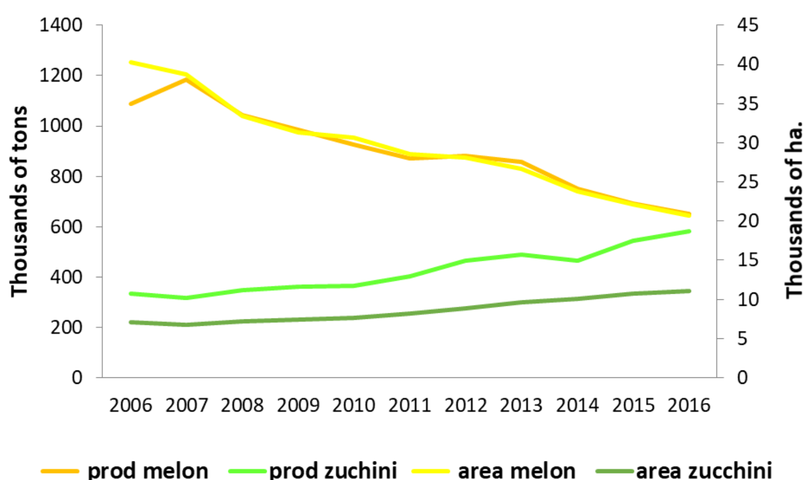

Figure 5. Evolution of the production and the surface dedicated to zucchini and melon in Spain between 2005 to 2016. Own elaboration through MAPA 2017.

Among the total zucchini produced in Spain in 2016, the vast majority came from the Autonomous Community of Andalusia, where 477,330 t were produced, corresponding to more than 82% of national production. Within Andalusia, the province that stands out for its production is Almería, which in the same year produced 434,195 t, corresponding to more than 90% of the total production in this community, and almost 75% of the total production of Spain (MAPA 2017).

The main community producing melon in 2016 was Castilla la Mancha with 222,096 t followed by the region of Murcia with 182,042 t and Andalusia with 152,236 t, accounting between the three of them for more than 85% of the total national production. In the province of Almería, 96,417 ha were dedicated to melon cultivation, which accounts for almost 15% of the total production of our country (MAPA 2017).

According to data from the Junta de Andalucía, zucchini was the fourth most important vegetable in Almería in 2016 respect to the area devoted to its cultivation, which were 7,630 hectares, corresponding to 13.2% of the total area devoted to horticultural cultivation (Figure 6), and the fifth in production, with almost 12% of the total horticultural production (Junta de Andalucía 2017). Melon was placed in seventh place representing 2.6% of the production and 4.2% of the total growing area of the province.

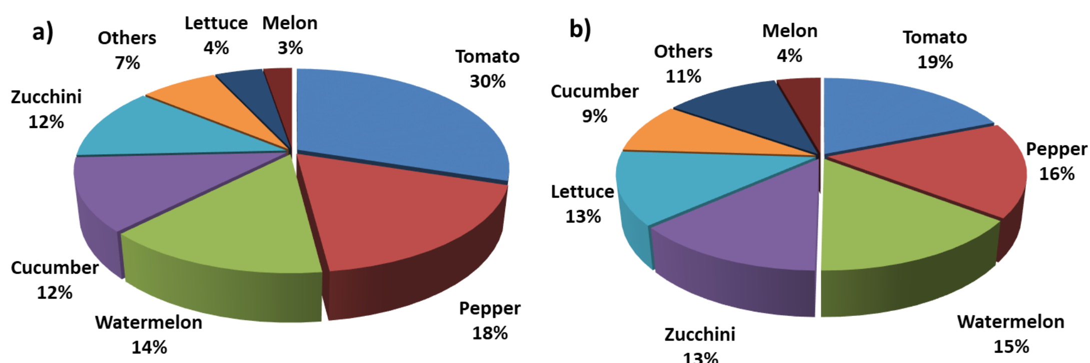

Figure 6. Distribution of a) production and b) area of the main horticultural crops produced in Almería in 2016. Own elaboration through Junta de Andalucía 2017.

1.4 Main soilborne diseases of zucchini and melon

Soilborne pathogens can significantly reduce yield and quality in vegetable crops. These pathogens are particularly challenging as they often are able to survive in the soil for several years. In addition, due to the similarity of the symptoms produced, many soilborne diseases are difficult to detect and diagnose (Koike et al. 2003). The most common diseases caused by soilborne pathogens are rots that affect belowground tissue, which may produce decay, damping-off, and root and crown rots; as well as vascular diseases initiated through root infections (Katan 2017, Koike et al. 2003). There are several organisms able to cause soilborne diseases such as several species of fungi, oomycetes, nematodes, viruses (carried by other organisms such as fungi or nematodes) and parasitic plants (Katan 2017). Among them, fungi cause most of soilborne diseases and therefore they are considered the main important pathogen group (Koike et al. 2003). The adoption of many new cultural practices in the last decades such as transplanting, plastic mulching, trickle irrigation, and increased plant density, among others, have contributed to increased yields and fruit quality but also to the increased incidence and severity of soilborne diseases (Bruton et al. 1998).

1.4.1 Soilborne diseases of zucchini

Zucchini production is greenhouses is heavily affected by fungal aerial diseases, such as oidium (*Podosphaera xanthii)* or grey mold (*Botrytis cinerea)* among others. However, it has been traditionally considered to show a low susceptibility to soilborne diseases compared to other cucurbits. In adult plants, the most important diseases affecting zucchini are the Foot and root rot caused by the oomycete *Phytophthora capsici*, able to cause disease in zucchini adult plants, and the Fusarium crown and foot rot caused by *F. solani* f. sp. *cucurbitae.* These diseases are explained below, however, as *F. solani* f. sp. *cucurbitae* is the main subject of this thesis, it will be explained in more detail in the following section.

- Phytophthora crown and root rot (*Phytophthora* spp.). *Phytophthora capsici* and other *Phytophthora* species can cause blight, crown and root rot, and fruit rot in several

vegetable hosts, including zucchini and melon. Phytophthora blight has become one of the most serious threats to production of cucurbits worldwide (Babadoost 2004) and is one of the most serious soilborne diseases on zucchini, which is probably the most susceptible host (Gubler and Davis 1996). The first symptom is usually a soft rot in the crown of the plant that quickly leads to wilting and collapse. The lesions are dark olive in the beginning and become dark brown in a few days. The rotting girdle the stem, resulting in rapid collapse and death of foliage above the lesion site (Babadoost 2004). Plants often have brown to black discolored roots and/or crowns. Fruits are very susceptible to this pathogen, and infected zucchini fruits initially exhibit dark, water-soaked lesions followed by a distinctive white layer of mycelia and spores on the surface of the fruit 2 to 3 days later (Hausbeck and Lamour 2004). Plants can be affected at any time during the growing season (Erwin and Ribeiro 1996).

P. capsici is able to produce thick-walled sexual spores called oospores. These are formed when mycelia of two opposite mating types grow together. Oospores are resistant to desiccation, cold temperatures, and other extreme environmental conditions, and can survive in the soil, in the absence of a host plant, for many years (Hausbeck and Lamour 2004, Gubler and Davis 1996). In Spain, the production of oospores has never been described. Soil moisture conditions are important for disease development (Hausbeck and Lamour 2004). *P. capsici* is able to produce zoospores, which are flagellated spores able to swim for several hours looking for a host to infect plant tissues. Due to the ability of this fungus to produce swimming zoospores, the water is one of the main sources for its dispersal (Babadoost 2004).
Sporangia form when the soil is at field capacity and they release zoospores when soil is saturated. The disease is usually associated with heavy rainfall, excessive-irrigation, or poorly drained soil. Frequent irrigation increases the incidence of the disease (Babadoost 2004).

Crop rotation is not easy due to the large host range of this pathogen, and removal of infected plant tissues may not be effective due to the ability of *P. capsici* to produce oospores that may survive in the soil in the absence of a host for extended periods of

time (Granke et al. 2012). Cultural practices, including irrigation management may limit disease but don't provide complete control. There are no commercial zucchini or melon cultivars resistant to *P. capsici*, hence, the most common control practice is the application of fungicides despite their limited efficacy (Babadoost and Islam 2003, Meyer and Hausbeck 2013).

1.4.2 Soilborne fungal diseases of melon

In contraposition to zucchini, there are several fungal soilborne diseases threatening melon production worldwide. The continuous and intensive cultivation of melon and the inadequate crop rotation has contributed to the increase of soilborne pathogens, particularly those causing vine declines (Bruton 1998). "Vine decline" is a general term applied to a group of soilborne diseases with similar symptoms, but with different causal agents (Bruton 1998, Miller et al. 1995). Initial symptoms include yellowing and death of the crown leaves, and wilting. Frequently, the disease symptoms can occur suddenly, normally when the plant is loaded with fruits approaching maturity stage, causing wilting or collapse of the whole vine (Bruton et al. 2000). Vine decline of melons may be provoked by vascular wilts caused primarily by *F. oxysporum* f. sp. *melonis* and *Verticillium dahliae*, crown rots caused by *P. capsici* or *F. solani* f. sp. *cucurbitae*, or root rots, being the most important *Monosporascus cannonballus* and *Plectosphaerella melonis;* which are considered the main causal agents of the sudden death (Bruton 1998, Bruton et al. 2000, García-Jiménez et al. 1990, Martyn and Miller 1996). In addition, the fungus *Olpidium bornovanus* (syn. *O. radicale*) is able to cause root rot and transmit *Melon necrotic spot virus* (MSNV), which has also been associated to sudden death in Spain (Cuadrado et al. 1993). In addition, melon plants can suffer damping-off in nurseries caused by several species of *Pythium* and *Phytophthora.*

- Plectosphaerella root and hypocotyl rot (*Plectosphaerella melonis* syn. *Acremonium cucurbitacearum*). *Plectosphaerella* has been described as a pathogen limiting melon production in the United States, Spain and Italy (Chilosi et al. 2008, García-Jiménez et al. 1994, Gubler 1996). The disease can affect the seedlings, with the first symptoms

being evident 7-10 days following germination. The infected area first shows chlorosis and yellowing, and 2-3 days later, it develops into a dry dark cortical rot. These lesions then enlarge in both directions, increasing in length without encircling the hypocotyl. The root system of these plants starts to turn brown and necrotic, remaining easily in the soil when the plant is directly pulled (Gubler 1996). In addition to this seedling disease, the pathogen can attack bigger plants. The first symptom consists in yellowing and necrosis of the root system, starting in the smaller roots. Affected roots generally are not water-soaked or macerated but exhibit a rough and corky appearance. In the aerial part, symptoms are evident when the root system is severely damaged. Aerial symptom consists in a wilting of the apex that rapidly evolves to the whole plant, which dies few days later (Bruton et al. 2000, García-Jiménez et al. 1994).

Plectosphaerella survives in soil as chlamydospores, and the infection occurs through the cortical tissue in the region where the seed coat attaches to the hypocotyl. Infection occurs over a wide range of temperatures, being the optimum comprised between 24-27°C.

Cultural practices can help the control of the pathogen. As the symptoms and collapse occurs due to a water deficit stress, measures that help to avoid this stress will help minimizing the negative effects of the infection. Soil disinfestation with chemicals and solarization as well as grafting on resistant rootstocks are efficient ways to control the disease (García-Jiménez et al. 1990, García-Jiménez et al. 1994)

- Fusarium wilt (*Fusarium oxysporum* f. sp. *melonis*). This disease can affect seedlings and adult plants. In young seedlings, it can cause hypocotyl rot and damping-off. In older plants, the older leaves turn yellow, and one or more runners wilt. In some cases, sudden wilting without any yellowing can be also observed. Mature infected plants are prone to collapse because of stress due to the fruit load. A longitudinal necrotic lesion may develop in the stem close to the crown and develop towards the main stem, and a gummy exudate may ooze from this lesion. Vascular necrosis is visible in longitudinal section. When the tissue dies, the pathogen may grow on the surface producing white

mycelium and sporodochia (Bruton 1998, González Torres et al. 1994, Martyn and Gordon 1996, Sharma et al. 2016).

Dissemination of the pathogen occurs primarily from the movement of infested soil and plant debris. The pathogen can also be seedborne, both internally and externally. The fungus can survive in the soil as chlamydospores that may be formed from macroconidia or on mycelium.
Penetration on the host occurs primarily in the elongation area of the root. Disease severity is maximum at soil temperatures ranging 18-25°C, and low moisture favors the pathogen and accentuates the wilting symptoms. Inoculum density play a key role in symptoms development (Martyn and Gordon 1996, Smith and Snyder 1971). High nitrogen, especially ammoniacal form, less than 25% soil moisture, and light, sandy, and slightly acidic soils (pH 5–5.5) favor disease development (Smith and Snyder 1971, Zitter 1998).

The most effective control tool is the use of resistant cultivars, however several races of the pathogen have been described, and the identification of the race present in the field is essential for an effective control. In addition, race 1,2 can overcome the known resistance genes. Crop rotation is normally not very effective due to the ability of chlamydospores to survive during a long time. Soil solarization or fumigation with disinfectants may reduce pathogen inoculum density, although recolonization of the soil occurs very quickly (Martyn and Gordon 1996).

- Verticillium wilt *(Verticillium dahliae)*. This fungus has a wide host-range, affecting all cucurbits and many other economically important plant species (Agrios 1995). The fungus usually infects the roots early in plant development, but symptoms may not be expressed until or following fruit set (Gubler 1996). Plants infected in the seedling stage often collapse once temperatures increase or the vines are drought stressed (Bruton 1998). Initial symptoms consist of a discoloration and wilting of the crown leaves. As the disease progress, leaf margins develop chlorotic lesions and later develop necrotic lesions, and eventually the crown collapses, and the disease progress along the runner. Wilt symptoms usually start in individual branches before affecting

the whole plant which may eventually die. Vascular necrosis at the crown of the plant is visible in longitudinal section. Symptoms may be confused with other vascular wilt pathogens (Gubler 1996).

V. dahliae may survive many years in the soil, due to the formation of long-surviving microscletoria. Infection usually occurs trough roots after plants are planted in infested soils. The fungus attacks the vascular system, causing plugging and limiting water transportation. Symptoms may be visible at any time, but usually are more common after fruit-set (Gubler 1996). Temperatures in the range of 18 to 24°C are conducive to disease development (Bruton 1998).

Crop rotation is difficult because of the wide host range of the fungus (Bruton 1998). Soil solarization have been proven to effectively reduce the incidence of Verticillium wilt in cotton, although disease incidence in the subsequent crop was not eradicated (Melero-Vara et al. 1995, Pullman 1981). Delaying the planting until the soil gets warm may help reduce disease symptoms. Although there are no resistant cultivars, grafting onto a resistant rootstock may also help provide control (Paplomatas et al. 2002).

- Olpidium root rot (*Olpidium bornovanus*). This obligate, soilborne, root inhabiting, zoosporic fungus has been described causing root rot in Southeastern Spain (Gómez Vázquez 1994) and the U.S.A (Stanghellini et al. 2010). This fungus can cause rot and necrosis in the roots of the affected plants, although its main importance is that it acts as a vector of the virus *Melon necrotic spot virus* (MNSV) causing sudden death of melon (Gómez Vázquez 1994, Stanghellini et al. 2010).

Olpidium produces zoospores that easily disseminate through irrigation systems and can also be transmitted through infested soils or substrates. In addition, it produces resting sporangia able to survive inside dead roots in the soil for at least 4 or 5 years (Guirado et al. 2009).

Soil disinfection with chemical fumigants or solarization is able to reduce the soil inoculum, but cannot completely eliminate it, and doesn’t reduce the incidence in subsequent melon crops (Gómez et al. 1993). Application of thiophanate-methyl on

lettuce or Agral on cucumber (Vanachter and Leuven 1995) or melon (Guirado 2015) allowed to maintain disease-free crops.

- Monosporascus root rot and vine decline (*Monosporascus* spp.). This pathogen is common in hot, semiarid melon-growing areas worldwide with soils that tend to be saline and alkaline (Martyn and Miller 1996). Aboveground symptoms are displayed as a result of root damage, and include stunting, yellowing and necrosis of the inner crown leaves, followed by a progressive death of the leaves. Symptoms evolve faster and are more evident in plants with concentrated fruit set, and when the fruits approach maturity. Plants may eventually collapse and die (García Jiménez et al. 1994b, Martyn and Miller 1996). The primary diagnostic character of the pathogen is the formation of black, spherical, emerging perithecia on the roots, easily visible with the unaided eye or hand lens (Martyn 2002)

No aerial stage of the disease is known, and no asexual spore stage of the fungus has been observed. Thus, ascospores are considered the primary inoculum and can survive in the soil or plant debris until the next planting season or, in the absence of a host, may survive for many years in a dormant state (Martyn 2002).

The best way to control this disease is the use of chemical fumigants either with preplant soil fumigations, or post plant application of fungicide to inhibit root colonization during the season. An immediate postharvest application of metam-sodium to prevent ascospore production is recommended (Martyn 2002). Studies using conventional soil solarization have been ineffective in controlling Monosporascus root rot and vine decline, presumably due to the ability of the fungus to grow at high temperatures (Martyn 2002). Nevertheless, solarization combined with the application of fungicides in combination with grafting in resistant rootstocks has shown potential for its control (Edelstein et al. 1999).

-Pythium and Phytophthora damping-off and root rot. This disease affects seedlings and poses a serious risk in seed nurseries worldwide. Although there are different fungi able to cause damping-off and root rot of seedlings, the most common are different species of oomycetes belonging to the genera *Phytophthora,* such as *P. dreschleri* or *P.*

capsici, and *Pythium,* such as *P. ultimum, P. irregulare, P. aphanidermatum* or *P. myriotylum,* among others. In addition, other fungi such as *Plectosphaerella melonis, Fusarium oxysporum* or *Rhizoctonia solani* can cause damping-off and root rot in seedlings (Rodríguez et al. 1994). These pathogens can cause rotting of seedlings prior to emergence (preemergence damping-off) and blighting of recently emerged seedlings (postemergence damping-off, Roberts and Kucharek 1994). After emergence, the main symptom observed in seedlings is a watery rot in the tap-root and the hypocotyl at the soil line, which normally produce plant collapse in green, without previous chlorosis. White mycelia growth may cover infected areas of blighted seedlings under moist conditions (Gubler and Davis 1996).

Conditions favoring the disease development vary depending on the pathogen involved. *P. ultimum* requires cool and wet soils for disease development, *P. irregulare* also prefers cooler temperatures, but can tolerate slightly higher temperatures than *P. ultimum,* while *P. aphanidermatum, P. myriotylum* and *P. capsici* require warmer soil temperatures for optimal disease development (Gubler and Davis 1996). Foliar symptoms of infection are intensified on hot, sunny days when transpiration is at its maximum, due to the inability of the rotten root system to provide the water requirements. When conditions are favorable for disease development, losses can be extremely high among susceptible seedlings. Infection of seedling roots occur soon after emergence (Roberts and Kucharek 1994). These oomycetes are waterborne organisms which produce swimming zoospores able to swim, so water is considered one of the primary sources of inoculum. They also produce long-surviving structures, the oospores which are able to survive in soils several years after a disease outbreak.

A good drainage in the substrate is a good means for disease control, applying short and frequent irrigations and avoiding substrates rich in clay. There are several biological organisms registered for control against *Pythium* and *Phytophthora*, and chemical control with effective fungicides is also recommended (Gubler and Davis 1996, Rodríguez et al. 1994).

2. THE GENUS *Fusarium*

Until the end of the twentieth century, fungi could be found in the taxonomic classifications within the Plant Kingdom, where they were considered "plants without chlorophyll". It was not until 1969, when the ecologist Robert H. Whittaker proposed a new taxonomic classification that divided the living beings into five kingdoms instead of two (animals and plants), including the Kingdom Fungi. Due to the biological importance and the role played by fungi in the ecosystem, the interest in their study has grown exponentially in recent decades.

Fungi are fundamental organisms in the maintenance of the biosphere, since they are the main decomposers of organic matter. In addition, another remarkable feature of fungi is their ability to produce secondary metabolites that in some cases are very useful for us, such as antibiotics and other substances used in the fields of medicine and biotechnology. On the other hand, these organisms stand out for their ability to produce mycotoxins and diseases in both plants and animals, including humans.

Among the fungal genera most studied today, *Fusarium* is one of the most important because of the role they play as pathogens. This genus comprises an extensive and heterogeneous group of filamentous fungi widely distributed in both natural and cultivated soils. Within this genus, there are species capable of causing a large number of diseases in plants, humans and animals, as well as producing mycotoxins and other secondary metabolites related to various types of cancers and other diseases.

2.1 History of the *Fusarium* taxonomy

The genus *Fusarium* was first described by Link in 1809, being the main characteristic of this group the presence of septate conidia in the shape of a banana or canoe, which are formed in the stroma. The first studies on the problems produced by *Fusarium* began with an investigation carried out by von Martius (1842) in the mid-nineteenth century, in which it was established as the causal agent of a potato tuber “dry rot” in Europe (Schroers et al. 2016, Zadoks 2008). A few years later, throughout the

nineteenth and early twentieth century, due to their important role as causal agents of diseases in a variety of host plants, and their ability to produce toxins, numerous strains started to be classified. Disease-causing strains in different hosts were described as distinct species, with numerous proposed characteristics and a series of different culture media, which made their identification and diagnosis very complicated tasks, until a moment when there were descriptions of more than 1000 species belonging to the genus *Fusarium* in the 1930s (Leslie and Summerell 2006, Nelson 1991).

In the year 1935, Wollenweber and Reinking published their monograph entitled “Die Fusarien”, in an attempt to put some order in the taxonomy of this genus. They reformulated the species concept within the genus and focused their identification on the basis of their mycological characteristics independently of the host plant of the pathogen. They reduced the number of species to 16 sections, 65 species, 55 varieties and 22 subspecific forms (Leslie and Summerell 2006, Nelson 1991).

This great work, despite being widely criticized for the difficulties involved in identification based on morphological criteria, laid the foundations for *Fusarium* modern taxonomy. The researchers who proposed taxonomic systems as of this moment did it mostly based on the techniques used by Wollenweber and Reinking. However, from this moment, the taxonomy of *Fusarium* took two opposite currents: on the one hand, the “splitters” or dividers, located mainly in Europe, which tended, like the German authors, to divide species according to small morphological differences; and on the other, the “lumpers” or groupers, of which Snyder and Hansen were predecessors, and who paid greater attention to the common characteristics of the isolates to describe their species and sections. Other researchers were considered moderate, being located between both currents (Nelson et al. 1994).

In the decade of the 40s Snyder and Hansen published their proposed classification of *Fusarium* in three manuscripts (Snyder and Hansen 1940, Snyder and Hansen 1941, Snyder and Hansen 1945). This system reduced the 16 sections of Wollenweber and Reinking to nine species, based mainly on the morphology of the macroconidia, and

based their classification system on the analysis of monosporic strains grown in identical environmental conditions. The species recognized by these American authors were *F. oxysporum, F. solani, F. moniliforme, F. roseum, F. lateritium, F. tricinctum, F. nivale, F. rigidiuscula,* and *F. episphaeria*. This system became popular between plant pathologists and diagnosticians because of its simplicity. However, the taxa circumscribed by these species' classification were polyphyletic, led to huge losses in information, and render much of the data generated during this time difficult to interpret or evaluate in any meaningful manner. Two of the species included by Snyder and Hansen, *F. oxysporum* and *F. solani*, are still widely used today, however, it is accepted that these taxa include more than one species, although their morphological characters are indistinguishable (Leslie and Summerell 2006, Nelson et al. 1994).

The next relevant contribution in the field of *Fusarium* taxonomy was made by Booth in the 60s and 70s when he published his monograph "The Genus *Fusarium*". This English researcher expanded the knowledge about the perfect states in *Fusarium*. He proposed to give greater taxonomic importance to the morphology of the conidiogenous cells, especially those producing the microconidia, than on the form of macroconidia in the asexual states, as a species-level diagnostic character. From this moment, the formation of polyphiallides has been accepted as a species separator in some sections of the genus *Fusarium*, and the morphology of conidiogenous cells is nowadays essential for the identification of species in the sections *Liseola* and *Sporotrichiella* (Booth 1971, Leslie and Summerell 2006). Booth was considered to belong to the current of moderates and made a real effort to bridge the gap between the taxonomic mycologists and plant pathologists and other groups that work with these organisms (Nelson et al. 1994). Also, Messiaen and Cassini in 1961 and Gerlach and Niremberg in 1982 continued working on the taxonomy of the genus, proposing new species and classifications (Nelson et al. 1994, Leslie and Summerell 2006).

The researchers Nelson, Tousson and Marasas published an identification manual in 1983. These authors tried to take advantage of the best characteristics of each system, to combine them together with their own discoveries in a system that was useful for

practical identification. This manual is still widely cited today (Nelson et al. 1983, Nelson et al. 1994, Leslie and Summerell 2006).

All these works about the taxonomy of *Fusarium* are characterized by having different criteria and methodologies in the identification of *Fusarium* that have been widely discussed during the last decades. Nevertheless, these systems have mainly a common characteristic, and it is that their taxonomic systems were based exclusively on morphology, and new species were described according to their morphological characteristics without taking into account other characteristics, such as interfertility among isolates or their genomic characteristics. Recently, Leslie and Summerell (2006) have published an extensive laboratory manual in which molecular, biological and morphological techniques for the description and identification of species are integrated for the first time. The authors have included a complete description of 70 species accompanied by photographs, information on the toxins they produce and the diseases they can cause together with protocols for their correct identification.

2.2 The species concepts in *Fusarium*

Over the years, the criteria used to delimit and identify species have been a controversial issue, and several concepts have been proposed for this purpose. This issue has not been less controversial in the case of mycology in general, or the genus *Fusarium* in particular (Booth 1984). Since Link made his first description of the genus in 1809, the main character to recognize *Fusarium* species was the presence of macroconidia in the shape of a banana or canoe. The main characteristics considered to classify species within the genus were the length and width of the macroconidia, as well as the curvature, the number of septa and the morphology of their foot cell; along with other characteristics such as the shape and length of the conidiogenous cells, the presence or absence of microconidia, the coloration of the colony, or the production of secondary metabolites (Leslie and Summerell 2006). For many years, the classifications were made mainly in terms of morphology, and to a lesser extent of physiological characters, and the controversy among taxonomists was based mainly on where to put

the limits of species, appearing two currents of opposing researchers, the aforementioned splitters and lumpers (Moretti 2009).

Subsequently, the concept of sexual fertility was introduced and used in the taxonomy. Gordon in 1944 was a pioneer in considering teleomorphs in his descriptions, work that was expanded and completed by Booth in 1971. This classification system had a special relevance in the case of the species complex of *Giberella fujikuroi* (Leslie 1991), where nine species have been described based on their sexual compatibility, and probably more will be described in the coming years (Leslie and Summerell 2006).

In the last years, with the development of the new molecular techniques, the agreement between different authors on the taxonomy of *Fusarium* returns to be a controversial subject. The use of the nucleotide sequences for studying *Fusarium* has led to both the description of new species and the division of some species into several that are morphologically indistinguishable, but whose phylogenetic studies reveal different origins.

The main cause of disagreements between researchers and taxonomists about the classification of *Fusarium* species is due to the “species concept” used and its practical implementation. Throughout history, the delimitation of species has been a controversial issue, just as it has been the definition of species itself. There is no consensus among biologists about the species concept, and in many cases, the concepts and criteria used to describe species are incompatible with each other (Mayden 1997). In his review in 1997, Mayden listed 24 different species concepts; however, the species concepts most used in mycology nowadays are the morphological concept, which studies the morphological and physiological divergence between isolates; the biological species concept, which refers to fertility between isolates; and the phylogenetic species concept, which emphasizes the nucleotide divergence between monophyletic lineages (Giraud et al. 2010, Taylor et al. 2000).

2.2.1 The morphological species concept

The main method used for species classification in mycology, and specifically in *Fusarium* has traditionally been the morphological species concept (Leslie and

Summerell 2006, Moretti 2009). A definition of species according to this criterion may be "a set of morphologically similar individuals, generally associated with each other by a defined geographical distribution and separated from other sets by morphological discontinuities" (Cain 1954).

The main advantage of this system is its wide application, in such a way that comparisons can be made between existing taxa, as well as between these and the new taxa that are being described (Taylor et al. 2000). However, its main disadvantage is that currently there are more species to classify than the number of morphological characters available to differentiate them, so it is possible that a species described according to the morphological species concept in fact includes several species according to other species concepts. In addition, morphological characters are subjective, easily misinterpretable and subject to variations depending on environmental conditions (Leslie et al. 2001, Taylor et al. 2000)

2.2.2 The biological species concept

Another important factor in the classification of filamentous fungi is their ability for sexual reproduction. In 1940, Mayr described the biological species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups". In the case of *Fusarium*, there is a great variability in the capacity for sexual reproduction, being known teleomorph species of known sexual reproduction, and anamorphic species of unknown sexual reproduction. The latter are also known as imperfect Deuteromycetes or fungi. The fact that not all species have reproductive capacity within the genus has made the implementation of this classification method difficult (Reynolds and Taylor 1993, Taylor et al. 2000). In addition, the sexual form of a species has been traditionally included in different groups and with different names from those without sexual reproduction (Coppin et al. 1997). Different teleomorphs have been associated with the *Fusarium* species: *Gibberella, Haemanectria* and *Albonectria* (Moretti 2009). *Gibberella* is the most commonly associated genus with most *Fusarium* species (Samuels et al. 2001) and includes phytopathogens such as *Gibberella zeae* (*F. graminearum*), *G. moniliformis* (*F.*

verticillioides) and other species included in the complex of species of *G. fujikuroi*. The genus *Haemanectria* includes *H. haematococca*, whose anamorph is *F. solani*; and the genus *Albonectria* is associated with a small number of *Fusarium* species, most notably *F. decemcellulare*, a pathogen of fruit species in tropical and subtropical regions (Kikot 2012). The implementation of The International Code of Nomenclature for algae, fungi and plants (ICN, McNeill et al. 2012), which arose out of the International Botanical Congress in Melbourne in 2011 and stipulated that only one scientific name should be used for a fungal species, resulted in the abolishment of this dual nomenclature (McNeill et al. 2012, Summerell 2019).

2.2.3 The phylogenetic species concept

In the recent years, especially since the decade of the 90s and 2000, as a consequence of the problems posed by the classification based exclusively on morphology or sexual compatibility, the molecular techniques of DNA analysis have allowed the study and analysis of species and their variability from a different point of view. The use of molecular markers has become an important tool for the identification of species, for estimating genetic variability among isolates, and for carrying out phylogenetic and molecular diagnostic studies within the genus.

According to the phylogenetic species concept developed by Carcraft, a species is the “smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestor-offspring” (Carcraft 1983). The application of the phylogenetic species concept is relatively new in the taxonomy of *Fusarium* and has been shown to be effective in clarifying taxonomic difficulties in some cases. However, if it is not applied correctly, it can lead to contradictions and create more confusion in the classification of species (Leslie and Summerell 2006). The taxonomic classifications may or may not represent the evolutionary history of the described taxa, as in the case of classifications based on morphology or sexual compatibility. The purpose of phylogenetic classifications is ordering the different taxa according to their evolutionary history. These differences explain why in many cases, the use of one or the other criterion leads to different results (Leslie and Summerell,2006).

The application of the phylogenetic species concept has led to the revision of the genus, and several changes have been made in its taxonomy with the introduction of molecular biology. For example, in 2002 the species *Acremonium falciforme* and *Cylindrocarpon lichenicola* were included in the clade of *F. solani*, being renamed as *F. falciforme* and *F. lichenicola* respectively, when analyzed from the evolutionary point of view (Summerbell and Schroers 2002). In addition, many of the *Fusarium* species that have been described in recent years have been discovered thanks to phylogenetic analysis techniques (Benyon et al. 2000, O'Donnell 2000, O'Donnell et al. 1998, Suga et al. 2002, Tan and Niessen 2003). The large amount of information generated in the last two decades has therefore shown that many of the classifications made exclusively on the basis of morphological characters did not contemplate the true diversity of species present in the genus (Gräfenhan et al. 2011). Phylogenetic analyses performed on 93 isolates belonging to *Fusarium* have revealed that the genus comprises at least 20 clades or "species complexes" that originated during the Cretaceous approximately 91.3 million years ago (O'Donnell et al. 2013).

Even when integrating morphological, biological and molecular techniques, the clear delimitation of species within the genus *Fusarium* is often complex, so the use of the "species complex" concept is widely accepted to refer to nearby and often morphologically indistinguishable species.

2.3. The special forms in *Fusarium*

Another controversial issue in the current *Fusarium* classification is the use of subspecific classifications such as varieties, cultivars, subspecies or special forms. During the history of the *Fusarium* taxonomy, several authors have made extensive use of these classifications to designate isolates that fit the definitions of species (Wollenweber and Reinking 1935, Snyder et al. 1957, Gerlach and Niremberg 1982), but whose differentiation based on morphological criteria of other isolates very closely related to them is almost impossible (Leslie and Summerell 2006). The most common subspecific nomenclature widely used by pathologists and mycologists are special forms or *formae speciales*. This denomination is used to distinguish pathogenic forms

belonging to a specific species, but morphologically indistinguishable from other non-pathogenic forms. Currently, more than 150 host-specific *formae speciales* have been described only in the *F. oxysporum* species complex (FOSC, Baayen et al. 2000), where most of the special forms described are found (for example *F. oxysporum* f. sp. *lycopersici* is specific to tomatoes, and *F. oxysporum* f. sp. *cubense* is specific to banana); followed by *F. solani;* species complexes to which a large part of the plant pathogens belong. This type of nomenclature is not exclusive to the genus *Fusarium*, as it is widely used in the case of aerial fungi such as *Puccinia* spp. or *Peronospora* spp. The simplicity of this system made its use widely accepted by phytopathologists and is the most common subspecific classification used nowadays.

Since the hosts of the different *formae speciales* usually correspond to closely related species, it has tended to assume that special forms pathogenic to the same host are also closely related. However, these have been classified throughout history only in terms of their pathogenicity, which has led that in many cases isolates belonging to the same special form have very different evolutionary origins and correspond to polyphyletic groups (Moretti 2009). In recent years, when *formae speciales* have been studied by molecular markers, it has often been found that strains belonging to the same *forma specialis* were distantly related to each other (Baayen et al. 2000, O'Donnell et al. 1998). This fact suggests that pathogenicity has evolved in a convergent manner, and that in many cases isolates currently included within the same species correspond to different lineages (O'Donnell et al. 1998). In fact, it has recently been shown that within the FOSC there are accessory chromosomes where specific virulence factors are encoded for specific hosts, and that these can be exchanged horizontally between different lineages and species (Baayen et al. 2000, Ma et al. 2010), so it is common to observe that isolates belonging to different special forms but from the same geographical region, are more closely related to each other than to other strains in the same *forma specialis* (Wang et al. 2004, Leslie and Summerell 2006). Several authors have recently suggested that the use of special forms tends to be confusing and discourage their use (Aoki et al. 2003, Aoki et al. 2014, Baayen et al. 2000, O'Donnell et al. 1998, O'Donnell et al. 2000, Sisic et al. 2018), which has

motivated that in recent years several special forms have been elevated to the rank of species (Aoki et al. 2003, Nalim et al. 2011, Short et al. 2013, Sisic et al. 2018), or that new descriptions of species belonging to the FSSC and FOSC are described with their own Latin binomial, and not as special forms of *F. solani* or *F. oxysporum* (Aoki et al. 2012, Costa et al. 2016, Dallé-Rosa et al. 2018, Papizadeh et al. 2018, Short et al. 2013).

2.4 Fusarium crown and foot rot of zucchini caused by *F. solani* f. sp. *cucurbitae*

Fusarium solani (Mart.) Sacc. f. sp. *cucurbitae* (Snyder and Hans), is an ascomycete fungus belonging to the genus *Fusarium* to which numerous economic losses worldwide are attributed (Fantino et al. 1989). This fungus is pathogenic to the cucurbit family and produces crown and foot rot that usually extends a few centimeters above the soil surface. The affected tissue adopts a brown and watery coloration, which under high humidity conditions develop mycelium on which numerous pinkish-orange sporodochia are formed (Hall et al. 1981, Martyn 1996). When the disease is very advanced, the tissues rot completely, leaving only vascular bundles visible. In the aerial part, wilt initially occurs, followed by chlorosis of the leaves and finally the death of the whole plant (Vannacci and Gambogi 1980, Martyn 1996). The disease was first described in the year 1930 in South Africa (Doidge and Kresfelder 1932) where the pathogenicity of a *Fusarium* isolate on squash was demonstrated, which was called *F. javanicum* Koord v. *theobromae* (App. & Strk.) Wr. A few years later the fungus was detected in the United States in the state of California in 1938 (Snyder 1938), and three years later it was described as *F. solani* f. sp. *cucurbitae* by Snyder and Hansen (1941). Later the fungus was detected in other states of the same country such as Georgia in 1970 (Sumner 1976), or Arkansas in 1978 (Boyette et al. 1984); and from that moment on it was detected in countries of very diverse places in the world such as Canada (Martyn 1996), Japan (Kinjo et al. 1987), Australia (Sherf and MacNab 1986), New Zealand (Pennycook 1989), Tunisia (Boughalleb et al. 2005) or Trinidad (Rampersad 2009). Recently, it has also been cited in other Asian countries

where until then the disease had not been observed such as Iran (Alymanesh et al. 2009), China (Li et al. 2010) or Iraq (Hussein and Juber 2014).

In Europe the disease was cited for the first time in the Netherlands on cucumber at the end of the 1950s, when it was suspected that the pathogen was introduced by infected seed of *Cucurbita ficifolia*, used as a rootstock for cucumber (Kerling and Bravenboer 1967). Later, in the decade of the 1980s, it was detected affecting zucchini crops in greenhouses in the same country (Paternotte 1987). In 1980, this disease was detected in Italy (Vannacci and Gambogi 1980) and a few years later in Greece (Bourbos and Skoudarakis 1993 in Bourbos et al. 1997). It was not until the 1995-1996 season when this pathogen was detected for the first time in Spain, specifically on pumpkin plants in the provinces of Valencia and Castellón (García-Jiménez et al. 1997). More than a decade later, in 2007, this disease was detected on zucchini in the province of Almería (Gómez et al. 2008), specifically in farms located in Las Norias de Daza, in the Campo de Dalías.

Fusarium solani f. sp. *cucurbitae* is a special form that has a host specificity on cucurbits and is able to attack plants in this family such as melon (Champaco et al. 1993), watermelon (Boughalleb et al. 2005), cucumber (Bourbos et al. 1997), zucchini (Paternotte 1985) or pumpkin (García-Jiménez et al. 1997) under field conditions.

Until a few years ago, it was considered that there were two races of *F. solani* f. sp. *cucurbitae* (Toussoun and Snyder 1961): the race 1, which was able to cause rot of the stem and the fruits independently of the state of development of the plants, and the race 2, which was only capable of producing rot in the fruits, and whose presence was limited to the United States and Tunisia (Martyn 1996, Boughalleb et al. 2005). Studies of sexual compatibility in the *F. solani* species complex (FSSC, Matuo and Snyder 1973) revealed that there were 7 mating populations named MPI-MPVII. While the race 1 (currently known as *F. solani* f. sp *cucurbitae*) belongs to the MPI, the former race 2 belonged to the MPV, indicating that they corresponded to two distinct biological species that could be differentiated quickly by "RAPD" technique (Random amplification of polymorphic DNA, Crowhurst et al. 1991). Subsequently, in a

phylogenetic study conducted with 39 isolates recovered from patients with ocular keratitis after an outbreak that occurred in the United States and Asia in 2006, it was discovered that several of the isolates belonged to the MPV and corresponded to a phylogenetic species conspecific with the race 2 of *F. solani* f. sp. *cucurbitae* (O'Donnell et al. 2000, Zhang et al. 2006). In 2007, Mehl and Epstein compared both races in a study where they evaluated the pathogenesis in pumpkin fruits, their sexual compatibility and their ability to grow and sporulate at 37°C, and concluded that their data supported the hypothesis that nosocomial isolates named as FSSC 1 and the isolates of *F. solani* f. sp. *cucurbitae* race 2 corresponded to a biological species, and they had the same virulence on fruits, and were able to tolerate the temperature of the human body (Mehl and Epstein 2007). Finally, in 2013, the race 2 of *F. solani* f. sp. *cucurbitae* together with some of the isolates that caused the outbreak of ocular keratitis were described as a single species that was renamed as *Fusarium petroliphillum* (Q.T. Chen & X.H. Fu) D. Geiser, O'Donnell, Short et Zhang, stat. nov. (Short et al. 2013).

Regarding its morphology, *F. solani* f. sp. *cucurbitae* belongs to the section *Martiella* whose main characteristics are the ability to produce abundant oval microconidia formed in false heads on elongated conidiophores, and macroconidia. Its mycelium may be sparse or dense, usually whitish in color, although in agar cultures it may develop bluish or brown colorations (Booth 1971). The microconidia develop abundantly within a few days of its cultivation *in vitro*. They are formed in lateral conidiophores that are elongated and sparsely branched and can measure up to 400 µm in length. These microconidiophores contrast with the short microconidiophores of *F. oxysporum*, being therefore the two species relatively easy to distinguish. Macroconidia are formed at 4-7 days on sporodochia that occur in the stroma of the fungus (Booth 1971). The production of macroconidia in sporodochia as well as its coloration is favored by ultraviolet light and by daily temperature oscillations, being the alternating temperature of 25°C during the day and 20°C during the night, with a photoperiod of 12 hours the optimal conditions for their formation (Windels 1992). *F. solani* f. sp. *cucurbitae* forms chlamydospores able to survive in the soil after the

incidence of the fungus (Schippers et al. 1982) thanks to their thick cell wall (Kraft 1984).

Regarding their sexuality, the existence of both homothallic and heterothallic isolates has been proven (Booth 1971). However, *F. solani* f. sp. *cucurbitae* requires the presence of compatible mating types for the formation of the perithecia which, in this case, may be reddish or white in color (Snyder et al. 1975).

Recently, several phylogenetic studies of the *Fusarium solani* species complex have been carried out, in which the isolates NRRL22098 and NRRL22153, described as *F. solani* f. sp. *cucurbitae* are included in clade 3 forming a monophyletic group with *F. solani* f. sp. *piperis* as the closest ancestor.

The available bibliography on the control of the disease caused by *F. solani* f. sp. *cucurbitae* is scarce. One of the effective methods described is the rotation of crops for 3 to 4 years with other non-host plants (Conroy 1953), that is, not belonging to the family of cucurbits. This is because the structures designed for persistence in the soil of the fungus, the chlamydospores, survive in the soil for short periods of time comparing to other *Fusaria* (Nash 1968), only remaining viable in the soil for two to three years (Garret 1956).

On the other hand, it has been shown that the pathogen can be transmitted by seed (Tousson and Snyder 1961), and that it can survive in it for 1 to 2 years (Watt 2006). However, it has been shown in pumpkin that *F. solani* f. sp. *cucurbitae* only colonizes the seeds when the fruit is visibly damaged (Mehl and Epstein 2007), so to produce seeds free of the pathogen it would be enough by selecting undamaged fruits. In addition, there are described methods of disinfection of seeds with heat, with various fungicides or with microorganisms (Takano et al 1985, Fantino et al. 1989).

Regarding chemical control methods, Sultana and Ghaffar were able to completely inhibit the growth of *Fusarium solani* in in vitro cultures on PDA medium supplemented with the fungicides Aliette, Benlate or carbendazim. In addition, they managed to eradicate the infection in plants born from bitter gourd seeds (*Momordica charantia*)

contaminated with the fungus and treated with Benlate and carbendazim (Sultana and Ghaffar, 2010). However, there are no studies available on the effect of fungicides on the disease in field conditions.

Few years ago, Roberti et al. (2012) showed an inhibition of this disease after treating plants with biological products based on fungi such as *Trichoderma* sp. and bacteria such as *Streptomyces* and *Pseudomonas* spp. In addition, recently Marín-Rodulfo (2019) demonstrated a reduction in the disease incidence and mortality rates of zucchini plants treated with *Trichoderma aggressivum* f. sp. *europeaeum* and *T. saturnisporum* when they were applied to the growing substrate (Marín-Rodulfo 2019).

On the other hand, in Greece, soil solarization has been cited in combination with the application of organic matter and calcium cyanamide as effective, reducing the amount of inoculum of the pathogen in the soil by 99% (Bourbos et al. 1997).

OBJECTIVES

Considering all the above, this thesis has been proposed based on two main objectives, for which several specific objectives are raised.

These are:

- **Objective 1: Characterization of *Fusarium solani* f. sp. *cucurbitae***.
    1.1 Assess the incidence and determine the geographic distribution of *Fusarium solani* f. sp. *cucurbitae* in the greenhouses of Almería Province, both in zucchini and in other cucurbits; and obtain a collection of isolates representative of the existing population in the area.
    1.2 Characterize the pathogenicity of several isolates to zucchini and melon, and to several other cucurbit and non-cucurbit crops.
    1.3 Describe the main morphological and cultural characters of the fungus.
    1.4 Determine the genetic variability between the isolates, and their phylogenetic relationships within the FSSC.

- **Objective 2: Control of the Fusarium crown and foot rot of zucchini.**
    2.1 Determine the possible transmission of *F. solani* f. sp. *cucurbitae* in commercial zucchini seeds.
    2.2 Study the survival of the fungus in perlite bags.
    2.3 Evaluate the susceptibility to the pathogen of some of the most commonly grown zucchini cultivars in southeast Andalusia.
    2.4 Determine the efficacy of several commercial fungicides and biocontrol organisms for the control of the Fusarium crown and foot rot of zucchini.
    2.5 Asses the efficacy of soil solarization and biosolarization in a greenhouse for eliminating the inoculum from the soil.

CHAPTER II

Pathogenic, Morphological, and Phylogenetic Characterization of *Fusarium solani* f. sp. *cucurbitae* Isolates from Cucurbits in Almería Province, Spain

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ABSTRACT

Fusarium solani f. sp. *cucurbitae* (syn. *Neocosmosporum cucurbitae)* is one of the most devastating soilborne pathogens affecting the production of cucurbits worldwide. Since its first detection in Almería Province in Spain in the spring of 2007, it has become one of the main soilborne pathogens affecting zucchini production. It has also been reported on melon, watermelon, and squash rootstocks in Spain, representing a high risk of dissemination in the area. The objectives of this study were to investigate the incidence and distribution of this disease in the southeastern Spain and to characterize isolates collected over 5 years. These strains were characterized on the basis of greenhouse aggressiveness assays on a range of cucurbit hosts, morphological characteristics, and *EF1-α* and *RPB2* phylogenies. All pathogenic isolates were highly aggressive on zucchini plants, causing a high mortality rate a few weeks after inoculation. The rest of the cucurbit hosts showed differential susceptibility to the pathogen, with cucumber being the least susceptible. Plants belonging to other families remained asymptomatic. Morphological characterization revealed the formation of verticilate monophialides and chlamydospores forming long chains, characteristics not described to date for this *forma specialis.* Phylogenetic studies of both the individual loci and combined datasets revealed that all pathogenic isolates clustered together with strong monophyletic support, nested within clade 3 in the *Fusarium solani* species complex (FSSC).

Keywords: disease development and spread, epidemiology, fungi, pathogen diversity

INTRODUCTION

Zucchini (*Cucurbita pepo* L.) is intensively cropped in the province of Almería, the main vegetable production area in Spain. In 2016, zucchini was produced on 11,081 ha in Spain, of which 7,490 ha were for greenhouse production in Almería, producing 434,195 metric tons of this crop. This production accounts for 74.7% of the total

production in Spain, making it one of the most economically important crops for this region (MAPA 2017).

In 2007 a new fungal disease of zucchini was detected in the province of Almería. The first symptom observed was a dark green coloration of the younger leaves, followed by wilt, crown rot and eventual plant death. The causal agent was *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* W.C. Snyder and H.N. Hansen (Gómez et al. 2008), which is host specific to cucurbits (Boughalleb et al. 2007; Messiaen et al. 1995; Paternotte 1987). This pathogen has been reported worldwide, causing disease in field production in pumpkin (Elmer et al. 2007), muskmelon (Champaco et al. 1993), cucumber (Bourbos 1997), watermelon, rootstock hybrids of *Cucurbita maxima* x *C. moschata* (Armengol et al. 2000), among other hosts.

In Almería zucchini crops have been considered for years to be highly resistant to soilborne fungal diseases. Until now, aside from *F. solani* f. sp. *cucurbitae*, only *Phytophthora capsici* Leonian has been detected as the causal agent of a soilborne disease in adult plants in this area (Gómez et al. 2013). Furthermore, since its detection, Fusarium crown, foot, and fruit rot of zucchini has been spreading to other cucurbits such as melon (Gómez et al. 2014) and watermelon (Porcel 2013), becoming an important threat to the cucurbit production in this region.

Several methods can be used for the control of Fusarium crown rot, such as fungicide applications with prochloraz, carbendazim, and thiophanate-methyl; crop rotation with non-cucurbits for at least 2 years; or soil solarization or biosolarization (Pérez-Hernández et al. 2017).

In recent decades, molecular tools have revolutionized the understanding of fungal taxonomy, including that of *Fusarium*. Recent phylogenetic studies have demonstrated that several species actually correspond to species complexes composed of different morphologically indistinguishable cryptic species. *F. solani,* known as the *F. solani* species complex (FSSC) comprises three major clades encompassing over 60 phylogenetic species (Nalim et al. 2011; O'Donnell 2000; O'Donnell et al. 2008; Short et

al. 2013; Zhang et al. 2006;). Members of clades 1 and 2 are known exclusively from diseased or dead plants, whereas members of clade 3 are frequently isolated from soil and as saprotrophs in other environments, causing diseases in immunocompromised humans (Zhang et al. 2006). In addition, clades 1 and 2 are composed of isolates limited to specific geographical origins (New Zealand and South America, respectively), while clade 3 includes most of the commonly encountered FSSC isolates associated with plant diseases and soil, with a cosmopolitan distribution (O'Donnell 2000). Lombard et al. (2015) proposed that the FSSC be redefined as *Neocosmospora* species based on morphology of the group, especially the sexual structures, and DNA phylogenetic placement. Geiser et al. (2013) argued that such a change was unnecessary, maintaining that the taxonomy better serves the community needs, especially with respect to plant biosecurity and human health, by retaining the species complex within the genus *Fusarium*. Additionally Summerell (2019) argued for the retention of *Fusarium* for the species complex until such a time that there is consensus of view. Consequently, we have retained the use of *Fusarium* for these fungi in this study.

Sandoval-Denis et al. (2019) has monographed the FSSC (as the genus *Neocosmospora*) and described *F. solani* f. sp. *cucurbitae* as *Neocosmospora cucurbitae* based on morphology and DNA phylogeny. Based on the available data we refer to the fungus as *F. solani* f. sp. *cucurbitae* throughout this paper.

Fusarium solani f. sp. *cucurbitae* (syn. Neocosmospora cucurbitae) was first described by Snyder and Hansen in 1941 as the causal agent of crown and foot rot of cucurbits. In 1961 Tousson and Snyder divided this *forma specialis* into two races on the basis of their ability to produce crown and foot rot in cucurbit plants, designated as *F. solani* f. sp. *cucurbitae* race 1, or only able to produce fruit rot, designated as *F. solani* f. sp. *cucurbitae* race 2. In 1973 Matuo and Snyder conducted fertility crosses between the different *formae speciales* described within *F. solani,* demonstrating that both races correspond to different biological species, assigned respectively to different mating populations, MP-I and MP-V. Some decades later, a comprehensive phylogenetic study

(O'Donnell et al. 2000) revealed that both races of *F. solani* f. sp. *cucurbitae* were polyphyletic, therefore representing two distinct phylogenetic species. Several years later, given the large amount of phylogenetic species lacking proper descriptions and Latin binomials in addition to the need to facilitate better communication, a haplotype nomenclatural system was introduced, in which each phylogenetic species received a numerical identifier, with unique sequence types within species designated by lower case letters (Chang et al. 2006; O'Donnell et al. 2008). In this study, race 1 was assigned to the phylogenetic species FSSC 10, with two haplotypes, FSSC 10a and FSSC 10b, while race 2 was assigned to FSSC 1. Zang et al. (2006) and Mehl and Epstein (2007) later demonstrated that race 2 was conspecific with the phylogenetic species FSSC 1, which was the most common phylogenetic species isolated in hospital environments and one of the predominant species in the 2005–2006 outbreaks of mycotic keratitis in the U.S. and Southeast Asia associated with contact lens wear (Chang et al. 2006; Khor et al. 2006). Further studies have confirmed this hypothesis, and in 2013, the former *forma specialis* was elevated to the species rank, *Fusarium petroliphilum* stat. nov. (Short et al. 2013).

In this context, the aim of this study was to investigate the incidence and distribution of Fusarium crown and foot rot of cucurbits in Almería Province and to characterize a collection of *F. solani* f. sp. *cucurbitae* isolates obtained over the years from different hosts. These isolates were analyzed on the basis of their pathogenicity to zucchini and other cucurbit hosts; a detailed description of their morphological characteristics; and their *EF1-α* and *RPB2* phylogenetic relationships within the FSSC.

MATERIALS AND METHODS

Disease survey

To assess the incidence and geographical distribution of Fusarium crown and foot rot of zucchini in the province of Almería, isolates were collected in zucchini commercial greenhouses situated in different growing areas. Crops with plants showing foot rot,

wilt, or death were visited in collaboration with the Production and Plant Health Laboratory, Almería, and agricultural technicians. In addition, five cucumber, one watermelon and one melon greenhouses with plants showing similar disease symptoms were included in this survey.

In each of the greenhouses visited, the percentage of plants showing any of the abovementioned symptoms was estimated, and plant samples were collected to diagnose the causal agent of the disease and to obtain a collection of isolates.

Collection of isolates

Basal stem samples were surface disinfected, placed on Komada medium (Komada 1975), potato dextrose agar medium (PDA; Rapilli 1968), and P5ARP (Jeffers and Martin 1986), and incubated at 25°C for 2 to 3 days. Colonies were obtained and subcultured on PDA or lima bean agar (LBA, Calvert et al. 1960). In addition, to detect possible chlamydospores or perithecia of *Chalara elegans* or *Monosporascus cannonbalus,* roots were observed under a stereoscopic microscope. The isolates classified as *F. solani,* according to their morphological characteristics, were maintained as single-spore cultures in the IFAPA Centro La Mojonera. Among a collection of a total of 83 *Fusarium* spp. isolates, 22 of them were selected for this study, and the isolate PCl-711 was deposited with the accession number CECT20831 in the Colección Española de Cultivos Tipo (CECT), Parque Científico de la Universidad de Valencia, Paterna, Spain.

Production of pathogen inoculum

For the pathogenicity tests, cultures of each isolate were grown on 90-mm PDA plates and incubated under indirect natural light at 25°C between 15 and 20 days until the plates were completely colonized. Conidial suspensions were then prepared by scraping the surface of the plates with a sterile loop and recovered by washing the plates with autoclaved water. The inoculum was then filtered through a 0.5-mm mesh to remove large portions of mycelia and adjusted with a haemocytometer to an

approximate concentration of 1.5×10^3 conidia ml^{-1}. Fifty ml of inoculum were poured around the stem base of each plant.

Plant growth and maintenance

The first pathogenicity trial of the fungus on various horticultural crops was conducted in an 80-m^2, multi-span polymethyl methacrylate-covered greenhouse equipped with ridge and side windows, in a semi-controlled environment with a cooling system as well as automated climate control and fertigation. The second pathogenicity trial on various horticultural crops was conducted in a 970-m^2, multi span polyethylene-covered greenhouse in order to maintain the plants for a longer period of time, along with the pathogenicity trials on zucchini plants. The greenhouse was equipped with ridge and side windows, and automated climate control and fertigation. All greenhouses were located in the IFAPA Centro La Mojonera, Almería Province, Spain (36° 47' 17", -2° 42' 14"). Fertigation was managed with a programmable system based on the leaching fraction, which was maintained close to 20%. Temperature for all crops was measured inside the bags of perlite and in the greenhouse environment above the plant canopies with P-108 temperature probes for 10-minute intervals and recorded with a Campbell CR10X datalogger (Campbell Scientific, Inc., Logan, UT).

Pathogenicity trials on zucchini plants and fruit

The pathogenicity of the isolates used in this study was tested on both zucchini plants and fruit. To evaluate the pathogenicity on zucchini plants, two trials were conducted: one commenced during the third week of February 2012 (first trial) and the other during the third week of May 2012 (second trial). The experiments were arranged as a randomized complete block design with four replicates. Each experimental unit within a block consisted of four bags of perlite with three plants per bag, totaling 48 plants per isolate in each trial. An uninoculated control was included for each block. Seeds of the cultivar Cónsul (Seminis Vegetable Seeds, St. Louis, MO) were sown in the bags, and the plants were inoculated at the 2-to-3 true leaf stage, as previously described. Plants were monitored twice a week for the presence of disease symptoms. Disease

incidence (DI) and mortality (M) were calculated as the percentages of symptomatic plants per isolate at the end of the experiment. In addition, disease severity was assessed by using a 0 to 2 rating scale, where: 0=plant without symptoms, 1=aerial disease symptoms, and 2=plant death. These data were plotted against time for each experiment, and the development of the disease was evaluated by calculating the area under the disease progress curve (AUDPC) using the trapezoidal method (Madden et al. 2007). The relative area under the disease progress curve (RAUDPC) was calculated as the proportion of the maximum possible AUDPC. Plants were maintained in the greenhouse over a period of 65 and 53 days, respectively, for the first and second trials. At the end of the experiments, 10% of the symptomatic plants were randomly selected for pathogen re-isolation to fulfill Koch's postulates.

To evaluate the pathogenicity of *F. solani* isolates on zucchini fruit, two consecutive trials were conducted. The experiments were arranged in a randomized complete block design with four replicates. Each experimental unit within a block consisted of one zucchini fruit with three wounds. Multiple fruits of approximately 200 g were surface-disinfected by spraying with 70% ethanol on the surface and allowed to dry. Wounds of a depth of approximately 5 mm were made in the fruit surface with a cork borer; 7-mm PDA plugs, excised from the actively growing margins of 5-day-old SNA cultures of each isolate, were placed mycelium-side down into the wounds. Three wounds per fruit and four fruits were inoculated with each isolate. Two fruits per block were inoculated with uncolonized PDA plugs to serve as negative controls. To maintain a humid environment, the fruits were placed on shelves and covered with unsealed plastic bags to allow air flow. Fruits were incubated in the laboratory at approximately 25°C. After 7 days of incubation, the diameter of the lesion produced on the fruit was obtained from an average of two perpendicular measures after subtracting the diameter of the wound.

Pathogenicity on other horticultural crops

After verifying the pathogenicity of 21 *Fusarium* isolates on both zucchini plants and fruit, two representative isolates were selected to study the pathogenicity of *F. solani* f. sp. *cucurbitae* to other horticultural crops. Two trials were conducted, one commenced during the first week of April 2013 (first trial) and the other during the second week of April 2014 (second trial). The experiments were arranged in a factorial design in three blocks, with each block comprising six plants. Factors studied were inoculation with the isolate PCl-511 obtained from zucchini, inoculation with the isolate PCl-1621 obtained from watermelon, and non-inoculated controls; the following horticultural species were evaluated: melon (*Cucumis melo*) cv. Amarillo Canario Jardiver, watermelon (*Citrullus lanatus)* cv. Mariola, tomato (*Solanum lycopersici*) cv. Pasadena, eggplant (*Solanum melongena*) cv. Alegría, pea *(Pisum sativum)* cv. Altesse and lettuce (*Lactuca sativa)* cv. Romana Jardiver (Ramiro Arnedo S.A., Calahorra, Spain); cucumber cvs. Borja (Enza Zaden Beheer, B.V., Enkhuizen, The Netherlands), Marketmore and Pepinillo Jardiver (Ramiro Arnedo S.A.), zucchini (*Cucurbita pepo*) cv. Cónsul (Seminis Vegetable Seeds, Woodland, CA), squash rootstocks (*Cucurbita máxima x C. moschata)* cvs. RS-841 (Akira Seeds S.L., Sant Boi de Llobregat, Spain) Hércules and Titán (Ramiro Arnedo S.A.)*,* pepper (*Capsicum annuum)* cv. Airen (Syngenta Seeds Inc., Boise, ID), snow pea (*Pisum sativum* sp. *arvense)* cv. Capuchino (Semillas Batlle S.A., Molins de Rei, Spain) and green bean *(Phaseolus vulgaris)* cv. Emerite (Vilmorin, La Ménitré, France). In the first trial, plants were sown in 1-liter pots with vermiculite and inoculated when the cotyledons were completely opened as previously described. In the second trial, seeds were sown in 32-liter perlite bags to maintain the plants for a longer period of time. Plants were monitored twice a week for the presence of disease symptoms, and the parameters DI, M, and RAUDPC were calculated per treatment, as previously described. At the end of the experiments, 10% of the symptomatic plants per species were randomly selected for pathogen re-isolation.

Determination of mycelial growth rates and morphological characteristics

Cardinal growth rates were determined by measuring the *in vitro* growth of four representative isolates (PCl-341, PCl-511, PCl-731 and PCl-1621) at different temperatures. Mycelial plugs with a diameter of 7 mm were excised from the actively growing margins of 4-day-old PDA cultures and placed mycelium-side down in the center of 90-mm PDA plates, sealed with laboratory film, and incubated for 6 days in complete darkness at each of the following temperatures: 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35°C. Mycelial growth data were obtained by calculating the average of two perpendicular measures of the colony diameter after 6 days of growth. Rates were calculated as mean values per day. Four repetitions of each temperature-isolate were included; this experiment was performed twice.

Morphological characteristics of the isolates were examined based on the criteria of Leslie and Summerell (2006). Single-spore isolates were grown using SNA (Niremberg 1976) for 10 days at 25°C, and subsequently, 7-mm plugs from actively growing edges of each culture were subcultured in plates with PDA, carnation leaf-piece agar (CLA; Leslie and Summerell 2006), and SNA in 90-mm plastic Petri dishes. Six repetitions per isolate were included, and the plates were incubated for 10 days at 25°C under indirect natural light or continuous Blacklight Blue (F40 BLB) fluorescent light. Colony color and morphology, together with the morphology and type of conidiogenous cells, were recorded from cultures grown on PDA plates under natural indirect light. The morphology, size, and shape of 30 randomly selected microconidia formed on SNA plates, and the presence or absence and shape of 30 chlamydospores, were determined under an Olympus BX41 microscope and a ColorView IIIu camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The production of sporodochia on CLA was induced on subcultures incubated at 25°C under continuous BLB light and checked within 15 days of incubation. The length and width of 30 randomly selected macroconidia of each septation class on CLA and the number of septa were measured and recorded. The reported measurements consisted of the minimum and maximum values in brackets and of the mean values plus and minus the standard deviation.

Genomic DNA extraction, PCR amplification, and sequencing

Fusarium cultures were grown on PDA for 5 days. Genomic DNA was extracted using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA), according to the manufacturer's instructions. The PCR amplifications of the isolates were performed for part of the translation elongation factor 1-α (*EF* 1-α), using the primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC -3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'; Carbone and Kohn 1999), and the RNA polymerase II *second largest* subunit (*RPB2*), using the primers 5f2 (5'-GGGGWGAYCAGAAGAAGGC-3'; Reeb et al. 2004) and 7cr (5'-CCCATRGCTTGTYYRCCCAT-3', Liu et al. 1999). Conditions for amplification for the *EF-1α* gene region were an initial denaturation step of 3 min at 95 °C, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (53 °C for 30 s), and elongation (72 °C for 45 s). The final elongation step was conducted at 72 °C for 7 min. For the *RPB2* loci, amplification consisted of 5 cycles of 45 s at 94 °C, 45 s at 60 °C, and 2 min at 72 °C, then 5 cycles of 45 s at 94 °C, 45 s at 58 °C, and 2 min at 72 °C, and 30 cycles of 45 s at 94 °C, 45 s at 54 °C, and 2 min at 72 °C (Woudenberg et al. 2013). The PCR amplicons were sequenced in both directions at the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales, Australia, or by the Sequencing Service, Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain.

Phylogenetic analyses

Sequences obtained were edited manually in Geneious software v.5.3.6 (Drummond 2011) and their classification as *Fusarium* was confirmed through the Basic Local Alignment Search Tool (BLAST) in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Fusarium-ID v. 1.0 database (Geiser et al. 2004). The sequences were aligned with reference sequences from the FSSC obtained from GenBank (Table 1) with ClustalW v.1.83 plug-in within Geneious. The alignments were edited, and polymorphisms were confirmed by re-examining the chromatograms. Sequences generated in this study were deposited in GenBank (Table 2). Phylogenetic analyses were performed using PAUP 4.0b10 (Swofford 2002) on the single and combined *EF-1α* and *RPB2* datasets.

Unweighted parsimony analysis was conducted using the heuristic search option with 1,000 random addition sequences and tree bisection reconnection branch swapping. Gaps were treated as missing data. The Consistency Index (CI) and the Retention Index (RI) were calculated to indicate the amount of homoplasy present. Clade stability was assessed via Maximum Parsimony Bootstrap Proportions (MPBS) in PAUP 4.0b10, using 1,000 heuristic search replications with random sequence addition. The *EF-1α* and *RPB2* datasets were rooted with *F. thapsinum* NRRL22045. Phylogenetic trees were visualized and edited using FigTree v.1.4 (Rambaut 2013).

Table 1. Reference strains obtained from the NCBI GenBank database used in the phylogenetic analysis of *F. solani* f. sp. *cucurbitae* isolates.

Species	**Collection**[z]	**GenBank accession number**		**Host**	**Origin**
		EF-1α	***RPB2***		
F. thapsinum **(Outgroup)**	NRRL 22045	AF160270	JX171600	*Sorghum bicolor*	Southafrica
F. virguliforme	NRRL 22825	AF178357	GU170615	*Glycine max*	USA
F. tucumaniae	NRRL 31096	GU170600	EU329557	*Glycine max*	Argentina
F. striatum	NRRL 22101	AF178333	EU329490	*Gossypium* sp.	Panama
F. solani sensu stricto	NRRL 25388	DQ246857	EU329535	Human eye	India
F. solani sensu stricto	NRRL 32737	DQ247056	EU329606	Human eye	USA
F. solani **f. sp.** ***xanthoxyli***	NRRL 22163	AF178328	EU329496	*Xanthoxylum piperitum*	Japan
F. solani **f. sp.** ***robiniae***	NRRL 22586	AF178353	EU329516	*Robinea pseudoacacia*	USA
F. solani **f. sp.** ***robiniae***	NRRL 22161	AF178330	EU329494	*Robinea pseudoacacia*	Japan
F. solani **f. sp.** ***robiniae***	NRRL 22162	DQ247599	EU329495	*Robinea pseudoacacia*	Japan
F. solani **f. sp.** ***piperis***	NRRL 22570	AF178360	EU329513	*Piper nigrum*	Brasil
F. solani **f. sp.** ***mori***	NRRL 22230	AF178358	EU329499	*Morus alba*	Japan
F. solani **f. sp.** ***mori***	NRRL 22157	AF178359	EU329493	*Morus alba*	Japan
F. solani **f. sp.** ***cucurbitae***	NRRL 22098	AF178327	EU329489	*Cucurbita* ssp.	USA
F. solani **f. sp.** ***cucurbitae***	NRRL 22153	AF178346	EU329492	*Cucurbita* spp.	USA
F. solani **f. sp.** ***batatas***	NRRL 22400	AF178343	EU329509	*Ipomoea batatas*	USA
F. solani 9a	NRRL 32755	DQ247072	EU329613	Turtle	USA
F. solani 6i	NRRL 28553	DQ246894	EU329548	Human	USA
F. solani 6g	NRRL 28016	DQ246873	EF470140	Human	USA
F. solani 6e	NRRL 32849	DQ247155	EU329628	Human eye	USA
F. solani 6d	NRRL 32785	DQ247094	EU329618	Human	USA
F. solani 6a	NRRL 43489	DQ790484	DQ790572	Human eye	USA
F. solani 34a	NRRL 46703	HM347126	EU329661	Nematode	Spain
F. solani 25a	NRRL 22389	AF178340	EU329506	*Liriodendron tulipfera*	USA

F. solani 22a	NRRL 32751	DQ247069	EU329611	Human eye	USA
F. solani 20a	NRRL 22608	DQ247622	EU329517	Human	USA
F. solani 14a	NRRL 22611	DQ246840	EU329518	Human eye	USA
F. solani 12d	NRRL 32309	DQ246937	EU329571	Human	USA
F. solani 12a	*NRRL 22642*	*DQ246844*	*EU329522*	*Fenneropenaeus* spp.	Japan
F. plagianthi	NRRL 22632	AF178354	JX171614	*Hoheria glabrata*	New Zealand
F. pisi	NRRL 22820	AF178355	EU329532	*Pisum sativum*	USA
F. pisi	NRRL 22278	AF178337	EU329501	*Pisum sativum*	USA
F. pisi	NRRL 45880	FJ240352	JX171655	*Pisum sativum*	USA
F. phaseoli	CBS 265.50	HE647964	KM232375	*Phaseolus* sp.	USA
F. petroliphilum	NRRL 28546	DQ246887	HM347152	Human eye	USA
F. petroliphilum	NRRL 43812	EF453054	EF470093	Contact lens solution	USA
F. petroliphilum	NRRL 32856	DQ247161	EU329629	Hospital	USA
F. neocosmosporiellum	NRRL 22166	AF178350	EU329497	*Heteroderma glycines*	USA
F. neocosmosporiellum	NRRL 43467	EF469979	EF469979	Human eye	USA
F. neocosmosporiellum	NRRL 22436	AF178348	EU329511	Soil	Southafrica
F. lichenicola	NRRL 34123	DQ247191	EU329635	Human eye	India
F. keratoplasticum	NRRL 43433	DQ790473	DQ790561	Human eye	USA
F. keratoplasticum	NRRL 43445	DQ790479	DQ790567	Human eye	USA
F. illudens	NRRL 22090	AF178326	AF178392	*Beilshmiedia tawa*	New Zealand
F. falciforme	NRRL 43441	DQ790478	DQ790566	Human eye	USA
F. euwallaceae	NRRL 54722	JQ038007	JQ038028	*Persea americana*	Israel
F. ambrosium	NRRL 20438	AF178332	JX171584	*Camellia sinensis*	India

[z] NRRL=Agricultural Research Service Culture Collection, Peoria, IL, United States; CBS=Centraalbureau voor Schimmelcultures- Fungal Biodiversity center, Utrecht, The Netherlands

Statistical analyses

Kruskal-Wallis nonparametric analysis of variance (ANOVA) tests were conducted for each of the experiments, of which the DI expressed as the percentage of symptomatic plants per treatment, M and RAUDPC were analyzed with Statistix 9.0 (Thallahassee, FL) owing to data non-normality and variance heterogeneity. The different repetitions of the experiments were included in the model and assayed for differences. In cases where ANOVA revealed no significant differences between experiments, data were pooled prior to analyses. In all cases, if the ANOVA results were significant for the main

effects, treatment means were compared using all-pairwise comparison tests (*P ≤ 0.05*).

RESULTS

Disease survey

A total of 26 zucchini, five cucumber, one melon, and one watermelon greenhouses with diseased plants were surveyed, where symptoms similar to those caused by *F. solani* f. sp. *cucurbitae* were observed, mainly leaf wilt, brown rot lesions at the base of the stem near the soil level, and plant death (Suppl. Fig. 1). In addition, 25 zucchini samples from the Production and Plant Health Laboratory, with similar disease symptoms, were included in this study. *Fusarium solani* was present in 92.3% of the zucchini greenhouses surveyed and in 100% of the zucchini samples received by the Plant Health Laboratory of Almería. Several of the affected greenhouses were located in the municipality of Las Norias de Daza, where the disease was first observed in zucchini (Gómez et al. 2008). In addition, the disease incidence was observed throughout the province, in at least five different municipalities. The disease incidence among the different greenhouses visited was variable. In some greenhouses, the disease affected a small number of plants distributed in one or few rows. In most of the cases, the percentage of affected plants ranged between 5 and 30%. In two greenhouses, between 50 and 60% of the plants showed crown and foot rot symptoms. In most of the cases, the disease was observed in spring and summer crops, but in some cases, it was detected in autumn crops in greenhouses which had been soil-disinfested during summer, a common practice in the growing area. In 7.7% of the greenhouses surveyed, the disease symptoms were associated with *Phytophthora capsici*. *Fusarium solani* was also detected in the melon and watermelon greenhouses, but not in any of the cucumber greenhouses inspected, where the disease symptoms were associated with *Monosporascus cannonballus* (data not sown).

Table 2. Collection details and GenBank accession numbers for the *EF-1α* and *RPB2* loci of the strains included in the phylogenetic analyses.

Isolate	Host	Year	GenBank accession number	
			EF-1α	***RPB2***
PCI-341	Zucchini	2007	MK048138	MK048118
PCI-511	Zucchini	2009	KF372878	MK048117
PCI-571	Zucchini	2009	MK048152	MK048116
PCI-711	Melon	2009	KC711040	MK048120
PCI-731	Melon	2009	KC711041	MK048119
PCI-1021	Zucchini	2010	MK048151	MK048132
PCI-1111	Zucchini	2011	MK048150	MK048123
PCI-1121	Zucchini	2011	MK048149	MK048122
PCI-1421	Zucchini	2011	MK048148	MK048115
PCI-1431	Zucchini	2011	MK048147	MK048114
PCI-1511	Zucchini	2011	MK048136	MK048131
PCI-1521	Zucchini	2011	MK048146	MK048129
PCI-1621	Zucchini	2011	MK048145	MK048124
PCI-1721	Watermelon	2011	MK048144	MK048125
PCI-1731	Watermelon	2011	MK048143	MK048126
PCI-1811	Zucchini	2011	MK048142	MK048127
PCI-1821	Zucchini	2011	MK048141	MK048128
PCI-1911	Zucchini	2011	MK048140	MK048130
PCI-2111	Zucchini	2011	MK048139	MK048133
PCI-2121	Zucchini	2011	MK048134	MK048110
PCI-2211	Zucchini	2012	MK048137	MK048113
PCI-2321	Zucchini	2012	MK048135	MK048121
PCI-25F1	Asparagus	2008	MK048154	MK048108
PCI-25F2	Pepper	2008	MK048156	MK048112
PCI-25F3	Green bean	2008	MK048155	MK048109
PCI-25F4	Orange tree	2009	MK048153	MK048111

zucchini greenhouses surveyed and in 100% of the zucchini samples received by the Plant Health Laboratory of Almería. Several of the affected greenhouses were located in the municipality of Las Norias de Daza, where the disease was first observed in

zucchini (Gómez et al. 2008). In addition, the disease incidence was observed throughout the province, in at least five different municipalities. The disease incidence among the different greenhouses visited was variable. In some greenhouses, the disease affected a small number of plants distributed in one or few rows. In most of the cases, the percentage of affected plants ranged between 5 and 30%. In two greenhouses, between 50 and 60% of the plants showed crown and foot rot symptoms. In most of the cases, the disease was observed in spring and summer crops, but in some cases, it was detected in autumn crops in greenhouses which had been soil-disinfested during summer, a common practice in the growing area. In 7.7% of the greenhouses surveyed, the disease symptoms were associated with *Phytophthora capsici*. *Fusarium solani* was also detected in the melon and watermelon greenhouses, but not in any of the cucumber greenhouses inspected, where the disease symptoms were associated with *Monosporascus cannonballus* (data not sown).

Suppl. Fig. 1. a, b, Symptoms observed in the field prospection on zucchini plants caused by *F. solani* f. sp. *cucurbitae*. **c**, **d**, symptoms observed in controlled inoculations in greenhouse trials.

Pathogenicity on zucchini plants and fruit

In the pathogenicity trials of *F. solani* on zucchini plants, all isolates tested, except PCl-2121, were pathogenic to zucchini, reproducing the crown and foot rot symptoms (suppl. Fig 1). The first disease symptom observed was plant wilt, observed at 16 and 9 days after inoculation (DAI) for the first and second trials, respectively. In the first trial, the most virulent isolates causing visible disease symptoms were PCl-511, PCl-711, PCl-1641, and PCl-2211, and the less virulent ones were PCl-341 and PCl-1911, which showed the first diseased plants by 22 DAI. By 27 DAI, all the pathogenic isolates had caused disease in at least 8% of the plants, and by 41 DAI, only 2% of the plants inoculated with PCl-1121 and 4% of the plants inoculated with PCl-1911 remained asymptomatic. Three isolates showed a 100% M rate by 41 DAI, namely PCl-1731, PCl-1811, and PCl-2111 (Table 3). In this trial, temperatures ranged from 6.4 to 34.9°C, with a mean of 18.1°C, in the greenhouse environment and from 14.4 to 28.7°C, with a mean of 21.4°C, in the substrate bags.

In the second trial, the most virulent isolates causing disease symptoms were PCl-511, PCl-731, and PCl-1731, and the least virulent one was PCl-1121, which caused visible disease symptoms at 17 DAI. At 27 DAI, all the pathogenic isolates had caused disease in at least 6% of the plants, and by 34 DAI, all the inoculated plants, except one plant inoculated with PCl-341 and another plant inoculated with PCl-1121, were diseased. By this date, all isolates had caused a 100% M rate, except PCl-341, PCl-1121, PCl-1431, PCl-1911, and PCl-2111. In this trial, temperatures ranged from 18.5 to 37.1°C, with a mean of 27.4°C, in the greenhouse environment and from 23.5 to 37.1°C, with a mean of 29.7°C, in the substrate bags.

In both trials, the isolate PCl-1731 was the most aggressive, showing an RAUDPC significantly higher than the rest and M rates of 100%, followed by the isolate PCl-1721, both obtained from watermelon. In contrast, the isolates PCl-1121 and PCl-341 from zucchini produced the lowest RAUDPC. Non-inoculated controls and plants

inoculated with the isolate PCl-2121 remained asymptomatic (DI=0%). In both trials, the pathogen was successfully recovered from all plants selected for re-isolation.

Table 3. Final disease parameters of the pathogenicity tests performed in zucchini plants and fruits of the strains of FSSC examined in the present study.

Isolate	Disease parameters						
	Zucchini Plants[y]						Zucchini fruits
	First trial			Second trial			Fruit lesion (mm·day^{-1})
	DI (%)	M (%)	RAUDPC	DI (%)	M (%)	RAUDPC	
PCI-341	100a	54.17a	0.28ef	97.92a	97.92a	0.42fgh	0.95 ± 0.28kl
PCI-511	100a	85.42a	0.50bcde	100a	100a	0.76ab	0.86 ± 0.12l
PCI-571	100a	91.67a	0.58abc	100a	100a	0.69abcde	1.13 ± 0.20jk[j]
PCI-711	100a	95.83a	0.55abcd	100a	100a	0.75abc	1.73 ± 0.49cd
PCI-731	100a	95.83a	0.58abc	100a	100a	0.77ab	1.54 ± 0.34de
PCI-1021	100a	93.75a	0.51bcde	100a	100a	0.70abcd	1.24 ± 0.27hij
PCI-1111	100a	83.33a	0.53bcde	100a	100a	0.81ab	1.80 ± 0.43bc
PCI-1121	97.92a	39.58b	0.27ef	97.92a	70.83b	0.28gh	1.18 ± 0.27ij
PCI-1421	100a	97.92a	0.66ab	100a	100a	0.69abcd	1.15 ± 0.18j
PCI-1431	100a	89.58a	0.59abc	100a	95.83[a]	0.51defgh	1.14 ± 0.19j
PCI-1511	100a	95.83a	0.56abcd	100a	100a	0.72abcd	1.96 ± 0.32b
PCI-1521	100a	72.92a	0.45cdef	100a	100a	0.55cdefgh	1.08 ± 0.24jk
PCI-1621	100a	93.75a	0.53bcde	100a	100a	0.61bcdefg	1.34 ± 0.28fghi
PCI-1721	100a	97.92a	0.70ab	100a	100a	0.81ab	0.90 ± 0.20l
PCI-1731	100a	100a	0.73a	100a	100a	0.84a	1.18 ± 0.25ij
PCI-1811	100a	100a	0.56abcd	100a	100a	0.62bcdefg	1.11 ± 0.21jk
PCI-1821	100a	95.83a	0.56abcd	100a	100a	0.71abcd	1.76 ± 0.49c
PCI-1911	95.83a	60.42a	0.35def	100a	89.58c	0.47efgh	1.51 ± 0.27ef
PCI-2111	100a	100a	0.60abc	100a	95.83a	0.64abcdef	1.43 ± 0.19efg
PCI-2121	0b	0b	0f	0b	0d	0h	2.64 ± 0.33a
PCI-2211	100a	95,83a	0.65ab	100a	100a	0,61bcdefg	1.41 ± 0.23efgh
PCI-2321	100a	97,91a	0.64ab	100a	100a	0,70abcd	1.25 ± 0.24ghij

[y] Disease parameters obtained at the end of the two trials conducted expressed as the percentage of plants at the end of the experiment. DI= disease incidence, M= mortality, RAUDPC= relative area under disease progress curve expressed as the proportion of the maximum potential AUDPC assessed by plotting the disease rates per plant on a basis of a 0-2 scale versus time with the trapezoidal method (Madden et al. 2007). Values in columns followed by different letters indicate significant differences according to the Kruskal-Wallis non parametric ANOVA ($P<0.05$).

[z] Measurements of lesions caused in zucchini fruits after 7 days expressed as (mm·day^{-1}). Values in columns followed by different letters indicate significant differences according to the least significant difference (LSD) method ($P<0.05$). Data of the two experiments were pooled prior to analyses.
n/a= not available

In fruits, all the isolates, including PCl-2121, caused lesions, which consisted of brown, water-soaked rot, sometimes forming white mycelium on the surface. The fruit lesion growth rate ranged from 0.9 to 2.64 mm day^{-1}, corresponding respectively to isolate PCl-341 and PCL-2121, the latter being the only isolate not pathogenic on zucchini stems. Fruits inoculated with uncolonized PDA plugs showed no symptoms (Table 3). *Fusarium solani* isolates were successfully recovered from the lesions.

Pathogenicity on other horticultural crops

In the first pathogenicity trial on the various horticultural crops, all plants belonging to different plant families except cucurbits remained asymptomatic (data not shown). All species in the cucurbit family, except cucumber, showed aerial disease symptoms (Table 4). The first disease symptoms were observed at 11 DAI, when melon, zucchini, and the three rootstock hybrids showed some diseased plants. Three days later, at 14 DAI, there was at least one wilted plant per cucurbit species, except cucumber. Plants were maintained up to 31 DAI, due to the height of the cucumber plants, which did not allow us to maintain the crop for a longer time. By that time, the species most affected was zucchini, followed by melon, watermelon and the three rootstock hybrids. Cucumber plants were asymptomatic, since no visible wilting or crown rot was observed (Table 4). A detailed evaluation of the root system revealed necrosis in the root system of 100% of the plants, indicating the ability of the isolates to cause disease in this species. Temperatures in this trial ranged from 9 to 39.7°C, with a mean of 21.5°C, in the greenhouse environment and from 9.4 to 34°C, with a mean of 21.2°C, in the pots.

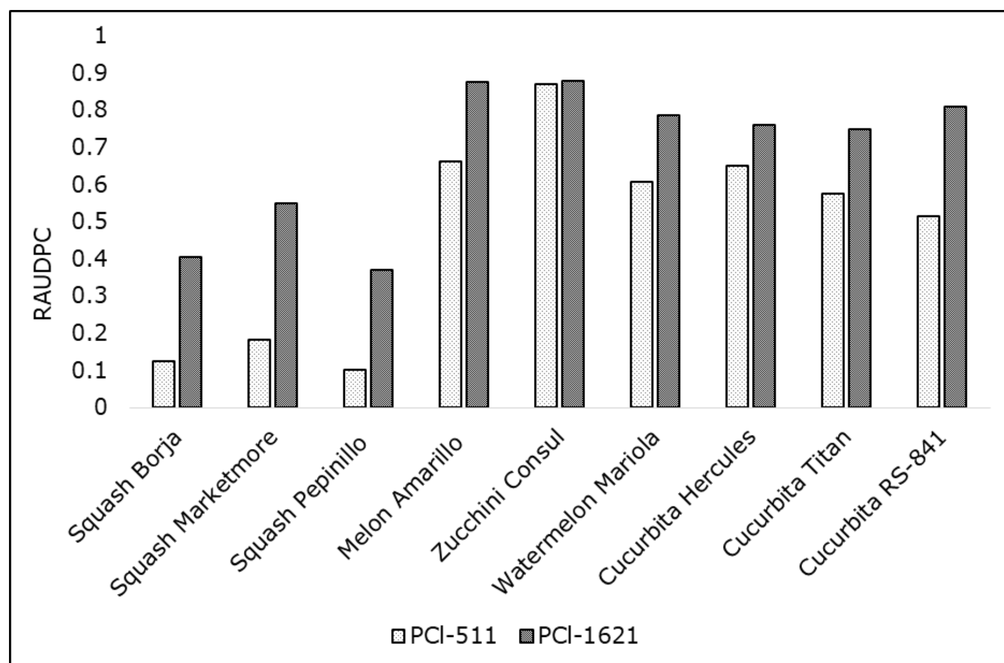


Fig. 1. Effect of *F. solani cucurbitae* inoculation with the isolates PCl-511 and PCl-1621 on different cucurbit species and hybrids. Data correspond to the final relative area under disease progress curve (RAUDPC) calculated as the proportion of the maximum possible AUDPC, assessed by plotting the rating on the basis of a 0-2 scale versus time, with the trapezoidal method (Madden et al. 2007) calculated 60 days after the inoculation in the second trial to evaluate the pathogenicity of *F. solani cucurbitae* to several horticultural crops.

In the second trial, as observed in the previous one, only plants belonging to the cucurbit family showed disease symptoms, with the isolates being non-pathogenic to the other families. The first disease symptoms were observed at 8 DAI, and as in the previous year, all species except cucumber and watermelon were the most susceptible. The first watermelon plants showed wilt symptoms at 12 DAI, and the cucumber cvs Marketmore and Borja displayed the first wilted plants at 26 DAI, while the cv. Pepinillo Jardiver displayed symptoms at 29 DAI. By 29 DAI, the DI for zucchini and the three *Cucurbita* hybrids was 100%, melon and watermelon displayed 91.7 and 86.1% DI, respectively, and the cucumber cvs. Marketmore, Borja, and Pepinillo Jardiver displayed 13.9, 41.7, and 11.1% DI, respectively. Plants were maintained in the greenhouse for a total of 60 DAI, when the DI for all the cucurbits tested, except cucumber, was 100%. M for melon, zucchini, and watermelon was 100%, being the M for the rest of the cucurbits comprised between 91.7 and 72.2% (Table 4). In this trial,

temperatures ranged from 12.3 to 39.5°C, with a mean of 23.6°C, in the greenhouse environment and from 21.3 to 35.4°C, with a mean of 27.5°C, in the substrate bags.

Table 4. Disease parameter values for 9 cucurbit species and cultivars tested for susceptibility to *F. solani* f. sp. *cucurbitae* isolates PCl-511 and PCl-1621 in greenhouse experiments during the two trials conducted.

Species	Trial 2013[w]			Trial 2014		
	DI(%)[x]	M(%)[y]	RAUDPC[z]	DI(%)	M(%)	RAUDPC
***Cucumis sativus* cv. Borja**	0	0c	0c	91.6	80.5	0.37c
***Cucumis sativus* cv. Marketmore**	0	0c	0c	88.9	77.8	0.26c
***Cucumis sativus* cv. Pepinillo JardiVer**	n/a	n/a	n/a	88.9	80.6	0.24c
***Cucumis melo* cv. Amarillo Jardiver**	97.2	97.2a	0.64a	100	100	0.77ab
***Cucurbita pepo* cv. Consul**	100	97.2a	0.62a	100	100	0.88a
***Citrullus lanatus* cv. Mariola**	97.2	63.9b	0.30b	100	100	0.70b
***C. maxima* x *C. moschata* cv. Hercules**	94.4	44.4b	0.41b	100	91.7	0.71ab
***C. maxima* x *C. moschata* cv. Titan**	86.1	16.7b	0.26b	100	83.3	0.66b
***C. maxima* x *C. moschata* cv. RS-841**	80	23.3b	0.24b	100	72.2	0.66b

[w] Values in columns followed by different letters indicate significant differences according to the Kruskal-Wallis non parametric ANOVA ($P<0.05$).
[x] DI= Disease incidence expressed as the percentage of the total number of plants.
[y] M= Mortality.
[z] RAUDPC= relative area under disease progress curve expressed as the proportion of the maximum potential AUDPC assessed by plotting the disease rates per plant on a basis of a 0-2 scale versus time with the trapezoidal method (Madden et al. 2007).
n/a= not available.

When comparing the ability of the two isolates to cause disease on the different cucurbit hosts, a higher aggressiveness was observed in PCl-1621 compared to PCl-511 in both experiments. In the first trial by 11 DAI and in second trial by 8 DAI, the isolate PCl-511 had only caused visible disease in zucchini, while PCl-1621 had caused disease symptoms in melon, zucchini, and the three *Cucurbita* hybrids. In watermelon, in the first trial, one plant inoculated with PCl-511 showed symptoms at 14 DAI, and no other plants were symptomatic until 21 DAI, when several plants inoculated with both isolates were wilted. In the second trial, the first disease symptoms were observed in watermelon plants by 12 DAI, and in cucumber by 26 and 29 DAI. In both cases, they were produced by PCl-1621, while in all cases, plants inoculated with PCl-511 remained asymptomatic by those dates. The final RAUDPC data per species and isolate of the second experiment are presented in Fig. 1.

Morphology

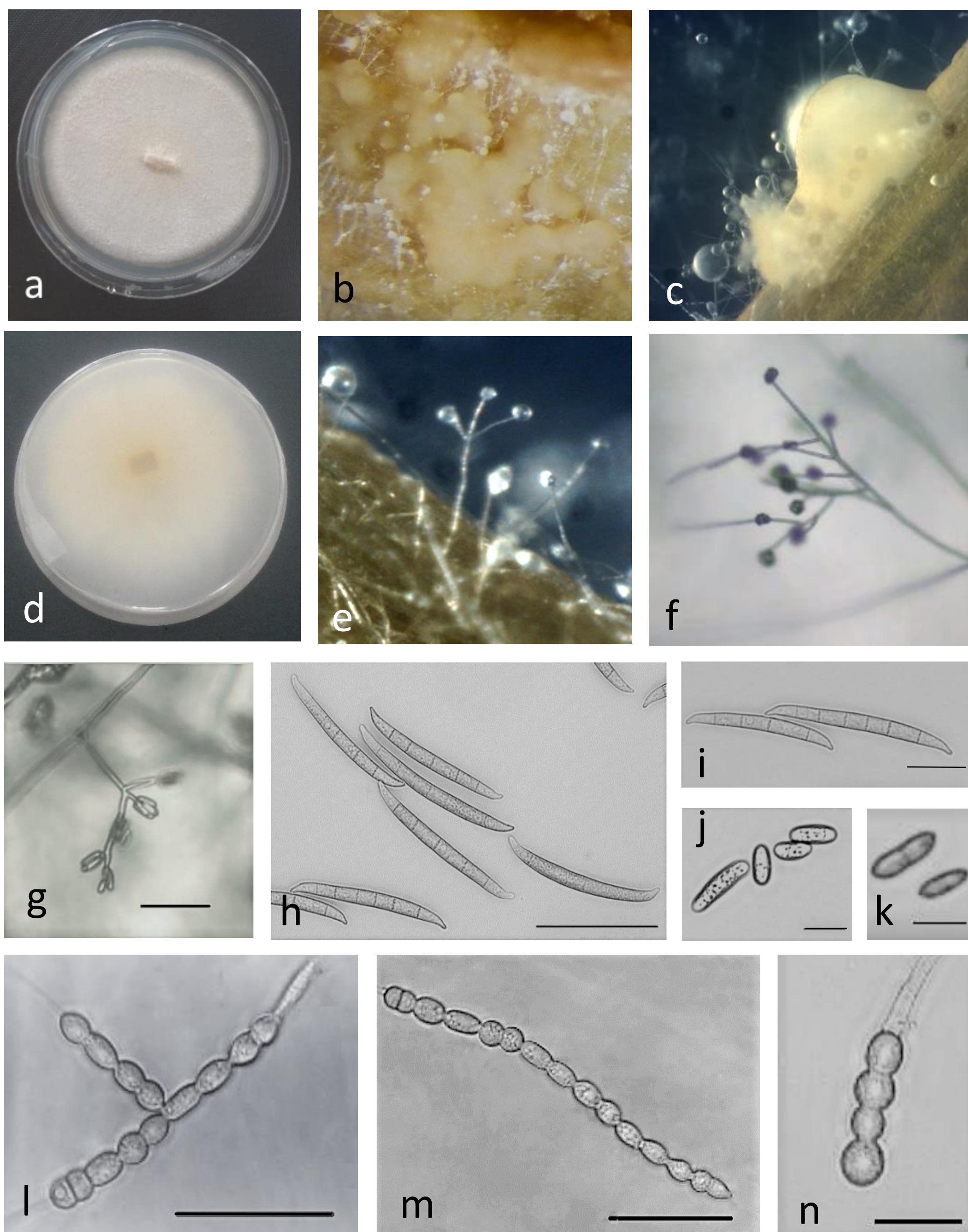


Fig. 2. Morphological description of *Fusarium solani* f. sp. *cucurbitae.* **a, d** growth on PDA; **b, c** yellow sporodochia after 10 days on pieces of carnation leaves on CLA; **e, f, g** long and branched conidiophores producing 0-1 septate microconidia; **h, i** macroconidia produced in CLA; **j, k** microconidia produced in PDA; **l, m** long chains of intercalary chlamydospores produced in PDA; **n** terminal chlamydospores produced in PDA. Scale bars: **h, l, m**=50 µm; **g, i, n**=20 µm; **j, k**=10 µm.

Culture characteristics: Colonies on PDA showing radial growth rates of 8-8.5 mm d^{-1} at 23°C and 7.8-9.1 mm d^{-1} at 29°C in the dark, reaching a diameter of 51-57 mm after 6 d at 26°C. Colony surface white, cottony, white to straw aerial mycelium and sporulation abundant; colony margins regular to slightly undulate. Reverse white to pale straw (Fig. 2).

Cardinal temperatures for growth: Minimum 8°C, maximum 35°C, optimum 23-29°C (Fig. 3).

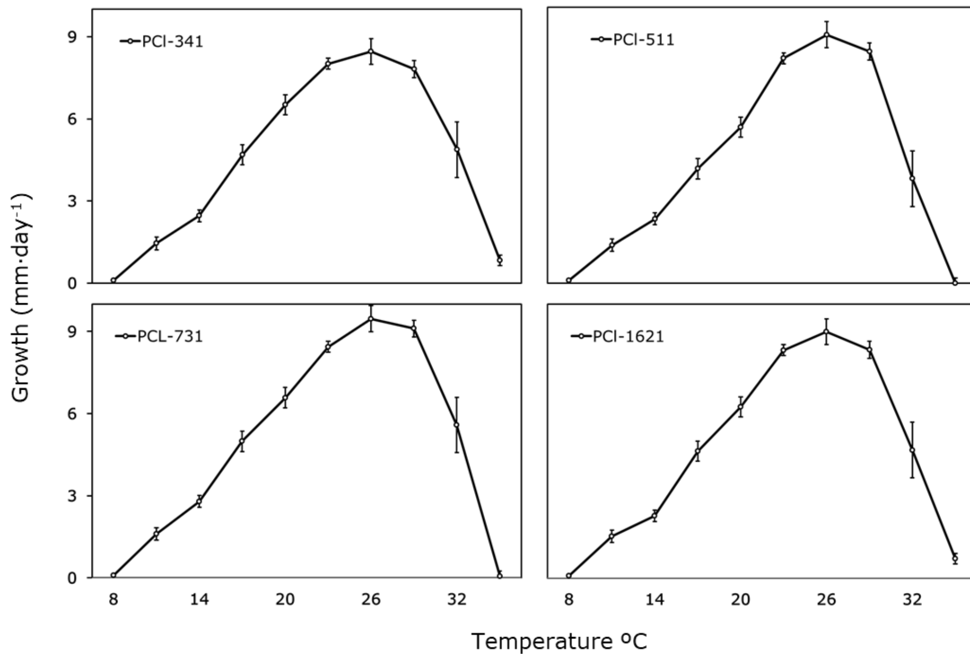


Fig. 3. Growth rates per day on PDA of isolates PCL-341, PCl-511, PCl-731 and PCl-1621 measured after 6 days of incubation in constant dark at temperatures ranging from 8 to 35°C, in 3°C intervals, and expressed in mm·day^{-1}.

Sporulation abundant from conidiophores formed on aerial mycelium, on the agar surface, and from sporodochia. Conidiophores straight, long, solitary, and simple or branched one to several times, verticillately or sympodially, each branch bearing a single terminal monophialide; microconidia formed on aerial conidiophores, hyaline, oval or obovoid with a truncate base, symmetrical or gently bent dorsoventrally, smooth- and thin-walled, 0–(1) septate. Zero-septate conidia (5.8) 7.0–10.4 (12.9) × (3.1) 3.9–5.3 (5.9) µm, one-septate conidia (11.1) 12.7–17.5 (21.0) × (3.9) 4.8–6.3 (6.7)

µm clustering in false heads at the tip of monophialides. Cream-colored sporodochia, formed abundantly on the surface of carnation leaves. Sporodochial macroconidia showing slight to moderate dorsal curvature, elongate and slender; apical cell blunt or papilate, sporadically hooked, more or less equally sized than the adjacent cell; basal cell barely or distinctly notched, (3) 4–5 (6-7)-septate, hyaline, thin- and smooth-walled. Three-septate conidia: (34.3) 37.2–46.5 (48.7) × (4.1) 4.5–5.6 (6) µm; four-septate conidia: (45.9) 49.4–58.8 (64.7) × (3.4) 4.5–5.8 (6.8) µm; five-septate conidia: (55) 57.9–64.1 (67.6) × (3.9) 4.6–5.8 (6.3) µm; six-septate conidia: (68.6) 74.6–90.7 (101.5) × (4.1) 4.8–6 (7.1) µm; overall (34.3) 44.2–75.7 (101.5) × (3.4) 4.6–5.8 (7.1) µm. Chlamydospores abundant and rapidly formed on agar media (approx. 10 d), hyaline to pale brown, spherical to subspherical (5.9) 8.3–10.8 (12) µm diam, solitary in pairs or in chains, terminal or intercalar, smooth- and thick-walled (Fig. 2).

Phylogeny

Phylogenetic analyses inferred from the *EF-1α* and the *RPB2* datasets resolved the position of all the isolates included in this study within the FSSC clade 3 in relation to currently recognized monophyletic species. The single and combined *EF-1α, RPB2* datasets included 76 strains. The combined *EF-1α* and *RPB2* dataset consisted of 1,011 nucleotides, of which 307 were parsimony-informative (Fig. 4, CI=0.61, RI=0.86); the *EF-1α* tree consisted of 436 characters, of which 142 characters were parsimony-informative (PIC) (Suppl. Fig. 2, CI=0.67, RI=0.89) and the *RPB2* tree consisted of 555 characters, of which 165 characters were parsimony-informative (Suppl. Fig. 3, CI=0.59, RI=0.85) and. Phylogenetic analysis resulted in 92 most parsimonious trees. No major topological differences were detected between Bayesian, neighbor-joining, and maximum parsimony phylogenetic inferences (data not shown).

In the three inferences, all the isolates pathogenic to zucchini clustered together with the only two available *F. solani* f. sp. *cucurbitae* sequences in GenBank, NRRL22098, and NRRL22153, forming a monophyletic group with 100% bootstrap support. The single *RPB2* and combined *EF1-α* and *RPB2* datasets generated two *F. solani* f. sp.

cucurbitae lineages, one of them containing eight isolates from Spain that are grouped with isolates NRRL22098 and NRRL22153 and another one containing isolates exclusively from Spain; however, only the second lineage presented bootstrap support. The isolate NRRL22570, corresponding to *F. solani* f. sp. *piperis,* was resolved as the sistergroup of the *F. solani* f. sp. *cucurbitae* group.

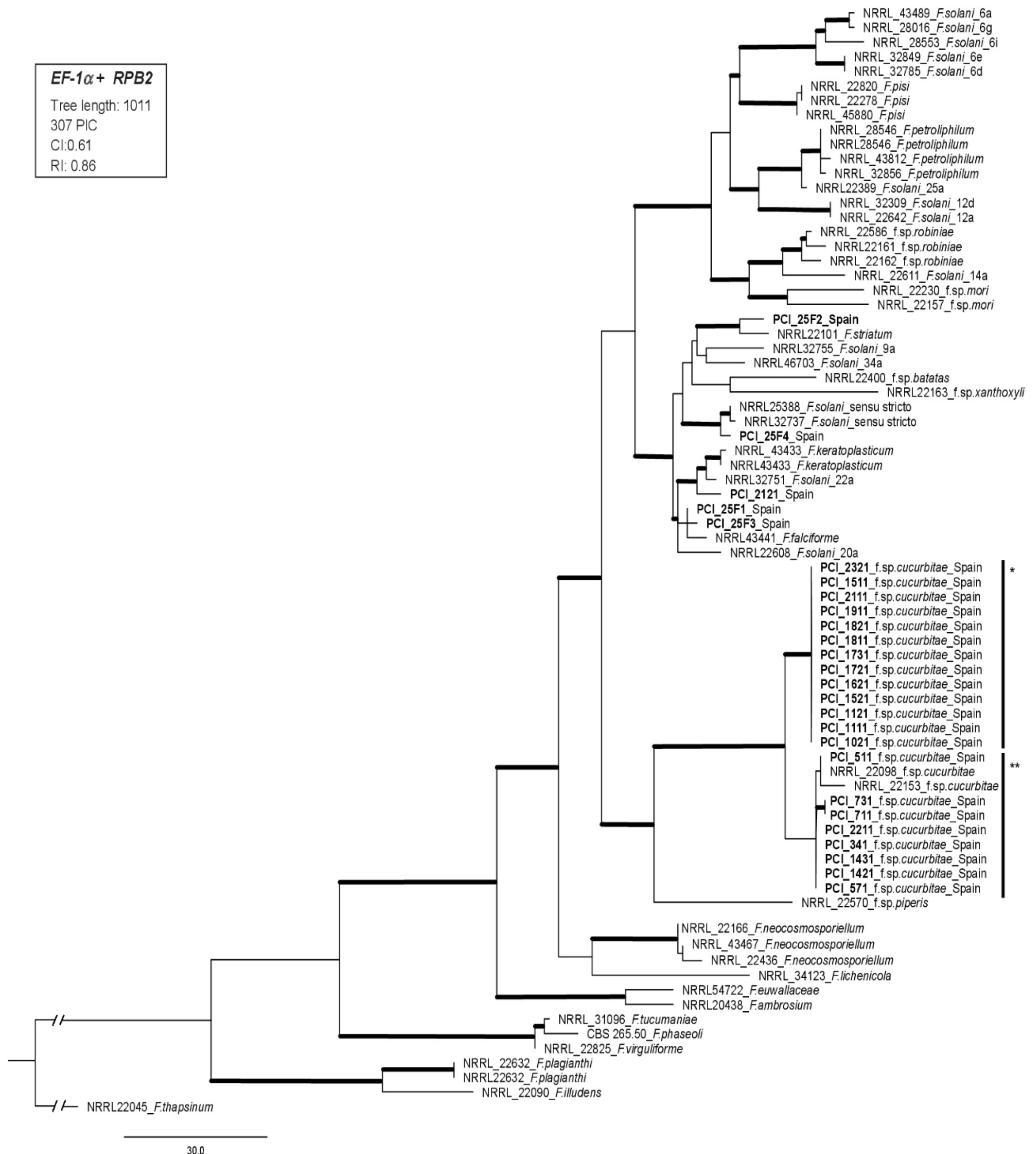
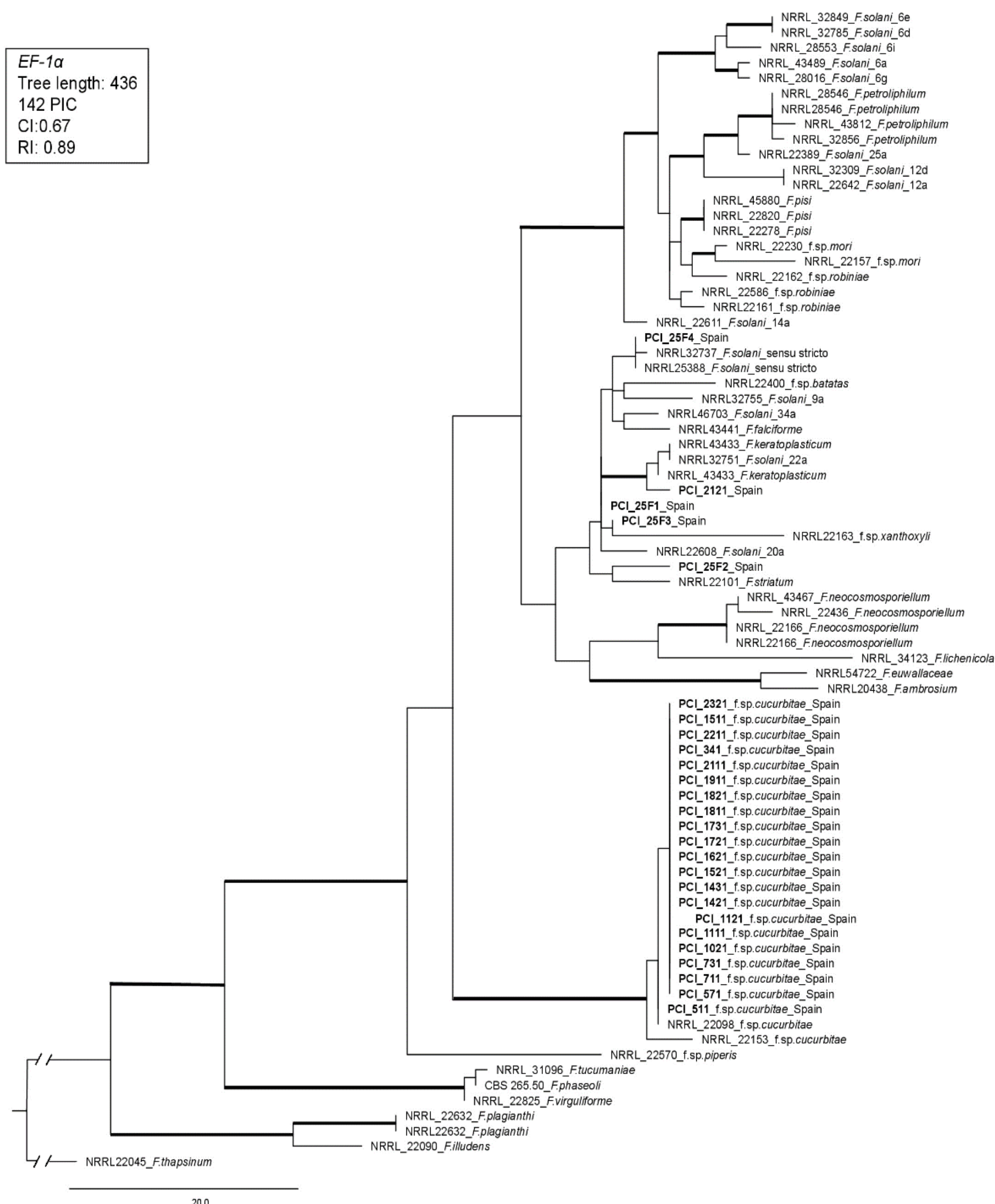


Fig. 4. One of the most parsimonious trees (MPTs) of the *Fusarium solani* species complex inferred from the *EF1-α* + *RPB2* combined gene sequences. The data set comprised 1011 bp of

aligned sequence data. The phylogram was rooted with *F. thapsinum* NRRL22045. Bold lines indicate bootstrap values above 70% based on 1000 pseudoreplicates. * Lineage composed of *F. solani* f. sp. *cucurbitae* isolates from Spain (bootstrap 70.9%). ** Lineage clustering isolates from Spain and the U.S.A. (no bootstrap support).



Suppl. Fig. 2. One of the most parsimonious trees (MPTs) of the *Fusarium solani* species complex inferred from the *EF-1α* gene sequences. The data set comprised 436 bp of aligned sequence data. The phylogram was rooted with *F. thapsinum* NRRL22045. Bold lines indicate bootstrap values above 70% based on 1000 pseudoreplicates.

The remaining isolates included in this study, nonpathogenic to zucchini, were also placed in the FSSC clade 3; however, their positions varied. According to the combined loci phylogeny, the isolate PCl-2121, pathogenic on zucchini fruit, was placed in the *F. keratoplasticum* linage with a bootstrap value of 81%. The isolates PCl-25F1 and PCl-25F3, isolated from asparagus and bean, respectively, were placed in the *F. falciforme* linage with a bootstrap value of 81%. The isolate PCl-25F2, isolated from pepper (*Capsicum annuum*), was clustered in the *F. striatum* linage with 100% bootstrap value; the isolate PCl-25F4, obtained from orange tree roots, clustered with *F. solani sensu stricto* strains NRRL253888 and NRRL32737; bootstrap support was only observed for the combined *EF1-α* and *RPB2* datasets (Fig. 4).

Suppl. Fig. 3. One of the most parsimonious trees (MPTs) of the *Fusarium solani* species complex inferred from the *RPB2* gene sequences. The data set comprised 555 bp of aligned sequence data. The phylogram was rooted with *F. thapsinum* NRRL22045. Bold lines indicate bootstrap values above 70% based on 1000 pseudoreplicates.

DISCUSSION

The results from our study indicate that *F. solani* f. sp. *cucurbitae* is the main soilborne fungus affecting adult zucchini plants in the province of Almería, being the problem of less importance in melon and watermelon crops. *F. solani* f. sp. *cucurbitae* was detected in 88.4% of the greenhouses surveyed. The disease caused by this fungus frequently associated with greenhouses dedicated to zucchini monoculture, as this is a common practice in the area. The symptoms observed both in the commercial greenhouses as well as in the artificial inoculations reproduced the typical brown crown rot described by previous authors (Bourbos et al. 1997; Champaco et al. 1993; Doidge and Kresfelder 1932; Fantino et al, 1989; García-Jiménez et al. 1997; Mehl and Epstein 2007; Snyder and Hansen 1941). *Phytophthora capsici* was also detected causing disease in adult zucchini plants (Gómez et al. 2013). In all the diseased cucumber plants found in commercial greenhouses, perithecia of *Monosporascus cannonballus* were consistently observed and isolated; however, after several pathogenicity tests on adult plants grown in perlite bags, the symptoms could not be reproduced (data not shown).

With respect to pathogenicity, all of the *F. solani* f. sp. *cucurbitae* isolates studied were highly virulent to zucchini. The isolate PCl-2121 was not pathogenic and was identified based on phylogenetic analysis as *F. keratoplasticum.* In our trials, almost 100% of the plants inoculated with *F. solani* f. sp. *cucurbitae* showed disease symptoms relatively early (by 44 and 38 DAI) in the first and second trials, respectively. In addition, a high number of plants were dead by those dates, with M rates of 67-100% in the first trial and of 73-100% in the second trial. The two trials were conducted consecutively in winter and spring 2013. Disease development was favored in the second trial, most likely because of the higher temperatures. While in the first experiment, the average temperature measured in the substrate bags was 21.4°C, in the second experiment, the mean temperature in the substrate bags was 29.7°C, which is closer to the optimal temperature range assessed in our *in vitro* experiments, i.e., between 26 and 29°C. These results are in agreement with those of Miguel (2001). Another study performed

previously to compare different *F. solani* isolates obtained from sweet pepper plants and *F. solani* f. sp. *cucurbitae* reported the highest growth rate at 30°C (Paternotte 1987). In our morphological studies, the daily growth at 23ºC was between 8-8.5 mm day^{-1} on PDA. Camilo (2001) reported a daily growth of 6.11-6.22 mm day^{-1} at the same temperature; however these data differ from the description of Sandoval-Denis et al. (2019) who observed an average daily growth of 2.4-4.2 mm day^{-1} at 24ºC.

In addition to studying the pathogenicity on whole zucchini plants, zucchini fruits cv. Cónsul were inoculated with the same strains to study the ability of the fungus to cause fruit rot. In this case, all isolates were able to produce visible fruit rot. In both trials, PCl-2121 caused the highest growth in the zucchini fruits, being significantly higher than the rot produced by the *F. solani* f. sp. *cucurbitae* strains. Although fruit rot was observed in laboratory experiments, these symptoms were not observed in the diseased plants in the field, probably because the fruits are normally harvested in the immature state and because of the lack of contact between the fruit and soil.

Our phylogenetic inference placed the isolate PCl-2121 in the *F. keratoplasticum* lineage, an opportunistic pathogen causing human eye infections (Chang et al. 2006). Recently Sisic et al. recovered *F. keratoplasticum* from naturally diseased pea, showing its potential pathogenicity to this plant species. This has clinical importance, as plant debris and soil may be a potential source of infection for farmers and crop workers (Sisic et al. 2018). *Fusarium keratoplasticum* commonly occurs in plumbing biofilms and is among the most frequent etiological agents of fusarial keratitis, together with *F. petroliphilum* (Short et al. 2013). Most known *F. keratoplasticum* and *F. petroliphilum* isolates have been obtained from plumbing, infected humans, or animals (Mehl and Epstein 2008; Short et al. 2011; Short et al. 2013; Zhang et al. 2006); however, *F. petroliphilum* was considered to be *F. solani* f. sp. *cucurbitae* race 2 due to its pathogenicity to cucurbit fruit in naturally infested fields until it was reclassified in 2013. In addition, *F. keratoplasticum* was isolated from zucchini in this study, and recently also from pea, indicating the plasticity of this FSSC member to colonize different environments and the potential threat to growers and field workers.

The greenhouse data on the host range of *F. solani* f. sp. *cucurbitae* are in agreement with previous reports that limit the host range of this forma specialis to cucurbits (Miguel 2001; Paternotte 1987; Tousson and Snyder 1961). In our trials, all plants evaluated, belonging to the cucurbit family, were susceptible to the pathogen. The rest of the plants, belonging to other families, were asymptomatic, and the fungus could not be recovered from these plants. Within the cucurbit species tested, differences in susceptibility were observed. Based on previous trials performed with zucchini, we conducted a first host range experiment in pots placed on benches, inoculating the plants in the seedling stage. The *F. solani* f. sp. *cucurbitae* isolates produced visible crown rot a few weeks after inoculation in several melon, watermelon, zucchini, and *Cucurbita* rootstock hybrids. Under the same conditions, cucumber plants were able to remain asymptomatic, but rot was observed below the soil level when the plants were removed and the root system evaluated for symptoms. This led us to repeat the trial with the same design, but in perlite bags in order to maintain the plants in the greenhouse for a longer period. In the second trial, the same tendency was observed, with melon, zucchini and watermelon plants being the most susceptible plants, closely followed by the *Cucurbita* hybrids. Cucumber plants remained asymptomatic for a longer period of time compared to the rest of the cucurbits. At 26 DAI the first wilted cucumber plants were observed, and by the end of the experiment, 20-31% of the plants had remained asymptomatic, indicating that although this species is also a suitable host for Fusarium crown and foot rot, it is a less susceptible host for the disease.

The phylogeny inferred from the combined *EF-1α* and *RPB2* datasets as well as the single gene phylogenies placed our isolates within clade 3 of the FSSC, as previously reported in other studies (Chang, et al. 2006; O'Donnell 2000; O'Donnell et al. 2008; Zhang et al. 2006; Sandoval-Denis et al. 2019). The isolates characterized as *F. solani* f. sp. *cucurbitae* were clustered together with NRRL22098 and NRRL22153, with 100% bootstrap support, suggesting that they correspond to the same phylogenetic species, with *F. solani* f. sp. *piperis* as the ancestral group, as previously reported in other FSSC

phylogenies (O'Donnell 2000; O'Donnell et al. 2008; Short et al. 2013; Sisic et al. 2018). The combined *EF1-α* and *RPB2* datasets separated the *F. solani* f. sp. *cucurbitae* into two lineages, one of them containing only Spanish isolates, with identical haplotypes (Bootstrap 85.2% for the *RPB2* and 70.9% for the combined dataset). The other linage contains the rest of the isolates together with NRRL22098 and NRRL22153; these isolates have previously been identified as pathogenic to cucurbits (O'Donnell et al. 2000; Elmer et al. 2007). This group is more diverse, containing different haplotypes, and did not receive bootstrap support. Our inference suggests that the first linage could have been diverged in our region after the introduction of *F. solani* f. sp. *cucurbitae*. To resolve this, more loci should be included for the phylogenetic inference, and a wider disease survey conducted in different regions of Spain to shed light on this hypothesis.

With respect to morphology, our study reveals some variations to the traditional description of *F. solani*. While *F. solani* produces relatively wide, straight, stout, and robust macroconidia, mostly 3- or 4-septate (Schroers et al. 2016), the *F. solani* f. sp. *cucurbitae* isolates produce relatively long and slender macroconidia, mostly 5-septate, measuring over 55 µm, and less frequently 6-septate, measuring over 75 µm. These results are concordant with the observations made by Paternotte (1987), who found that conidia from *F. solani* f. sp. *cucurbitae* were up to 5-septate and more slender than conidia of *F. solani* from pepper, which were up to 3-septate. These observations are in agreement with the description of Sandoval-Denis et al. (2019) who described the macroconidia as long and narrow. These authors, however, reported macroconidia up to 9-septate, and we have never observed macroconidia containing over 7 septa in any of the isolates included in this study.

In addition, in our *F. solani* f. sp. *cucurbitae* isolates, the conidiophores were often verticillate, which is not common in the *F. solani* morphological description (Leslie and Summerell, 2006; Nelson, et al. 1983; Schroers et al. 2016), and was not reported by Sandoval-Denis et al. (2019), who described conidiophores rarely sparingly branched. We also observed chlamydospores forming long chains, which is a characteristic that

has never been described for *F. solani* f. sp. *cucurbitae*. While our chlamydospores ranged between 5.9 to 12 µm in diameter, Sandoval-Denis et al. (2019) described smaller chlamydospores, between 4-5 to 9 µm in diameter.

Fusarium taxonomy has been controversial since the first description of this fungus by Link in 1908. The initial species descriptions were based exclusively on morphological or physiological characteristics, and the use of new approaches in the last decades, such as sexual compatibility or phylogenetic analysis, have challenged the morphological species description of *Fusarium*, as it has been demonstrated that many of those descriptions correspond to species complexes composed of distinct species that are morphologically indistinguishable (Leslie and Summerell, 2006; Nelson et al. 1994; Zhang et al. 2006). The *forma specialis* nomenclature is an informal taxonomic rank which is also controversial (Amstrong and Amstrong 1981; Jacobson and Gordon 1991; Rossman and Palm-Hernández 2008). In several cases, species have been described and assigned to a *forma specialis* without proper host range tests, and this taxonomic classification provides no insights into the phylogeny or the evolutionary history of the pathogen (Al-Hatmi et al. 2018; Fourie et al. 2011; Sisic et al. 2018). This fact leads us to consider that there were two races of *F. solani* f. sp. *cucurbitae* for decades, until vegetative compatibility and phylogeny demonstrated that they correspond to two very distinct species (Matuo and Snyder 1973; Mehl and Epstein; 2007; O'Donnell, 2000; Zang et al. 2006). Recently, Sisic et al. (2018) have re-evaluated *F. solani* f. sp. *pisi* and assigned it to *F. pisi* comb. nov. The authors confirmed the pathogenicity of this species to 33 hosts corresponding to 10 legume genera, which is in agreement with previous studies, expanding the host range of this *forma specialis* to chickpea (*Cicer auriantum* L.) and other non-legume hosts (Matuo and Snyder 1972; Westerlund et al. 1974). Years before, Kolander et al. (2012) have expanded the host range of *F. virguliforme* (formerly *F. solani* f. sp. *glycines*) to several legumes, and the host range of *Fusarium oxysporum* f. sp. *chrysanthemi* has recently been expanded to orange coneflower (*Rudbeckia fulgida; Matic et al. 2018*). Sisic et al. (2018) stress the confusion that the *forma specialis* nomenclature has caused in several cases and challenge this nomenclatural system, stating that it might deserve revision and formal

taxonomic treatment. Similarly, several authors stress the necessity for proper species descriptions and nomenclatures to designate cryptic speciation within the FSSC (Debourgogne et al. 2010; O'Donnell et al. 2000; Wang et al. 2011; Zhang et al. 2006).

Based on the phylogenetic data and the morphological differences between *F. solani* and *F. solani* f. sp. *cucurbitae,* the definition of the latter to species rank within the FSSC by Sandoval-Denis et al. (2019) is supported.

ACKNOWLEDGEMENTS

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CHAPTER III

Fusarium solani f. sp. *cucurbitae*, Affecting Melon in Almería Province, Spain

J. Gómez, Y. Serrano, A. Pérez, E. Porcel, R. Gómez and M. I. Aguilar.

Australasian Plant Disease Notes 2014, 9:136

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ABSTRACT

During surveys carried out for assessing the occurrence of cucurbit-infecting soil fungi, melon plants exhibited necrosis on the basal stem, wilt and death. Mycological analysis and experimental inoculations showed the causal agent to be *Fusarium solani* f. sp. *cucurbitae*. This is the first report of *F. solani* f. sp. *cucurbitae* as the causal agent of crown rot of melon in Europe.

Keywords

Fusarium solani f. sp. *cucurbitae*, melon, zucchini

MAIN TEXT

Melon (*Cucumis melo*) is a widely cultivated crop in the south of Spain, covering 14,500 ha of which approximately 5,500 ha are grown in plastic-houses in the southeast (Anonymous 2009). In the spring of 2009, melon plants cv. Timon, cultivated in a commercial plastic-house near the village of San Agustín (36’71 N, 2’69 W) in Almería Province, exhibited necrosis on the basal stem, wilt and death (Fig. 1). The incidence of dead plants was 10-15%. *Fusarium* sp. was consistently isolated on potato dextrose agar (PDA) from the basal stems of symptomatic plants.

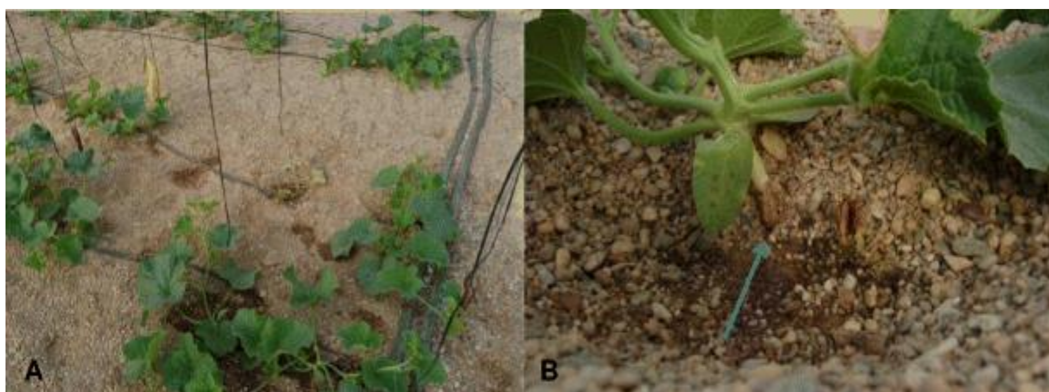

Fig. 1. Melon plants affected by *Fusarium solani* f. sp. *cucurbitae* causing basal stem necrosis and wilting in a commercial plastic house in Almeria province, Spain. (a) Comparison of diseased and symptomless plants; (b) Basal stem necrosis

Three single spore isolates (Fsm711, Fsm721 and Fsm731) were identified on PDA, carnation leaf-piece agar medium (CLA) and Spezieller Nährstoffarmer agar medium (SNA, Leslie and Summerell 2006) as *Fusarium solani* species complex (FSSC) due to their production of long and slightly curved or straight macroconidia, containing between three to six septa (four and five septa more frequent) with distinctly or barely notched basal cells. Microconidia formed on false heads on long or intermediate, branched and often verticillate monophialides (Fig. 2). Chlamydospores formed in conidia and in hyphae, often in chains. DNA was extracted from three isolates and the identity of these isolates was confirmed by comparing a portion of the elongation translation factor 1-alpha at the Fusarium-ID database, Pennsylvania State University (Geiser et al. 2004). The pathogenic isolates had a 99.85% homology with the isolates NRRL 43315 (Elmer et al. 2007) and NRRL22098 (O'Donnell et al. 2008), corresponding to *F. solani* f. sp. *cucurbitae* MPI, isolated from cucurbits in the United States. The sequences of two isolates, Fsm711 and Fsm731, were deposited in GenBank with accession Nos: KC711040 and KC711041, respectively, and the isolate Fsm711 has been deposited with the accession CECT20831 in the Colección Española de Cultivos Tipo (CECT), Parc Científic Universitat de Valéncia, Paterna, Spain. The pathogenicity of the three isolates was tested in two experiments conducted in a greenhouse in Almería. Pre-germinated seeds of melon cv. Timon and zucchini cv. Consul were sown in 1L-containers filled with vermiculite in the fall 2010 and spring 2011 (experiments 1 and 2, respectively). Melon plants at the 1–3 true-leaf stage and zucchini plants at the 3–4 true-leaf stage were inoculated by irrigating plants with a suspension (50 ml/plant) obtained by blending and homogenizing the colonies when they fully covered each Petri dish of PDA in 600 ml sterile distilled water. Inoculum concentrations for different isolates and experiments ranged 7.3 × 106 to 4.4 × 107 propagules/ml. A nutrient solution of 1.9–2.1 dS m−1 EC was prepared for fertigation using water of 0.6 dS m−1 EC, and distributed to each plant with a drip irrigation system. An experimental design of two-factor randomized complete blocks with three replicates, each plot comprising six plants (three plants per container) was established. In experiments, 18 uninoculated melon and zucchini plants of the same cultivars were used as controls. The plants were

maintained along 35 and 43 days following inoculation in the greenhouse, for experiments 1 and 2, respectively. During the experiments, sequential observations of the uninoculated plants were performed for the detection of symptoms development. The mean temperatures ranged from 15.2 to 26.1°C, maximum ranged from 21.3 to 44.9°C and minimum ranged from 8.5 to 18.0°C for experiment 1, and mean temperatures ranged from 19.0 to 26.0°C, maximum ranged from 27.9 to 41.1°C and minimum ranged from 12.9 to 18.3°C for experiment 2. In experiment 1, the first wilting occurred in both species 17 days after inoculation and at the end of the experiment, 83.3% melon and 61.1% zucchini plants inoculated with Fsm721 and 100% of inoculated plants with Fsm711 and Fsm731 died. In experiment 2, the first wilting occurred 7 days after inoculation in zucchini plants, and 36 days later, 100% of melon and zucchini plants inoculated with the three isolates died. Inoculated plants exhibited lesions in the crown but had no secondary root rot (Fig. 3), while all uninoculated plants remained asymptomatic. The pathogen was recovered from symptomatic plants in both experiments, fulfilling Koch's postulates.

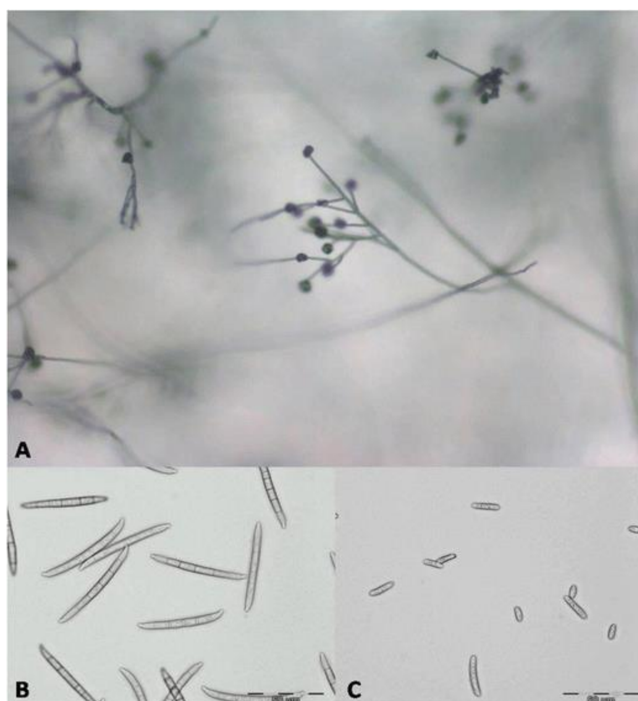

Fig. 2. Morphological characteristics of the pathogen on carnation leaf-piece agar medium: branched monophialides (a), macroconidia (b), microconidia (c)

Fig. 3. Wilt symptoms in melon plants inoculated with *Fusarium solani* f. sp. *cucurbitae* in pathogenicity test

Fusarium solani f. sp. *cucurbitae* race 1 was reported in a squash field (*Cucurbita maxima*) in the province of Valencia, east-central Spain (García-Jiménez et al. 1997), and in zucchini greenhouses (Gómez et al. 2008) in Almería. However, this is the first report of *F. solani* f. sp. *cucurbitae* race 1 as the causal agent of crown rot of melon in Europe, specifically in southern Spain, which is one of the world's largest concentrations of plastic-houses. In this area, other susceptible species belonging to the Cucurbitaceae family such as zucchini, melon, cucumber, watermelon and rootstocks for watermelon are cropped on greater than 16,700 ha (Sanjuán 2001). These cucurbits are potential hosts of the pathogen. There is therefore a reasonable risk that once introduced, the pathogen can spread easily in the area and cause significant economic damage.

ACKNOWLEDGMENTS

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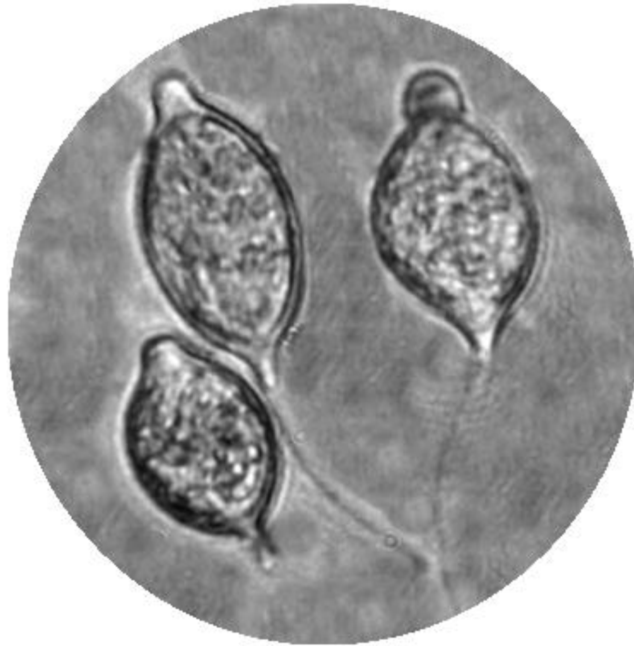
CHAPTER IV



Phytophthora Crown and Root Rot of Zucchini Squash in Almería, Spain

J. Gómez, A. Pérez, Y. Serrano, M. I. Aguilar and R. Gómez. Plant Disease 2013,97(9):1249

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ABSTRACT

Zucchini (*Cucurbita pepo*) is intensively cropped in approximately 4,500 ha of plastic houses in southern Spain. In 2008 to 2009, Consul, Cronos, and Tosca zucchini plants showed symptoms of leaf wilting, basal stem necrosis, and plant death. Incidences of dead plants were 20 to 30% and these plants were distributed in clusters. *Phytophthora capsici* Leonian was isolated from the basal stems of symptomatic plants, using PDA and cornmeal agar amended with a pimaricin, ampicillin, and rifampicin. Five resultant isolates (PCl-211, PCl-221, PCl-611, PCl-612, and PCl-811) on lima beans agar (LBA) produced white mycelia with lemon-shaped and papillate sporangia borne on long pedicels, but no oospores or chlamydospores. These isolates had an identical ribosomal DNA ITS sequence, matching with that of *P. capsici* in GenBank. The sequences of two representative isolates, PCl-211 and PCl-811, were deposited in GenBank with accession nos. KC662328 and KC688317, respectively. The pathogenicity of these five isolates was tested on zucchini cv. Consul in 1-liter containers filled with vermiculite in May and September of 2009. Plants were inoculated at the 2 to 3 true-leaf stage. Plates with LBA fully covered with colony of each isolate were separately blended and homogenized with 300 ml of sterile distilled water. Inocula were poured around stem at 50 ml per plant. Each experiment had three replicates and four plants per replicate. Treatments with different isolates were arranged in a randomized complete block design. In both experiments, 12 uninoculated plants served as controls. Test plants were maintained for a month following inoculation in a greenhouse with mean temperatures ranging from 21.9 to 27.9°C and from 20.7 to 24.6°C for the May and September experiments, respectively. The first wilting occurred 5 days after inoculation. At the end of the May experiment, all control plants and those inoculated with PCl-221 remained asymptomatic while 83.3% of those inoculated with PCl-211 and 100% of those with the other isolates were dead. Inoculated plants exhibited crown and root rots, excluding the secondary roots. In the September experiment, 83.3% and 33.3% of plants inoculated with PCl-211 and PCl-221, respectively, were symptomatic, while all plants inoculated with the other isolates

were dead. The control plants remained healthy. The pathogen was consistently recovered from symptomatic plants in both experiments. Although *P. capsici* was reported in peppers (*Capsicum annuum*) in several provinces of Spain (Andrés et al. 2005), to our knowledge, this is the first report of *P. capsici* as the causal agent of crown rot in zucchini plants in plastic houses in the Almería Province of Spain, one of the world's largest concentrations of greenhouses.

CHAPTER V

Survival of *Fusarium solani* f. sp. *cucurbitae* and Fungicide Application, Soil Solarization, and Biosolarization for Control of Crown and Foot Rot of Zucchini Squash

Ana Pérez-Hernández, Elena Porcel-Rodríguez, and Julio Gómez-Vázquez. Plant Disease 2017, 101(8): 1507-1514

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ABSTRACT

Fusarium crown and foot rot of zucchini squash *(Cucurbita pepo* L.) caused by *Fusarium solani* f. sp. *cucurbitae* is one of the major diseases affecting zucchini squash production in Almería, Spain. Experiments were conducted to determine the pathogen's ability to survive in infested bags of perlite and to test several control methods under greenhouse conditions. The pathogen survived in the bags for at least 20 months with enough inoculum at that time to produce disease symptoms in zucchini plants, although disease severity was significantly reduced after 14 months. A total of 14 zucchini cultivars were inoculated with F. solani f. sp. cucurbitae, and all were highly susceptible to the disease. Eight fungicides and two microbial products, Trichoderma harzianum and Rhizophagus irregularis, were tested to determine their efficacy for the control of this disease. Prochloraz, carbendazim, and thiophanate-methyl, which are not labeled for use in zucchini in Spain, were highly effective for the control of the disease, while the other products were ineffective. Two soil solarization and biosolarization experiments were conducted in a greenhouse for 45-day periods during the summer. Inocula in the soil samples decreased by more than 99%, indicating the efficacy of completely closing the greenhouse windows, solarization, and biosolarization in reducing inoculum. Fungicide applications, crop rotation for at least two years, and soil solarization or biosolarization are promising control methods for this disease.

INTRODUCTION

Zucchini squash (*Cucurbita pepo* L.) is a predominant crop grown in Almería Province, southeastern Spain, where the largest concentration of greenhouses worldwide is found. In 2014, 7,116 ha of greenhouse space were used to produce 347,160 metric tons of zucchini squash. This production accounts for 74.7% of the total production in Spain, making it one of the most economically important crops for this country (Spanish Ministry of Agriculture, Food and Environment, MAGRAMA 2015).

In this area, around 80% of crops are grown in a soil management system known as "enarenado." It consists of adding to the soil surface a 10 to 15-cm layer of siliceous sand with a particle size of less than 3 mm in diameter. This sand layer helps increase the soil temperature in the coldest months, retains the irrigated water for a longer period of time, avoids the concentration of salts near the rhizosphere, and promotes aeration and superficial root development (Valera et al. 2014, Wolosin 2008). Another important crop system in the region is the use of soilless culture, used by almost 14% of growers, in which the most commonly used substrates are perlite and coco peat (Valera et al. 2014). Soilless cultures provide some advantages, such as greater production of crops, reduced energy consumption, better control of growth, and independence of soil quality (Vallance et al. 2011).

In 2008, a new fungal disease of zucchini squash was reported in Almería Province (Gómez et al. 2008) and was found to be caused by *F. solani* (Mart.) Sacc. f. sp. *cucurbitae* W. C. Snyder and H. N. Hansen. The high incidence in the affected greenhouses and the aggressiveness of this pathogen had a large economic impact on growers. Since its detection, Fusarium crown and foot rot of zucchini squash has spread rapidly within the province and to other cucurbits, such as melon (Gómez et al. 2014) and watermelon (Porcel 2013).

Fusarium solani f. sp. *cucurbitae* has a host specificity for cucurbits (Boughalleb et al. 2007; Messiaen et al. 1995; Paternotte 1987). This pathogen has been reported to cause disease in pumpkin (Matta and Garibaldi 1981), muskmelon (Champaco et al. 1993), cucumber (Bourbos et al. 1997), watermelon, and rootstock hybrids of *Cucurbita maxima* × *Cucurbita moschata* (Armengol et al. 2000), among others. In addition, it has been demonstrated that the pathogen can be transmitted by seed, at least in watermelon and pumpkin seeds (Boughalleb and El Mahjoub 2006; Mehl and Epstein 2007).

Genetic resistance against this pathogen has not been found, and the most common control method for the disease caused by *F. solani* f. sp. *cucurbitae* is the rotation of

crops with nonhosts (i.e., plants not belonging to the cucurbit family) for 3 or 4 years (Zitter et al. 1996). However, there are few reports on the survival of *F. solani* f. sp. *cucurbitae* in the soil or in substrates after a disease occurrence. Some plant-pathogenic *Fusaria* produce chlamydospores that permit the survival of the organism in the soil. Although *F. solani* f. sp. *cucurbitae* produces chlamydospores, these seem to be less suited for survival than those of other *Fusarium* species, such as *F. solani* f. sp. *phaseoli* (Nash and Alexander 1965). In the Almería region, it is common to reuse the substrates used for soilless cultures for several years in order to improve crop profitability (Bonachela et al. 2008), and usually parts of the roots from previous crops remain in the bags. Elucidation of the survival of this pathogen after a disease incidence in the substrates would provide useful information for the implementation of a good management strategy.

There is a lack of research on the efficacy of available products for the chemical control of this disease. Sultana and Ghaffar (2010) demonstrated an inhibition of the growth of *F. solani* on potato dextrose agar (PDA) supplemented with the fungicides fosetyl-Al, benomyl, carbendazim, mancozeb, metalaxyl-m, thiophanate-methyl, and carboxin with concentrations greater than 500 ppm. They were also able to inhibit infection in bitter gourd plants (*Momordica charantia*) grown from naturally infested seeds by treating them with benomyl and carbendazim. In addition, Ben Salem et al. (2011) achieved total inhibition of the in vitro growth of *F. solani* in culture media amended with the fungicides hymexazol, carbendazim, and thiophanate-methyl. However, despite evidence of the efficacy of these chemicals for the control of zucchini crown and foot rot caused by *F. solani* f. sp. *cucurbitae*, some of them are subjected to restrictions for their use in cucurbit crops. For example, benomyl's registration for agricultural use was cancelled in the last decade in the United States (EPA-738-R-02-011, Environmental Protection Agency 2002), the European Union (2002/928/EC, European Communities 2002), and other countries.

There are few reports regarding the biocontrol of this disease. Recently, several commercial microbial products containing various species of *Trichoderma* spp. or

bacteria-like *Streptomyces* sp. and *Pseudomonas* sp. were found to reduce the severity of Fusarium crown and foot rot of zucchini squash (Roberti et al. 2012), establishing the potential of these microorganisms for controlling this disease, as well as the need for further research into microbial antagonists as biocontrol agents for commercial crops.

There are also cultural practices with potential for controlling this disease. Soil solarization is a technique based on the utilization of solar radiation to raise soil temperatures close to a level that may be lethal or sublethal to pathogens in moist soils using plastic mulching (Katan 1981). Its efficacy in reducing inoculum density of soilborne pathogens such as *Fusarium* spp. has been demonstrated in several studies (Chellemi et al. 1997; González-Torres et al. 1993; Matheron and Porchas 2010). Solarization can be performed in greenhouses by completely closing the windows to retain heat. Temperatures reached in solarized soils inside greenhouses are significantly higher than those obtained in open fields and can therefore be more effective (Elmore et al. 1997). In Greece, soil solarization in combination with calcium cyanamide and organic matter was reported to be effective at reducing *F. solani* f. sp. *cucurbitae* soil inoculum by 99% in greenhouse experiments (Bourbos et al. 1997). Biofumigation is yet another disinfestation method and refers to the suppression of soil-borne pathogens or pests by decomposing organic materials, such as agricultural byproducts or manure. Fermentation of these materials releases volatile compounds that may have a fumigant action against plant pathogens (Bello et al. 1999). When soil solarization and biofumigation are combined, the resulting technique is known as biosolarization. This disinfestation method is relatively new and, therefore, information in the scientific literature is scarce. Recently, Martínez et al. (2011) observed a reduction in *F. oxysporum*, *F. solani*, and *F. equiseti* populations in greenhouse soils after repeated biosolarization treatments with diminishing doses of manure and pepper crop residues. In addition, Núñez-Zofio et al. (2011) demonstrated a high reduction in the oospore viability of *Phytophthora capsici* in greenhouse pepper (*Capsicum annuum*) crops after applying organic amendments followed by soil plastic mulching.

On the basis of its ability to produce chlamydospores, we hypothesized that *F. solani* f. sp. *cucurbitae* would survive in the soil and in soilless substrates after a disease outbreak, and a control strategy would be needed to avoid occurrence of this disease in future crops. The objectives of this study were to examine (i) the survival of the fungus in artificially infested bags of perlite and (ii) the efficacy of five possible control methods for Fusarium crown and foot rot of zucchini squash: host resistance; chemical control; biological control; soil solarization; and soil biosolarization.

MATERIALS AND METHODS

Isolate identification

An isolate of *F. solani* f. sp. *cucurbitae* (PCl-511), which was collected in 2009 from zucchini in a commercial greenhouse in Almería Province and whose pathogenicity on zucchini plants grown on bags of perlite was previously demonstrated (Porcel 2013), was used for the experiments. A pure culture was isolated from a single conidium and identified based on morphological and molecular characteristics. Polymerase chain reaction (PCR) amplification of the translation elongation factor 1-α (TEF 1-α) using primers EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) was purified and submitted to the Universitat Politécnica de Valencia (Spain) for sequencing. Both DNA strands of the TEF1-α gene were sequenced. The resulting sequence of the isolate was compared with those available in GenBank, and the sequence had 100% homology with GenBank Accession No. DQ986152, corresponding to *Nectria haematococca* MP-I. This sequence was submitted as GenBank Accession No. KF372878.

Production of pathogen inoculum

For every experiment, plates with the isolate were produced on PDA medium. PDA was prepared by mixing infusion of potatoes (200 g), 20 g of dextrose, and 17 g of agar per liter. Plates were incubated under indirect natural light at 25°C and maintained between 15 and 20 days until they were completely colonized. Conidial suspensions were prepared by scraping the surface of the plates with a sterile loop and recovered

by washing the plates with autoclaved water. The inoculum was then filtered through a 0.5-mm-opening mesh to remove large portions of mycelia and adjusted with a Thoma cell counting chamber to an approximate concentration of 1.1×10^5 conidia/ml. Fifty milliliters of inoculum were poured around the basal stem of each plant. For soil solarization and biosolarization experiments, inoculum from 30 PDA plates was recovered in 200 ml of sterile water and blended to homogenize the solution. This conidial suspension was then used to produce artificially infested soil samples.

Methods for growing plants

The experiments to assess survival of the fungus in bags of perlite, to evaluate the susceptibility of several zucchini squash cultivars, and to evaluate the efficacy of some fungicides were conducted in a 970-m^2, multispan polyethylene-covered greenhouse located at IFAPA Centro La Mojonera in Almería Province, Spain (36°47′17′′N; 2°42′14′′W). The greenhouse was equipped with ridge and side windows as well as automated fertigation. Plants were grown in soilless cultures in 32-liter bags of perlite (Agroperl GL-3-6, S&B Industrial Minerals, Berwyn, PA) after direct seed sowing. Fertigation was managed with a programmable system based on the leaching fraction, which was maintained close to 20%. The experiments to evaluate the efficacy of soil solarization and biosolarization were conducted in a 1,144-m^2 sloping roof-type polyethylene-covered greenhouse also located at IFAPA Centro La Mojonera. The traditional “enarenado” soil was used in the greenhouse, as it is the most representative of this area.

Temperature data acquisition

Temperatures for all crops were measured inside the bags of perlite and in the greenhouse environment above the plant canopies with P-108 temperature probes for 10-min intervals and recorded with Campbell CR10X dataloggers (Campbell Scientific, Inc., Logan, UT). For solarization experiments, environmental and soil temperatures were measured for the different treatments at a 10 to 15-cm depth and additionally at a 30 to 35-cm depth in the second trial.

Soil-borne inocula survival assessment

The effect of time on inocula contained in bags of perlite was tested based on the ability of the inocula to infect and produce disease symptoms in adult plants. For this purpose, prior to the assessment of inocula survival, a zucchini crop was grown in soilless culture using 32-liter bags of perlite. Three plants per bag were grown in 124 bags of perlite. Ninety-six of the bags of perlite were inoculated with *F. solani* f. sp. *cucurbitae* as described previously at the 2 to 3 true leaf stage, and 16 bags of perlite were used as noninoculated controls. The plants were maintained in the greenhouse until 60 days after inoculation (dai) when all of the inoculated plants had developed severe leaf wilting or died. Following standard growing practice, the plants were cut and removed at the crown, leaving a portion of the root systems inside the bags of perlite. The bags were left in the greenhouse with the ridge and side windows opened.

For studying the survival of soil-borne inocula, a completely randomized design was used. Four different time intervals during which survival was assessed were considered as treatments. Each treatment was assayed in 24 bags of perlite, and seven noninoculated bags served as controls that were used to evaluate viability decline in propagule survival in vivo over time. After each after-harvest interval, the randomly selected bags were sown with zucchini plants for their evaluation. The bags were grouped in a different part of the greenhouse for better irrigation management. The plants were harvested on 20 July 2012. Bags corresponding to the first treatment were sowed with zucchini seeds of cv. Cónsul in September 2012, as the 2-months-after-harvest (2 mah) interval; bags corresponding to the second treatment were sowed in March 2013, 8 months after harvest (8 mah); bags corresponding to the third treatment in September 2013, 14 months after harvest (14 mah); and the bags corresponding to the fourth treatment in March 2014, 20 months after harvest (20 mah). At 20 mah, plants corresponding to 14 mah were re-evaluated, and these plants are referred to as 14 mah-b. Plants were monitored twice a week for the presence of disease symptoms, and the area under the disease progress curve (AUDPC) was calculated by plotting the number of diseased plants versus time by the trapezoidal

method (Madden et al. 2007), and the relative AUDPC (RAUDPC) was expressed as the proportion of the maximum possible AUDPC. In addition, data of the percentage of wilted plants (wilting, W), dead plants (mortality, M) and plants showing crown rot were recorded. After each block was evaluated, 10% of symptomatic plants were randomly selected for pathogen reisolation.

Fungicide efficacy greenhouse evaluation

Two experiments were conducted to evaluate the efficacy of several chemical and biological products to control the crown and foot rot of zucchini squash. Seeds were directly sowed into bags of perlite during the last week of February 2012 for the first trial and during the first week of May 2012 for the second trial. The experimental design was a randomized complete block with four replicates. Experimental units consisted of three bags of perlite with three plants of cv. Cónsul per bag. Plants were inoculated with the isolate PCl-511 at the 2 to 3 true leaf stage, as previously described. The commercial fungicides were applied to the basal stems of the plants with 50 ml of each product per plant according to the recommended doses listed in the manufacturers' instructions (Table 1). An initial treatment was applied 2 days before inoculation with *F. solani* f. sp. *cucurbitae*, simulating a preventive treatment, and a second treatment 15 days after the first in both experiments. In addition to fungicides, two fungi were used as biological treatments. The first was a commercial formulation of *Trichoderma harzianum*, which was inoculated as recommended at the time of sowing with a granular formulation (Trianum-G, *T. harzianum* strain T-22 spore powder, 1.15% w/w, 1.5 × 10^8 spores/g; Koppert B.V., Berkel en Rodenrijs, The Netherlands) of 50 ml containing 2 g/liter and inoculated again the same day that fungicides were applied with a wettable powder formulation (Trianum-P, *T. harzianum* strain T-22 spore powder 1.15% w/w, 1 × 10^9 spores/g, Koppert B.V.) with 50 ml at a concentration of 2 g/liter. Another treatment was applied with the vesicular arbuscular mycorrhizal fungus *Rhizophagus irregularis* (registered in the International Bank for the Glomeromycota as *Glomus intraradices* BEG 72 [Blaszk., Wubet, Renker & Buscot] C. Walker & A. Shüßler comb. nov.), a fungus isolated from a citrus (*Citrus auriantum* L.)

nursery on the northeastern Spanish coast line (Camprubí and Calvet 1996). The inoculum consisted of rhizosphere, soil, and leek (*Allium porrum* L.) roots from 1-year-old pot cultures inoculated with the *R. irregularis* strain and grown in calcined montmorillonite clay (Pro's Choice, Oil-Dri Corp., Chicago, IL). Zucchini plants were previously germinated and transplanted to a seedbed with vermiculite and 5 g of bulk inoculum per plant. Plants were maintained to at least two true leaves, after which they were transplanted to the bags of perlite. In each experiment, a noninoculated control and a nontreated inoculated control were included. To evaluate disease development, plants were monitored twice a week for the presence of disease symptoms. Disease incidence (DI) was calculated as the percentage of symptomatic plants per treatment at the end of the experiment, and the AUDPC and M were calculated as previously described. During the duration of the experiments, no phytosanitary treatments were applied to avoid interference among products. After experiments concluded, 10% of symptomatic plants were randomly selected for pathogen reisolation.

Table 1. Features and rates of fungicides and fungal antagonists used for greenhouse experiments, and their influence on Fusarium crown and foot rot of zucchini squash plants cultivar Cónsul caused by *F. solani* f. sp. *cucurbitae* in greenhouse experiments.

Trade Name or Treatment	Active Ingredient	Manufacturer	Concentration (%)[u]	Chemical Group[v]	Rates (g or ml per liter)[w]
Vimar	Metalaxyl	Sarabia S.A.	25% w/w	Acylalanines	1.2
Previcur	Propamocarb	Bayer CropScience	60,5% w/v	Carbamates	1.5
Octagon	Prochloraz	BASF	45% w/v	Imidazoles	3
Kemdazim	Carbendazim	Agroproser S.L.	50% w/w	Benzimidazoles	0.75
Terrazole	Etridiazole	Dow AgroSicences	48% w/v	1,2,4-thiadiazoles	2
Trotis	Pencycuron	Bayer CropScience	25% w/v	Phenylureas	5
Topsin	Thiophanate-methyl	Bayer CropScience	70% w/v	Thiophanates	1
Moncut	Flutolanil	Massó	50% w/w	Phenyl-benzamides	1
Trianum	*Trichoderma harzianum* Strain T22	Koppert B.V	1.25% w/w	-	2
R. irregularis	Rhizophagus irregularis Strain BEG 72	-	-	-	-
Control +[Y]	-	-	-	-	-
Control -[z]	-	-	-	-	-

[u] Percentage of active ingredient in the product formulation: w/w=weight/weight; w/v=weight/volume.
[b] Chemical group according to Fungicide Resistance Action Committee, FRAC Code List 2013.
[w] Doses were applied to the crown of the plants according to manufacturer's recommendations and legislation. A first treatment was applied two days before the inoculation with the isolate PCl-511 and a second applied 13 days later.

Table 1. Continues from previous page

Trade Name or Treatment	Disease Parameters[x]					
	First Trial			Second Trial		
	DI (%)	M (%)	AUDPC	DI (%)	M (%)	AUDPC
Vimar	100a	100a	626.5a	100a	75ab	529.5a
Previcur	100a	97.2a	567a	100a	86.1a	595a
Octagon	0b	0b	0b	0b	0b	0b
Kemdazim	0b	0b	0b	0b	0b	0b
Terrazole	97.2a	86.1a	640.5a	100a	100a	758.5a
Trotis	97.2a	69.4ab	548.5a	100a	88.9a	593a
Topsin	2.8b	0b	7b	0b	0b	0b
Moncut	100a	75a	545.5a	100a	88.9a	597a
Trianum	97.2a	88.9a	676.5a	100a	97.2a	722a
R. irregularis	100a	100a	733.5a	100a	97.2a	882a
Control +[y]	100a	91.7a	676.5a	100a	86.1a	627a
Control -[z]	0b	0b	0b	0b	0b	0b

[x] Disease parameters were evaluated twice a week on the basis of a 0 to 2 rating scale for the period of the trials and are expressed as percentages of the total plants. DI=disease incidence, M= mortality, AUDPC=area under disease progress curves assessed by plotting the number of diseased plants per bag versus time, with the trapezoidal method (Madden et al. 2007). Values in columns followed by different letter indicate significant differences ($P \leq 0.05$).
[y] Inoculated control not treated with any product.
[z] Non-inoculated control not treated with any product.

Cultivar susceptibility evaluation

To evaluate the susceptibility of zucchini squash cultivars to *F. solani* f. sp. *cucurbitae*, two trials were conducted: one started during the last week of April 2009 (first trial)

and the other during the first week of May 2011 (second trial). The experiments were arranged as a randomized complete block design with three replicates. Each experimental unit within a block consisted of two bags of perlite with three plants per bag, thus there were 18 plants per treatment in each trial. The cultivars studied were 14 of the most commonly used commercial cultivars in the province: Milenio (Semillas Fitó S.A., Barcelona, Spain); Cónsul, Otelo, Platinum, and Senator (Seminis Vegetable Seeds, St. Louis, MO); Capea, Cora, Tosca, and Sinatra (HM-Clause, Davis, CA); Candela (Semillas Fitó S.A.); Cronos (Syngenta Seeds, Basel, Switzerland); Cassiopée (Gautier Semences, Eyragues, France); Natura (Enza Zaden Beheer B.V., Enkhuizen, The Netherlands); and Vesul (Semillas Fitó S.A.). Seeds were directly sowed in the bags, and plants were inoculated at the 2 to 3 true leaf stage, as previously described. Plants were monitored twice a week for the presence of disease symptoms. DI, M, and the AUDPC were calculated per cultivar as previously described. After the experiments concluded, 10% of the symptomatic plants were randomly selected for pathogen reisolation.

Soil solarization and biosolarization

For the efficacy evaluation of soil solarization and biosolarization in “enarenado” soil, viability of propagules of *F. solani* f. sp. *cucurbitae* contained in a portion of soil was assessed after being exposed to 6-week solarization periods in a greenhouse in the summers of 2012 and 2013. For preparing infested soil samples, methodology was adapted from Cebolla and Maroto (2005). Two hundred milliliters of a conidial suspension were transferred to 200 g of sterile talc to promote the transformation of conidia and mycelia to chlamydospores due to desiccation. After 17 days, the dried talc was added to 3.36 liters of a blend containing 33% siliceous sand, 33% natural sandy loam soil, and 33% peat moss (Comercial Projar S.A., Valencia, Spain) that was previously filtered through a #20 sieve (840-µm openings) and autoclaved for 1 h at 121°C and 1 atm for 3 consecutive days. The mixture was then thoroughly homogenized. Samples were prepared by placing 40 ml of this mixture into polyester and polypropylene 100-µm mesh sacks and sealed with tie wraps. Samples were then

buried in the different blocks at two different depths, 10 to 15 cm and 30 to 35 cm respectively. In addition, 24 sample bags were conserved at 4 to 8°C to serve as controls: 12 samples to assure the viability of the fungus and the detection method and another 12 soil samples without the addition of the pathogen.

Solarization and biosolarization were performed with a factorial design with two factors (disinfestation treatment and depth) and two blocks. Each block was composed of three treatments (solarization, biosolarization, and a nonsolarized control) and the two depths, 10 to 15 cm and 30 to 35 cm. Each experimental unit consisted of a 5 × 10-m plot composed of six subsamples per depth and treatment. Solarization and biosolarization were performed during 45-day periods by completely closing the ridge and side windows of the greenhouse and covering the different 50-m^2 plots with transparent 50-µm-thick polyethylene plastic. Control plots were left uncovered. Biosolarization was conducted by adding organic matter consisting of fresh pepper plant debris at 5.4 kg/m^2 and 5 kg/m^2 during the first and second years, respectively. Organic matter in the second year was reduced due to European and local limitations on nitrogen applications to soil. The soil sand covering was removed prior to the addition of organic matter, which was homogenized with a rototiller, and then the sand was redistributed before irrigating with water at 60 $liters/m^2$.

After the solarization period, soil samples were recovered from soil for colony-forming units (CFU) quantification. Propagule viability was assessed by suspending a portion of the soil samples contained in the bags in Komada's semiselective medium. Fifteen milliliters of Komada medium (Komada 1975) cooled to 50°C were poured in 90-mm Petri dishes, and then an aliquot of approximately 30 to 35 mg of each soil subsample was transferred and dispersed by gentle rotation before the medium solidified (Tello et al. 1991). Three replicates per soil subsample were assayed. Plates were incubated at 25°C, as previously described, and after 3 to 7 days of incubation, all the colonies were counted and checked to verify that they corresponded to *F. solani*, and a concentration of CFU per gram was estimated.

Statistical analyses

Kruskal-Wallis nonparametric analyses of variance (ANOVA) tests were conducted for each of the experiments, of which the percentage of symptomatic plants per treatment (DI), mortality rate (M), AUDPC, and RAUDPCs were analyzed with Statistix 9 (Analytical Software, Tallahassee, FL) due to data non-normality and variance heterogeneity. The different repetitions of the experiments were included in the model and assayed for differences. In cases where ANOVA revealed no significant differences between experiments, data were pooled prior to analyses. For the solarization and biosolarization experiments, a Kruskal-Wallis ANOVA was performed by comparing the number of CFU per gram estimated per treatment. In all cases, if ANOVA results were significant for main effects, treatment means were compared using all-pairwise comparison tests ($P \leq 0.05$).

RESULTS

Pathogen survival assessment in bags of perlite

During the summer months, at 2 mah, temperatures ranged from 17.4 to 47.8°C with a mean of 29.7°C in the greenhouse environment, and from 23.8 to 42.5°C with a mean of 31.9°C inside the bags of perlite. Means of the maximum daily temperatures achieved were 41.7°C in the greenhouse environment and 38.1°C in the substrates. Among plants, 97.2% showed disease symptoms, and, subsequently, 70.8% of the plants were dead. In the control bags, 14.3% of the plants showed disease symptoms at the end of the cropping system.

At 8 mah, temperatures ranged from 8.6 to 39.8°C with a mean of 17.5°C in the greenhouse environment, and from 10 to 36.4°C with a mean of 19.5°C in the substrate bags. Means of the maximum daily temperatures achieved were 27.8°C in the greenhouse environment and 24.2°C in the substrates. In this after-harvest interval, 91.7% of the plants showed disease symptoms, resulting in 87.5% plant death after 71 days of growth. The incidence in this period was slightly lower than that at 2 mah; nevertheless, this decrease was not statistically significant. In the control bags, 95.2%

of the plants remained asymptomatic, and 4.8% of the plants showed leaf wilting and crown rot.

The following spring months (March to June) and the summer months (June to September) then comprised 14 mah. During the spring months, temperatures in the greenhouse environment ranged from 8.6 to 44.9°C with a mean of 22.8°C, and from 16.1 to 42.7°C with a mean of 26.6°C in the substrate bags. Means of the maximum daily temperatures achieved were 34.6°C in the greenhouse environment and 33.6°C in the substrates. During the summer months, temperatures in the greenhouse environment ranged from 18.0 to 45.9°C with a mean of 29.7°C, and from 24.7 to 43.9°C with a mean of 32.7°C, in the substrate bags. Means of the maximum daily temperatures achieved were 41.6°C in the greenhouse environment and 40.4°C in the substrates. After a month of growth, no disease symptoms of Fusarium crown and foot rot were observed in any of the plants. During this crop there was a high outbreak of a newly described virus to this area, *Tomato leaf curl New Delhi* virus (ToLCNDV, Juárez et al. 2014). The incidence of this virus was very high, affecting all the plants and strongly reducing their development and fitness. This fact could have an implication for the lack of crown and foot rot symptoms detected in these plants, and for this reason bags corresponding to 14 mah were reevaluated together with 20 mah.

At 20 mah, temperatures ranged from 5 to 40.7°C with a mean of 16.9°C in the greenhouse environment, and from 8.5 to 36.4°C with a mean of 18.1°C in the substrate bags. Means of the maximum daily temperatures achieved were 25.9°C in the greenhouse environment and 21.7°C in the substrates. By 79 days after sowing, only 13.8% of plants showed wilting symptoms for plants corresponding to 14 mah-b and only 1.4% at 20 mah. After evaluating the crown rot, the number of diseased plants increased from 13.8 to 22.2% of the plants for 14 mah-b and from 1.4 to 26.3% of the plants for 20 mah. There was no plant death in the 14 mah-b group or at 20 mah. Control plants showed no disease symptoms. Final DI data are presented in Figure 1.

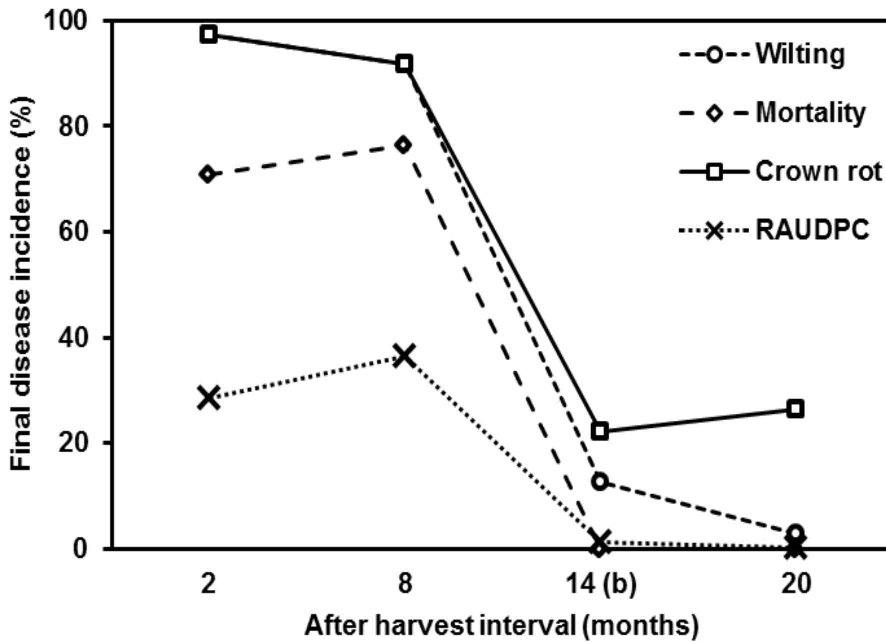

Fig. 1. Final disease incidence curves for each after harvest interval on perlite bags infested with *Fusarium solani* f. sp. *cucurbitae* expressed as the percentage of plants showing final wilting, mortality, crown rot and relative area under disease-progress curves (RAUDPC). Data corresponding to 14 (b) months after harvest were obtained by re-evaluating this plot at 20 months after harvest (mah) due to the incidence of ToLCNDV that strongly affected plant growth and development at 14 mah.

Statistical analyses did not reveal significant differences between 14 mah-b and 20 mah, but the DI was statistically lower (P=0) than at 2 mah and 8 mah. After 8 mah, a reduction in disease development was observed. Compared with 2 mah and 8 mah, aerial disease symptoms were delayed at 14 mah and 20 mah. While at 2 mah and 8 mah disease symptoms were first observed 2 weeks after sowing, at 14 mah-b and 20 mah they were observed 9 weeks after sowing (Fig. 2). Consequently, the RAUDPC for 2 mah and 8 mah was significantly higher than the RAUDPC for 14 mah-b and 20 mah (Fig. 1). For 2 mah, 8 mah, 14 mah-b, and 20 mah, the pathogen was successfully recovered from all plants selected for reisolation.

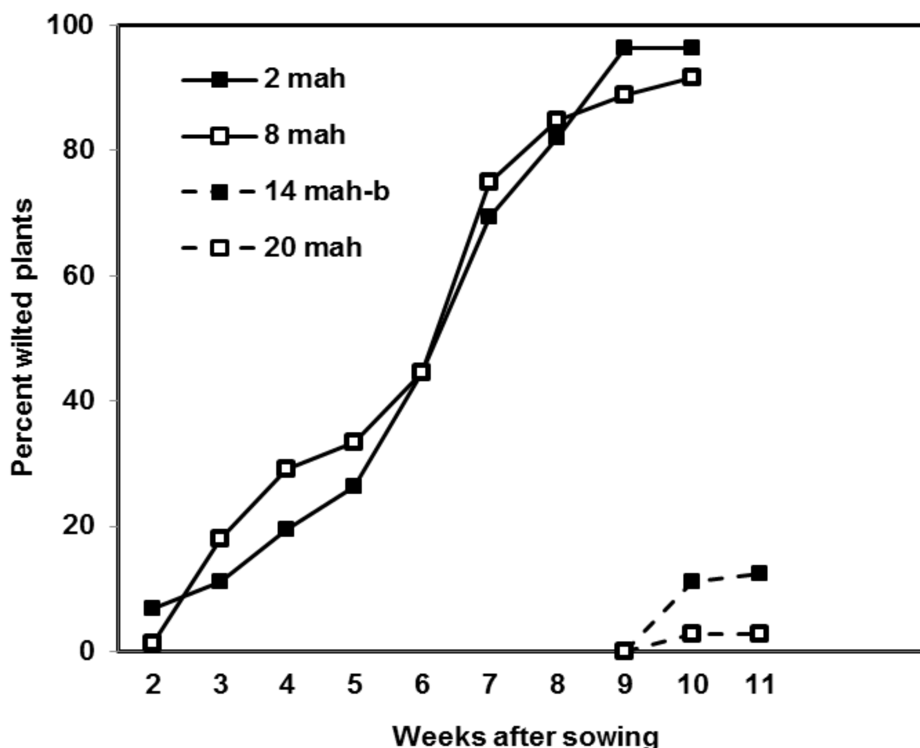

Fig. 2. Disease incidence on zucchini plants grown in previously artificially infested perlite bags for the different after-harvest intervals where bags were left unused expressed as percentage of wilted plants per week after sowing. Data corresponding to 14 mah-b were obtained by re-evaluating this plot at 20 mah due to the incidence of ToLCNDV that strongly affected plant growth and development at 14 mah.

Fungicide efficacy greenhouse evaluation.

In the first trial, temperatures in the greenhouse environment ranged from 6.4 to 34.9°C with a mean of 18.6°C, and from 14.3 to 24.5°C with a mean of 20.8°C in the culture substrates. In the second trial, temperatures ranged from 12.5 to 44.1°C with a mean of 27.4°C in the greenhouse environment, and from 11.9 to 46.1°C with a mean of 27.9°C in the substrates. Disease symptoms were first observed at 14 and 13 dai for the first and second trials, respectively. Noninoculated controls and plants treated with prochloraz or carbendazim remained asymptomatic (DI=0%), and only 2.8% of the plants treated with thiophanate-methyl in the first trial showed disease symptoms. There were no statistical differences among these three treatments. All the inoculated controls showed disease symptoms, and the DI of the rest of the treatments ranged

from 97.2 to 100% (Suppl. Fig. 1), with no statistically significant differences compared with the inoculated controls (Table 1). In both trials, the pathogen was successfully recovered from all plants selected for reisolation.

Suppl. Fig. 1. Appearance of the greenhouse at the end of the second fungicide efficacy evaluation experiment, 35 days after inoculation. A. Plot treated with etridiazole; B. Plot of plants treated with thiophanate-methyl; C. Non inoculated control plot.

Cultivar experiments

In the first trial, zucchini squash plants were maintained in the greenhouse for 53 days, during which temperatures ranged from 12.6 to 47.1°C with a mean of 25.7°C in the greenhouse environment, and from 14.1 to 35.9°C with a mean of 27.2°C in the substrates. The first disease symptoms were observed at 14 dai. At 18 dai, only the cvs. Otelo, Platinum, Capea, and Tosca remained asymptomatic and, at 21 dai, only the cv. Capea was asymptomatic. At 23 dai, all cultivars had a 100% DI, with the exceptions of cvs. Cónsul, Otelo, and Platinum, which had a DI of 94.4%, and cv. Capea, which had a DI of 77.8%. These differences were not statistically significant (P=0.26).

In the second trial, plants were kept for 52 days, during which temperatures ranged from 14.1 to 42.9°C with a mean of 24.6°C in the greenhouse environment, and from

13.6 to 45.3°C with a mean of 26.9°C in the substrates. In this experiment, disease symptoms where first observed at 12 dai, when every cultivar presented some diseased plants, with the exceptions of cvs. Platinum and Tosca, which remained asymptomatic. Three days later, every cultivar showed disease symptoms in at least some of the plants, and by 41 dai, 100% of the plants of all cultivars exhibited disease symptoms (Table 2). Statistical analyses revealed differences between the two experiments performed. In both trials, the pathogen was successfully recovered from all plants selected for reisolation.

Table 2. Disease parameter values of 14 zucchini squash cultivars inoculated with *F. solani* f. sp. *cucurbitae* in greenhouse experiments during the two trials conducted

Cultivar	First Trial			Second Trial		
	DI (%)[a]	M (%)[b]	AUDPC[c]	DI (%)	M (%)	AUDPC
Milenio	100	100	393.5 a	100	100	442 ab
Consul	94.4	94.4	368 ab	100	94.4	396 ab
Otelo	94.4	88.9	286.5 abc	100	100	401.5 ab
Platinum	94.4	61.1	196.5 bc	100	100	338.5 b
Senator	100	55.6	286 abc	100	100	415 ab
Capea	77.8	22.2	96.5 c	100	94.4	445.5 ab
Cora	100	94.4	245.5 abc	100	100	437 ab
Tosca	100	100	247.5 abc	100	100	365.5 ab
Sinatra	100	66.7	322 abc	100	100	376.5 ab
Candela	100	100	352 a	100	100	457.5 a
Cronos	100	94.4	364.5 a	100	100	451.5 ab
Cassiopee	100	100	337.5 ab	100	100	418 ab
Natura	100	100	303.5 abc	100	100	445.5 ab
Vesul	100	100	306 abc	100	94.4	414.5 ab

[a] DI=Disease Incidence expressed as the percentage of the total number of plants.
[b] M=Mortality.
[c] AUDPC=Area Under Disease Progress Curves assessed by plotting the rating on the basis of a 0 to 2 scale versus time, with the trapezoidal method (Madden et al. 2007). Values in columns followed by different letters indicate significant differences ($P \leq 0.05$).

Solarization and biosolarization

An increase in the temperature of the solarized and biosolarized plots was achieved during both years compared with the control plots, which did not have plastic mulching. In 2012, while control plots had maximum absolute temperatures under 45°C, solarization treatment resulted in plots measuring a maximum absolute temperature of 52.7°C, and biosolarization treatment resulted in plots measuring 49.8°C. In 2013, two depths were measured. At 10 to 15 cm, the maximum absolute temperature reached 47.1°C in the solarization plots and 44°C in the biosolarization plots. At 30 to 35 cm, temperatures reached 42.1°C in the solarization plots and 41.6°C in the biosolarization plots. Control plots had temperatures measuring below 40°C at both depths (Table 3).

Table 3. Temperature data during the solarization and biosolarization six-week periods in the summers of 2012 and 2013 measured in the different treatments, buried at different depths and exposed to the solarization and biosolarization periods[z]

	Year 2012						Year 2013					
	Mean		Maximum Mean		Absolute Maximum		Mean		Maximum Mean		Absolute Maximum	
	10-15 cm	30-35 cm	10-15 cm	30-35 cm	10-15 cm	30-35 cm	10-15 cm	30-35 cm	10-15 cm	30-35 cm	10-15 cm	30-35 cm
Solarization	44.1	n/a	49.5	n/a	52.7	n/a	42.1	39.5	45.1	40.1	47.1	42.1
Biosolarization	43.3	n/a	47.0	n/a	49.8	n/a	40.7	39.0	42.1	39.4	44.0	41.6
Control	39.5	n/a	42.4	n/a	44.5	n/a	36.9	35.9	38.3	36.2	39.8	37.9

[z] Temperatures were measured with Campbell Temperature probes P-108 and registered each 10 minutes with a Campbell CR10X datalogger. Temperature probes were buried at 10 to 15 cm. In 2013, additional temperature probes were buried at 30 to 35 cm. Mean=mean temperatures for the whole period; Maximum Mean=mean of the maximum daily temperatures; Absolute Maximum=Maximum temperature registered for the whole period; n/a: not available.

The analysis of the soil samples stored at 4 to 8°C as positive controls revealed a high inoculum concentration in the soil contained in the bags with the samples. Propagules were detected in a range of 1.6 to 3.7 × 10^3 CFU/g in 2012 and 1.8 to 4 × 10^3 CFU/g in 2013. No propagules were detected in the negative controls.

The analysis of the solarized, biosolarized, and control samples compared with the positive controls showed a high reduction in the detection rates of the pathogen. The pathogen could not be detected in any of the solarized or biosolarized samples in 2012, but it was detected in two out of 48 samples at the 30 to 35-cm depth measured in 2013. One sample corresponded to solarization treatment that presented a 97.35% reduction (77 CFU/g), and the other corresponded to biosolarization treatment with a 99.4% reduction (17 CFU/g). Even in the control plots, the inoculum was substantially reduced. Two samples in 2012 had estimated pathogen inocula reductions of 99.6% at 30 to 35 cm (9 CFU/g). In 2013, one sample at 10 to 15 cm showed a 99.5% reduction (12 CFU/g), and two samples at 30 to 35 cm showed 99.5 and 99.1% reductions (12 CFU/g and 24 CFU/g, respectively; Table 4). Statistical analyses indicated no significant differences in pathogen survival between solarization, biosolarization and the control plots (P=0.674).

Table 4. Pathogen survival of *Fusarium solani* f. sp. *cucurbitae* buried at different depths and exposed to the solarization and biosolarization periods [z]

	Year 2012				**Year 2013**			
	10-15 cm		**30-35 cm**		**10-15 cm**		**30-35 cm**	
	B1	**B2**	**B1**	**B2**	**B1**	**B2**	**B1**	**B2**
Solarization	0	0	0	0	0	0	0	**12.8**
Biosolarization	0	0	0	0	0	0	0	**2.8**
Control	0	0	**1.5**	**1.5**	0	**2**	**2**	**4**

[z] Number of CFU/g detected per sample after six-week solarization periods in artificially infested soils buried at 10 to 15 and 30 to 35 cm depth. Samples with the infested soil were placed in polyester fabric and polypropylene net bags and buried in plots at the start of the periods. Soil analyses were done by dispersing three aliquots of each soil sample on Komada's medium Petri plates. Values correspond to the average number of colonies counted per gram of soil of the six samples included on each replicate. There were not statistically significant differences among treatments (P=0.675). B=Block.

DISCUSSION

Results of the different experiments revealed severe disease development in adult zucchini squash plants grown in bags of perlite, when mean temperatures in the

substrates ranged between 20 and 28°C. These temperatures are optimal for the in vitro growth of *F. solani* f. sp. *cucurbitae* (Pérez-Hernández et al. *unpublished*), and this could explain why in our experiments there was such a rapid development of the disease after the first appearance of symptoms, resulting in the death of a high percentage of the plants. The lack of symptoms in most of the noninoculated plants in our experiments reveals the low aerial dispersal rate of the pathogen, and the success of the basic hygienic measures implemented in this soilless growth system to avoid disease dispersion through the greenhouse.

The experiments performed to study the survival of the fungus in the culture substrates revealed its resistance during July and August, when it was exposed to daily maximum temperatures averaging 38.1°C. Although the fungus was exposed to maximum temperatures greater than 42°C, there was no apparent loss of viability. In addition, plants continued to show severe disease symptoms for an additional six months, indicating the survival of a high number of propagules in the substrate bags for a total of eight months after the disease outbreak. At 14 mah, a large number of plants were affected by the recently detected *Tomato leaf curl New Delhi* virus (Juárez et al. 2014). This disease strongly affected plant development and fitness, and could have an implication for the lack of crown and foot rot symptoms detected in these plants. For this reason, at 20 mah we reevaluated the 14 mah specimens, and data corresponding to this group of bags were referred to as 14 mah-b. The presences of symptoms in the 14 mah-b and 20 mah groups indicates that there were likely surviving inocula at 14 mah. For 14 mah-b and 20 mah, aerial disease symptoms were observed in 12.5 and 2.78% of the plants, respectively. This DI was statistically lower than those observed at 2 mah and 8 mah, revealing an inoculum reduction in the bags of perlite 14 and 20 months after harvesting. An evaluation for crown rot in the 14 mah-b and 20 mah groups revealed necrosis and pathogen reisolation from some of the plants, indicating that some inocula remained; however, the quantity was not high enough to produce wilting after more than 60 days. These results are in agreement with previous observations by Nash and Alexander (1965) which revealed a large

decrease in *F. solani* f. sp. *cucurbitae* survival in artificial soils after 586 days, which is a shorter period than other *Fusarium* species, which could survive for up to 10 years in sterile soil (Nash and Alexander 1965; Windels et al. 1993). It is important to consider that part of the root systems were left in the bags, as this is common practice in the growing area due to the high cost and difficulty associated with completely removing them from the substrate. This fact could have extended the pathogen's survival, since some reports indicate that root tissue may reduce the effect of adverse soil conditions and provide protection to pathogens (English and Mitchell 1994), aiding the survival of long-lasting structures such as chlamydospores of *Fusarium* spp. (Sharma 2004; Leslie and Summerell 2007) or oospores of *Phytophthora cinnamomi* (Jung et al. 2013). Our results indicate that *F. solani* f. sp. *cucurbitae* is able to survive in the substrates after a disease outbreak, and that inoculum is significantly reduced after 14 months. Crop rotations with nonhost crops may be a good alternative way to avoid this disease. Tomatoes, peppers, eggplants, and beans are important crops commonly cultivated in Almería Province, and can be grown in different crop cycles and conditions under the same type of greenhouse structures. There are therefore many possible combinations that make rotations a viable alternative for growers.

The utilization of cultivars less-susceptible or resistant against certain diseases is a broadly implemented measure, and is recommended for integrated pest management strategies where possible (Jiménez-Díaz and Jiménez-Gascó 2011). In the first of the cultivar susceptibility trials, cultivar Capea appeared to be less susceptible to the disease than the others tested, with a final DI of 77.2% and M of 22.2%. However, this result was not repeated in the second experiment, where 100% of the plants showed disease symptoms. The statistical analyses revealed differences between experiments; however, these differences do not seem to be due to temperature, since mean temperatures in the substrates were similar in both experiments. The other zucchini squash cultivars tested in these experiments were very susceptible to crown and foot rot caused by *F. solani* f. sp. *cucurbitae*. These results suggest that none of the tested cultivars are effective options for controlling the disease. Therefore, the search for

resistant or tolerant zucchini squash accessions should continue due to the aggressive nature of this pathogen to zucchini and other cucurbits, including melon, watermelon, cucumber, and *Cucurbita* spp. rootstocks (Pérez-Hernández et al. *unpublished*).

With respect to the fungicide efficacy evaluation, three of the products tested, prochloraz, carbendazim, and thiophanate-methyl, were highly effective at controlling disease up to 40 dai. No disease symptoms were detected in any of the plants treated with these products, with the exception of one plant treated with thiophanate-methyl in the first trial. This efficacy is consistent with previous studies carried out in vitro, where *F. solani* growth was totally inhibited in culture media supplemented with carbendazim and thiophanate-methyl (Ben Salem et al. 2011; Sultana and Ghaffar 2010). However, this is the first study to demonstrate the efficacy of these fungicides against this disease under greenhouse conditions with adult plants. In contrast, the other tested fungicides and biological products showed no significant differences in disease severity compared with the nontreated inoculated controls, suggesting that they are ineffective for the control of Fusarium crown and foot rot of zucchini squash. The use of chemical products is subjected to constant regulatory modifications. Recently, the use of carbendazim has been banned in the European Union and the United States, among other countries. Prochloraz and thiophanate-methyl are currently included in the Annex 1 list of permitted products in the European Union (Regulation 1107/2009/EC). Both products are currently labeled for their use in Spain and other countries in the European Union to control diseases caused by *F. oxysporum* in watermelon, cucumber, and melon. However, its use is not currently allowed in zucchini squash, probably because of the lack of other *Fusarium* diseases affecting it in Spain. Our results demonstrate the interest of the registration of prochloraz and thiophanate-methyl for the control of Fusarium crown and foot rot of zucchini squash, as they are used in several other cucurbits.

Soil solarization and biosolarization effectiveness in greenhouses depends on a great variety of factors, such as incoming solar radiation, soil humidity, and temperatures reached. In Almería Province, these techniques are frequently performed in summer,

when the high temperatures are difficult for the cultivation in the greenhouses of most horticultural crops. Our experiments were performed in the months of July and August, keeping the greenhouse completely sealed for 45 days. The analysis of the samples exposed to solarization, biosolarization, and the nontreated control, revealed that closing the greenhouse windows during the hottest months even without the addition of a plastic mulch had a significant impact on the pathogen's survival, and that temperatures reached in the soil were high enough to reduce the population of *F. solani* f. sp. *cucurbitae.* In addition, the application of plastic mulch increased the temperatures measured in the soil compared with the nontreated control. However, the effect on pathogen's survival of this temperature increase could not be validated with these experiments, in contrast to what was previously observed in similar experiments with other pathogens such as *F. oxysporum* ff. spp. *radicis-lycopersici* and *melonis,* and *Pythium aphanidermatum* (Pérez et al. 2014).

Several measures can be applied for the control of crown and foot rot of zucchini squash caused by *F. solani* f. sp. *cucurbitae.* We tested several control strategies including host resistance, chemical, and biological control, and soil solarization and biosolarization. Based on our results, the most promising management strategy would be the application of prochloraz or thiophanate-methyl to the stems of the plants. However, because their use is not currently allowed in zucchini squash, manufacturers should consider registering them for their use in this crop. At least two years of crop rotation without cucurbits is also expected to be effective. Although soil solarization and biosolarization have proven to reduce inoculum in the soil, additional research is needed to document their efficacy to control the disease in greenhouse conditions.

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Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not necessarily imply recommendation by the IFAPA.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis has focused in the study of Fusarium crown and foot rot of zucchini caused by *Fusarium solani* f. sp. *cucurbitae*, one of the most important soilborne diseases affecting zucchini production worldwide. Our main goals were to study the incidence and distribution of the disease in zucchini and other cucurbits grown in Almería Province, the characterization of the causal agent *Fusarium solani* f. sp. *cucurbitae* and the evaluation of several methods for disease control.

Before the 2007 outbreak in Almería (Gómez et al. 2008), the incidence of this disease in cucurbits in Spain was restricted to the Valencian Community, where it had been described several years before causing disease in pumpkin plants and in *Cucurbita* hybrids used as rootstocks for growing watermelon (García-Jiménez *et al.* 1997). However, as our survey indicate, the disease has spread throughout El Campo de Dalías, the main growing area in Almería Province, becoming the most important soilborne disease affecting zucchini. Although our main focus was to study the disease in zucchini, we were also interested in studying the incidence in other cucurbit crops in our area. We only found a watermelon greenhouse affected by this disease and also a melon greenhouse. This was the first time that *F. solani* f. sp. *cucurbitae* was detected in commercial melon crops in Spain (Gómez et al. 2014).
Our disease survey started in 2007, when the pathogen was first reported in Almería, and continued for a total of 6 years, until 2012. During the following years after the first outbreak, the disease spread throughout the province, being 2011 the year when we received more notifications of infected greenhouses. This sudden outbreak happened in the province of Almería in 2007, after over 40 years of intensive zucchini production in greenhouses, suggests a possible introduction of the pathogen through seeds followed by its dissemination throughout the area.

In addition to *F. solani* f. sp. *cucurbitae*, we detected in the survey *Phytophthora capsici* L. for the first time causing root rot and wilting in adult plants in Almería, being this one the first report of the disease in Spain. This is an important fact, as there are

several areas in the region well-known for suffering recurrent problems of *P. capsici,* which has been traditionally considered a pathogen affecting mainly pepper crops in our area (De Cara et al. 2017, De Cara et al. 2018, Gomez et al. 2013, Herrero et al. 2002). The confirmation of the pathogenicity of *P. capsici* to adult zucchini plants is a valuable information for growers affected by the pathogen, who should take it into account in order to avoid the incidence of the disease in their crops.

The several experiments performed in greenhouse conditions in adult zucchini plants revealed the high virulence of this *forma specialis*. Our results showed that this fungus was able to cause disease in over 94% of the inoculated plants in all cases except in the cultivar Capea in the first trial to evaluate the susceptibility of several cultivars against the disease, and the mortality rates were in all the experiments over 50% with the same exception. According to our expectations, all the cucurbits tested were also susceptible to the disease, nevertheless, cucumber plants behaved as more tolerant. In the first experiment to study the host range of the fungus, we didn't detect visible wilting or foot rot in cucumber plants after 31 days post-inoculation. However, when we analyzed their root systems, we observed that all of them had a severe rot under the soil layer, mainly in the crown. The development of adventitious roots and the lack of fruit setting by that time probably let plants remain visibly asymptomatic. For this reason, we decided to repeat the experiment in 32-liter perlite bags, in order to let them grow for a longer time. The tendency in this experiment was the same, as the cucumber plants remained asymptomatic for a longer time, showing no aerial symptoms when all the other cucurbits had disease incidences over 80%. However, when the plants started to show disease symptoms, these evolved very quickly reaching mortality rates over 75% for the three cultivars tested by 60 dai, 30 days after the first wilted plant was observed. In several cases the disease led to a swift death of the plants, passing few days between the first aerial disease symptoms and the plant death (data not shown).

According to our results, we can affirm that high temperatures favor disease development. There were two greenhouse experiments that were executed in two

consecutive trials: the pathogenicity of several isolates to zucchini, and the fungicide experiments. In both cases, the first trial was performed during the winter-spring season, and the second immediately after in the spring-summer season. In the two experiments, temperatures measured were higher in the second trials, when the disease evolved much faster in both cases. This fact is in agreement with our field observations in the survey, since the incidence of the disease was in most cases observed in spring-summer crops, and only few cases in autumn crops.

By the time of starting this thesis, it was considered that there existed two races within *F. solani* f. sp. *cucurbitae:* the race 1, which was pathogenic to both cucurbit plants and fruits; and the race 2, which could only cause disease in cucurbit fruits which were in contact with the soil, and was observed mainly in pumpkin fields (Tousson and Snyder 1961). In this context, experiments with fruit inoculations were performed to study the ability of the isolates to infect zucchini fruits. All the isolates were able to grow in the fruits, colonizing the surface and producing a high amount of aerial mycelium, but also producing rot in the tissues under the fruit epidermis. Remarkably, the only isolate which was not pathogenic to zucchini plants, PCl-2121, was the one producing the highest growth and rot in the fruits. The phylogenetic analyses identified this isolate as *F. keratoplasticum*, an opportunistic pathogen which has been recently identified in numerous countries as a causal agent to ocular keratitis diseases mainly in immunocompromised patients together with other species within the FSSC (Short et al. 2013). In a recent study of pea and legume diseases (Sisic et al. 218), a strain of *F. keratoplasticum* isolated from winter vetch (*Vicia villosa)* was weakly pathogenic to pea. Several studies indicate that keratitis caused by different *Fusarium* species occurs especially among farmers and workers with agricultural occupations, existing several reported cases of corneal lesions happened during harvest or while handling decayed and dried plant debris (Al-Hatmi et al. 2014, Chander and Sharma 1994, Homa et al. 2013, Polack et al. 1971). Our findings together with these reports remark the potential of plant and agricultural debris as a source of fusarial keratitis in humans, which may result in loss of eyesight and require corneal transplants (Short et al. 2013).

Traditional species description methods are based in morphological characters. There are several differences observed between the characters of our isolates comparing with the traditional *F. solani* description. Our *F. solani* f. sp. *cucurbitae* isolates have long phialides as described in *F. solani,* however these are frequently branched and verticillate, character that to our knowledge, has not been previously described for this species. In addition, macroconidia in the *forma specialis cucurbitae* are long and slender, mostly 5-septate, while in *F. solani* they are relatively short and robust, mostly 3-septate (Schroers et al. 2016). These differences were already reported in a study comparing *F. solani* isolates obtained from sweet pepper plants and *F. solani* f. sp. *cucurbitae* isolates from the Netherlands (Paternotte 1987). We have also observed long chlamydospore chains formed intercalary or terminally in the mycelium, which are not described for this fungal species.

Respect to *Fusarium* nomenclature, before 2012, pleomorphic *Fusaria* (those that produce both sexual and asexual stages) were assigned different names for both the teleomorph (perfect or sexual) state and the anamorph (imperfect or asexual) state of their life cycles, and it was stated that the teleomorph name had preference when referring to the holomorph (the whole fungus including both stages). In 2011, a modification in the International Code of Botanical Nomenclature stipulated that only one scientific name should be used for a fungal species, and resulted in the abolishment of this dual nomenclature (McNeil et al. 2012, Summerell 2019) and stated that all names, whether they were typified by an anamorph or a teleomorph, would be on equal footing in terms of priority (McNeil et al. 2012), allowing scientists to conserve anamorph names such as *Fusarium* (Geiser et al. 2013). In 2013, several researchers working in this genus got to a consensus and proposed to use the famous anamorph name *Fusarium* for species belonging to this genus in order to facilitate communication between the scientific community and create nomenclatural stability (Geiser et al. 2013). Countering this proposal since then have been a number of publications recommending splitting the genus into a number of genera based on phylogenetic considerations as well as the morphology of the sexual structures (Summerell 2019). For example, Lombard et al. (2015) proposed that the FSSC be

redefined as *Neocosmospora* species based on morphology of the group, especially the sexual structures, and DNA phylogenetic placement, and only retain the name *Fusarium* for members previously assigned to a *Gibberella* teleomorph. These changes have been controversial within researchers who didn't follow this idea and preferred to maintain the name *Fusarium* for species belonging to FSSC in recent descriptions (e.g. Al-Hatmi et al. 2018, Aoki et al. 2018, Dalle-Rosa et al. 2018, Sisic et al. 2018, Sisic et al. 2018b, Papizadeh et al. 2018). Recently, the same research team (Sandoval-Denis et al. 2019) published a reappraisal of the FSSC and reassigned 68 species as *Neoscosmospora*, including *F. solani* f. sp. *cucurbitae* as *N. cucurbitae.*

Our phylogenetic studies carried out demonstrate that *F. solani* f. sp. *cucurbitae* consists in a monophyletic group within clade 3 of FSSC, corresponding to a unique phylogenetic species with a common evolutionary origin. The phylogenetic inference based in *EF 1-α* indicate that all our isolates are highly similar among them, including the isolates available in GenBank from the United States. However, the analysis of the *RPB2* or the combined dataset phylogenies reveal the possibility of the existence of two different linages, one including some of the Spanish isolates together with the isolates from the United States, and another group that could have diverged from this one in Spain, since it only contains Spanish isolates. The data that was available for this study, however, is not enough for being able to get to this conclusion, and more isolates from different years and geographical origins would be required for a deeper population study. Both unique morphological characters we observed in the isolates studied as well as their monophyletic origin revealed in the phylogenetic analyses, support the elevation of the *forma specialis* to species rank, however, we believe there is no consensus with the reassortment of the FSSC species complex as *Neocosmospora*, and we are in favor of maintaining this species within the genus *Fusarium* until such a time that there is consensus of view in agreement to what was recently stated by Summerell (2019). For this reason, we consider that the most appropriate name for this species would be *Fusarium cucurbitae.*

After studying the Fusarium crown and foot rot of zucchini, and characterizing its causal agent, the second main objective of this thesis was to study several methods for the control of the disease. For this, the first step was to study the survival of the fungus in substrate bags after the incidence of the disease. The available literature on this subject is very limited. Although there are plenty of divulgate articles or fact sheets on the disease which recommend crop rotation for two or three years with non-host plants as a control measure, there is only one study performed in 1965 by Nash and Alexander where they compare the ultrastructure of chlamydospores of *F. solani* f. sp. *cucurbitae* and *F. solani* f. sp. *phaseoli*. The authors concluded that *F. solani* f. sp. *cucurbitae* showed chlamydospores with a thinner wall than f. sp. *phaseoli,* and therefore they were less well suited for long term survival. Based on these results, we decided to study the survival of the fungus in artificially infested perlite bags, the substrate we normally use for our adult plant greenhouse tests. In our experiments, the fungus was able to survive over 20 months after removing the infested plants from the bags, however, the fungal viability was severely affected after 8 months. It is important to remark that during the third period to evaluate the survival of the fungus there was a high incidence of ToLCNDV which severely affected the crop producing severe stunting, leaf curl, chlorosis and leaf deformation. During this crop, no wilting or necrosis was observed in the plants, which could have been a consequence of the defense response initiated in the plant upon the infection with the virus. For this reason, we repeated the evaluation of these perlite bags in the fourth period. In this evaluation, the first disease symptoms appeared later comparing with the first two periods, and the severity of the disease was also lower, however, the disease was visible in both groups of bags, demonstrating the viability of the fungus 20 months after harvesting the plants.

Genetic resistance is one of the most effective methods for disease control caused by *Fusarium* or several other diseases. To get an idea of the level of susceptibility of zucchini crops in our area to *F. solani* f. sp. *cucurbitae,* 14 of the most popular cultivars were selected. Our experiments demonstrated that all cultivars were highly susceptible to the disease, and no resistant or tolerant varieties were identified. In the last

decades, seed companies and public research institutions have released commercial varieties of several cucurbits harboring genes that confer them resistance to several *formae speciales* and races, mainly of *F. oxysporum*. However, despite the many reports of *F. solani* f. sp. *cucurbitae* in many different areas around the world, to our knowledge, there are no resistant varieties of any cucurbit to this pathogen. It would be desirable that seed companies and public institutions tried to identify resistances in order to breed resistant varieties available for growers, providing environmentally-friendly and effective means for disease control.

Another interesting technique for controlling soilborne diseases in cucurbits is grafting on non-host species. A common example is grafting of watermelon plants on *Cucurbita* rootstocks to avoid *F. oxysporum* f. sp. *niveum.* This technique, when properly performed is highly effective for controlling the disease and is widely implemented in many areas, including Almería Province. Our host range experiments have demonstrated that the fungus affects also the most common *Cucurbita* rootstocks, discarding this technique as a possible control measure to avoid the disease.

Three fungicides, prochloraz, carbendazim and thiophanate-methyl were effective for the control of the disease when applying them two weeks before inoculation simulating a preventive treatment, and again two days after inoculation simulating a curative treatment. In the case of the three products, plants remained asymptomatic with the exception of a plant treated with thiophanate-methyl in the first experiment. Despite of the efficacy of these products, none of them is currently labeled for their use in zucchini. Th use of carbendazim has recently been banished in the European Union and the United States, however, prochloraz and thiophanate-methyl are registered for their use in other horticultural and cucurbit crops for controlling *Fusarium* diseases. Probably these products are not labeled for zucchini because no other *Fusarium* diseases associated to this crop have been described in our country. Respect to the biological control agents tested, *Trichoderma harzianum* and *Rhizophagus irregularis,* they were not able to control or reduce the disease, as there were no statistical differences with the infected, non-treated control. It is important to

remark that the tests were performed in perlite bags with fertigation, and these conditions may not the best for the fungal colonization. Plants were grown in a nutrient-rich solution which probably difficult the fungal colonization of the root system in the case of *R. irregularis* as this is more effective in low phosphorous rhizospheres (Grant et al. 2005).

There are several reports demonstrating the efficacy of soil solarization and biosolarization for soil disinfestation. However, there are few of them studying this technique in Almería, where most growers perform their crops in a system called "enarenado". This system consists in the addition of a 10-cm sand layer above the soil surface with the main objective of water use optimization. Since this method is based in temperature increase of the soil, it is a possibility that the sand layer may have a buffer effect in the soil heating, and therefore make the technique less effective. Our results demonstrated that both techniques were very effective in reducing the inoculum in the soil. Even in the case of the control plot, which was inside the greenhouse but with the plastic covering it, the inoculum was severely reduced, in contraposition with our observations in similar experiments with *F. oxysporum* ff. spp. *radicis-lycopersici* and *melonis,* and *Pythium aphanidermatum*, which were able to survive in control plots (Pérez et al. 2014). These results agree with the observations of Nash and Alexander and seem to corroborate that *F. solani* f. sp. *cucurbitae* has a lower ability to survive in the soil after a disease outbreak comparing to other *Fusaria,* fact that makes its management easier for growers affected with the disease.

Based in our results, we can conclude that there are several effective measures for the control of the Fusarium crown and foot rot of zucchini caused by *F. solani* f. sp. *cucurbitae.* Our recommendation would be implementing an integrated pest management strategy consisting in the use of pathogen-free seeds, together with several prophylactic measures to avoid the introduction of the pathogen from other infected lands, and once introduced, to avoid pathogen dispersal within the crop. Once the disease is detected, it would be advisable to apply prochloraz or thiophanate-methyl in the crop, but it would be necessary the previous authorization of the product

by the European Commission. After the crop finishes, we recommend performing a solarization or biosolarization during the summer months, and if possible, planting a non-host crop after to avoid the multiplication of the few inocula that may be left after the soil disinfestation.

CHAPTER VII

CONCLUSIONS

CONCLUSIONS

1. *F. solani* f. sp. *cucurbitae* has spread within the province of Almería, being detected for the first-time causing disease in melon plants in Spain. It has become the main soilborne disease affecting zucchini production in the region and has also been detected in watermelon. The pathogen was not detected in any diseased cucumber crop (Chapters II, III and IV).

2. The *F. solani* f. sp. *cucurbitae* isolates tested were very aggressive to zucchini, being most of the isolates able to cause aerial disease symptoms in all inoculated plants and produce high mortality rates. The disease was favored with higher temperatures (Chapters II and V).

3. *F. solani* f. sp. *cucurbitae* was pathogenic to all the cucurbits tested, and it was not to the rest of the plants belonging to other families. Among the cucurbits tested, cucumber was significantly less susceptible to the disease (Chapter II).

4. The morphological characters of the pathogenic isolates studied revealed important differences respect to the classical description of *F. solani*, primarily the production of long and slender macroconidia, the production of branched and verticilate monophialides, and the production of long chains of chlamydospores (Chapter II).

5. The phylogenetic studies reveal a monophyletic group including all the pathogenic isolates together with accessions from the United States indicating a common evolutionary origin of the *forma specialis,* being *F. solani* f. sp. *piperis* its closest ancestor (Chapter II).

6. Based on the phylogenetic data and the morphological differences between *F. solani* and *F. solani* f. sp. *cucurbitae* the latter could possibly be elevated to species rank within FSSC as *Fusarium cucurbitae* (Chapter II).

7. The pathogen was not detected in any of the commercial seeds tested (Chapter V).

8. *F. solani* f. sp. *cucurbitae* survived in perlite bags for 20 months after harvesting the previous crop, although its survival was significantly reduced after 14 months (Chapter V).

9. The 14 cultivars tested resulted highly susceptible to the disease (Chapter V).

10. Prochloraz, carbendazim and thiophanate-methyl resulted highly effective in controlling the Fusarium crown and foot rot of zucchini, however, these products are not registered for their use in zucchini in the European Union. The rest of the fungicides and biological agents tested resulted ineffective (Chapter V).

11. Soil solarization and biosolarization resulted highly effective in reducing the inoculum from the soil after 6 weeks. Even in the control plots not covered with a plastic, the amount of pathogen propagules was reduced (Chapter V).

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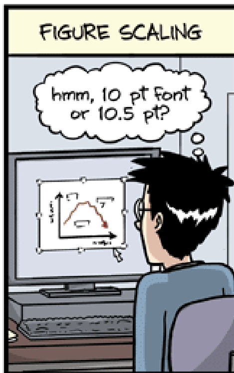

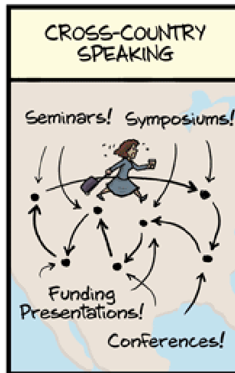

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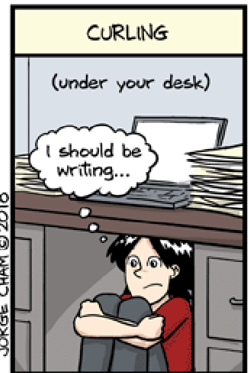

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