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Departamento de Agroquímica
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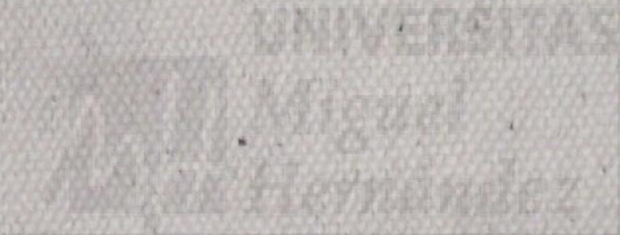
Molecular diagnosis and biological control of Phytophthora root rot in pepper and Fusarium wilt in muskmelon



Josefa Blaya Fernández

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Diagnóstico molecular y control biológico de la tristeza seca del pimiento y la fusariosis vascular del melón

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Josefa Blaya Fernández

Trabajo realizado para obtener el grado de Doctor
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Departamento de Conservación de Suelos y Agua
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CENTRO DE EDAFOLOGÍA Y BIOLOGÍA
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CERTIFICAN,

Que la presente Tesis Doctoral titulada, "Diagnóstico molecular y control biológico de la tristeza seca del pimiento y la fusariosis vascular del melón" ha sido realizada por Dª. Josefa Blaya Fernández, bajo nuestra dirección y supervisión, en el Departamento de Conservación de Suelos y Agua y Manejo de Residuos Orgánicos del CEBAS-CSIC, para la obtención del Grado de Doctor por la Universidad Miguel Hernández de Elche.

Y, para que conste a los efectos oportunos, firman el presente certificado en Murcia a 27 de Febrero de 2015

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DA SU CONFORMIDAD a la lectura de la tesis doctoral titulada: "Diagnóstico molecular y control biológico de la tristeza seca del pimiento y la fusariosis vascular del melón" presentada por Dª. Josefa Blaya Fernández.

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Many of life's failures are people who did not realize

how close they were to success when they gave up

Thomas Edison 1847-1931

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Abbreviations

Lists including the most used abbreviations

ABA	Abcisic acid
ADN	Deoxiribonucleic acid
BCA	Biological control agent
BDL	Below detection limit
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumine
CECT	Colección española de cultivos tipo
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxiribonucleic acid
dNTP	Dinucleotide
dPCR	Digital polymerase chain reaction
EC	Electrical conductivity
FAM	6-carboxyfluorescein
FOM	*Fusarium oxysporum* f.sp. *melonis*
GC-MS	Gas chromatography-mass spectrometry
JA	Jasmonic acid
ISR	Induced systemic resistance
ITS	Internal transcribed spacer

MGB	Minor grove binder
NAGase	N-acetyl-β-glucosaminidase
NCBI	National Center of Biotechnology Information
ND	Non-detected
NMR	Nuclear magnetic resonance
NS	Not specified
OTU	Operational taxonomic unit
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PLFAs	Phospholipid fatty acids profile
PR	Pathogenesis-related
qPCR	Real time polymerase chain reaction
RDP	Ribosomal database project
RFLP	Restriction fragment length polymorphism
SA	Salicylic acid
SAR	Systemic acquired resistance
SNP	Single nucleotide polymorphism
TOC	Total organic carbon

Preface

The present Doctoral Thesis entitled "Molecular diagnosis and biological control of Phytophthora root rot in pepper and Fusarium wilt in muskmelon" has been written according to the rules included in "Normativa de la UMH para la presentación de tesis doctorales como un conjunto de publicaciones" and "Normas para la obtención de la Mención de Doctor Internacional según R.D. 99/2011".

This work includes different parts:

- Abstract summarizing this Doctoral Thesis
- A review of literature (Chapter 1)
- The aims and objectives of study (Chapter 2)
- The compendium of articles included in the Doctoral Thesis (Chapter 3)
- A general discussion of the main results achieved (Chapter 4)
- Conclusions obtained from this Doctoral Thesis (Chapter 5)
- References cited in Chapter 1, 2 and 4 (Chapter 6)

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Abstract

The southeast Spain is localized in an area with an important agricultural sector that is well-known for its high production of pepper and melon. These two crops are affected by Phytophthora root rot and Fusarium wilt, respectively. An early detection of the causal agents of these diseases is of paramount relevance to avoid their expansion in new fields and, to improve the management strategies for their control. For this reason, we developed a TaqMan system in order to detect and quantify *Phytophthora nicotianae* specifically by real time PCR (qPCR) in pure culture and environmental matrices such as soil, compost and plant tissues. With this system, we identified *P. nicotianae* as the current causal agent of Phytophthora root rot in "El Campo de Cartagena" and we characterized its population through the use of mitochondrial molecular markers and phenotypic traits. Moreover, the application of the TaqMan system along with a new approach, namely digital PCR (dPCR), proved to be an attractive alternative to quantify *P. nicotianae* accurately. The dPCR was not only able to detect the presence of low levels of *P. nicotianae* in environmental samples, but was also less susceptible than the qPCR to inhibitors present in the DNA plant extracts.

We also evaluated the ability of a batch of composts made from agro-industrial waste to control Fusarium wilt and Phytophthora root rot under greenhouse nursery conditions. The results showed positive correlations between enzymatic activities such as NAGase, chitinase and protease regarding Fusarium wilt suppression, and dehydrogenase activity related to Phytophthora root rot suppression. Besides, a deeper study was carried out in

order to elucidate the mechanisms involved in Phytophthora root rot control. This was accomplished studying the physical and chemical characteristics of composts, including their organic matter composition, and analyzing their metagenome and metabolome. We observed that the involvement of certain antagonistic microbes such as *Streptomyces*, *Bacillus*, *Zopfiella* or *Fusarium* in the phenomenon of disease suppression was related to the degree of stabilization of the materials and thus, to the microbial activity achieved. These parameters provided an array of outcomes whose integration may be a promising approach to improve not only the characterization but also the identification of suppressive composts.

Furthermore, vineyard pruning waste compost amended with *Trichoderma hazianum* showed better results controlling Fusarium wilt than the same compost without this BCA and compared with peat. The control exerted by *T. harzianum* itself as well as the changes induced by its presence in the bacterial community and in some chemical parameters may explain these results. The use of composts fortified with certain strains of BCA can be a good alternative controlling Fusarium wilt of melon seedlings at greenhouse nursery level. The use of *in vivo* tests to characterize the suppressive effect of composts was vital to obtain accurate conclusions due to the inability of *in vitro* tests to provide them.

Resumen

El sureste español se encuentra localizado en un área que destaca por su importante sector agrícola, reconocido por su alta producción de cultivos como el pimiento y el melón, los cuales se ven afectados por enfermedades causadas por patógenos del suelo, como la tristeza y la fusariosis vascular respectivamente. Una detección temprana de los agentes causantes de estas enfermedades es de gran importancia, para evitar su expansión a nuevos terrenos y mejorar su control mediante una gestión integrada. Por esta razón, desarrollamos un sistema TaqMan para detectar y cuantificar *Phytophthora nicotianae* específicamente mediante PCR a tiempo real (qPCR) en cultivo puro y en matrices ambientales como suelo, compost y tejido vegetal. Con este sistema se identificó *P. nicotianae* como el actual causante de la tristeza del pimiento en el "Campo de Cartagena" y su población fue caracterizada mediante el uso de marcadores moleculares y un estudio fenotípico. Además, la aplicación del sistema TaqMan junto con la nueva técnica de la PCR digital (dPCR), ha mostrado ser una alternativa interesante para cuantificar *P. nicotianae* con gran precisión. La PCR digital no solo fue capaz de detectar bajos niveles de *P. nicotianae* en muestras ambientales, sino que también mostró ser menos susceptible que la qPCR a los inhibidores presentes en las extractos de ADN de plantas.

Por otra parte, evaluamos la capacidad de un grupo de compost de origen agroindustrial para controlar la fusariosis vascular del melón y la tristeza del pimiento bajo condiciones de semillero. Los resultados mostraron correlaciones positivas entre las actividades enzimáticas NAGasa, chitinase y proteasa con el control de la fusariosis vascular, y entre la actividad

dehydrogenasa y la supresión de la tristeza del pimiento. Con el objetivo de clarificar los mecanismos de los compost involucrados en la supresión de la tristeza del pimiento, se llevó a cabo un estudio más profundo donde se analizaron las características físicas y químicas de los compost, incluyendo la composición de la materia orgánica, y también, su metagenoma y metaboloma. La involucración de ciertos antagonistas como *Streptomyces*, *Bacillus*, *Zopfiella* o *Fusarium* en el fenómeno de supresión estuvo influenciada por el grado de estabilización de los materiales y por tanto, los niveles de actividad microbiana observados. La integración de los resultados obtenidos mediante estas técnicas podría ser de gran interés para mejorar la caracterización e identificación de compost supresivos.

Además, compost de poda de vid enmendados con *Trichoderma harzianum* mostraron mejores resultados para controlar la fusariosis vascular del melón en comparación con el mismo compost sin enmendar y la turba. El control ejercido por *T. harzianum per se* así como los cambios inducidos por su presencia en la comunidad bacteriana y en algunos parámetros químicos del compost, podría explicar los resultados obtenidos. El uso de compost fortificados con ciertas cepas de ACBs puede ser una buena alternativa para controlar la fusariosis vascular en semillero. El uso de test *in vivo* para caracterizar la capacidad supresividad de los compost fue vital para obtener conclusiones realistas debido a la incapacidad de los test *in vitro* para proporcionarlas.

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I

Chapter I. Introduction

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I. INTRODUCTION

1. Socioeconomic importance of pepper and muskmelon

Pepper, *Capsicum annuum*, is a plant included in Solanaceae family which is cultivated around the world. The production of pepper is leaded by Mexico followed by Turkey and China (FAOSTAT, 2011). Spain is the first pepper producer in Europe and the fifth in the world. The trade market of pepper in Spain is also of high relevance since today Spain is the second country in pepper exportations, preceded by Mexico (Figure 1). Aproximately 50% of the pepper produced in Spain (921,089 tonnes) is exported, and the rest is mainly sold on the market for fresh products. The production of pepper in Spain is concentrated in the southeast, which comprises more than 63% of the total production, mostly localized under greenhouses. Almeria with 7 300 ha and a production of 470,000 tonnes, is the main producer region, followed by Murcia with 1,300 ha and 111,000 tonnes (Table 1).

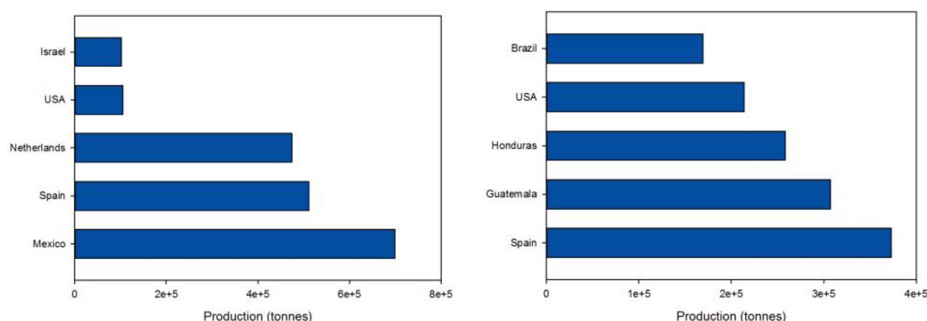

Figure 1. Exportations of the five main exporting countries of pepper (left) and melon (rigt) (FAOSTAT, 2011)

Murcia, which production is concentrated in El Campo de Cartagena, has specialized its production to be competitive in the market. Pepper recolection in Murcia starts in March-April and finishes in July or August, which is complementary to the collection time in Almeria. It is worth mentioning that during the last 10 years, pepper cultivation is a mono-crop in 90% of the greenhouses in “El Campo del Cartagena” (Guerrero, 2012).

Table 1. Cultivated area, yield and production data of pepper in Spain in 2011 (MAGRAMA, 2012)

Region	Cultivated area (ha)	Yield (kg ha^{-1})	Production (tonnes)
Cantabria	10	16,000	160
P. de Asturias	18	60,000	260
Madrid	30	25,000	750
Aragón	178	40,685	2,449
Balerares	87	64,300	2,548
La Rioja	172	77,000	4,351
País Vasco	299	56,271	4,915
Castilla-León	282	99,846	6,162
Cataluña	396	66,104	9,213
Canarias	165	131,731	10,078
Extremadura	471	284,891	19,230
Navarra	776	46,997	19,876
Comunidad Valenciana	654	100,102	38,549
Castilla-La Mancha	1,238	73,076	46,563
Galicia	1,337	117,050	70,259
Murcia	1,334	132,000	110,968
Andalucia	10,148	104,413	572,218
TOTAL	17,595	108,647	918,549

Nowadays, melon, *Cucumis melo* L., a plant within Cucurbitaceae family, is an important horticulture crop around the world. China is the main producer followed by Turkey and Iran (FAOSTAT, 2011). It is worthy mentioning that Spain is the main producer of melon in Europe with an annual production of 871,996 tonnes in 2011 (Table 2) as well as the main exportator of melon in the world (Figure 1). Around 40% is exported, the rest remaining mainly for the domestic market. In some regions of Spain, the relevance of melon market is even higher, since the production of melon is mainly localized in few of them: Castilla-La Mancha, followed by Murcia and Andalucia (Table 2).

Table 2. Cultivated area, yield and production data of melon in Spain in 2011 (MAGRAMA, 2012)

Region	Cultivated area (ha)	Yield (kg ha^{-1})	Production (tonnes)
Aragón	2	7000	14
La Rioja	6	20000	120
Navarra	8	30,000	240
Castilla-León	134	30,417	2,135
Balerares	132	52,000	2,208
Madrid	260	28,750	2,250
Canarias	146	74,252	3,738
Cataluña	349	27,607	6,277
Comunidad Valenciana	1,716	53,381	35,425
Extremadura	2,166	69,326	62,630
Andalucia	6,885	69,824	208,557
Murcia	5,989	96,500	219,774
Castilla-La Mancha	10,768	36,865	328,618
TOTAL	28,561	72,842	871,996

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2. Soil-borne plant pathogens: *Phytophthora nicotianae* and *Fusarium oxysporum* f.sp. *melonis*

Soil-borne fungi and oomycetes are the causal agents of many diseases that cause serious losses in a large number of vegetable crops from the initial stages of the production process up to harvest (Lievens *et al*., 2006; Colla *et al.,* 2012).

In the last decades, several plant-pathogenic *Phytophthora* species have caused huge damage to a wide range of agriculturally and ornamentally important crops as well as to landscape plants, forests and ecosysmtens (Wang *et al.,* 2011; Laurens *et al.,* 2012). Phytophthora root rot is considered the most potentially destructive disease of cultivated pepper (Pomar *et al.,* 2001), *Phytophthora capsici* being considered the main collar and root rot pathogen in Europe (Messiaen *et al.,* 1991; Erwin & Ribeiro, 1996; Pomar *et al.,* 2001; Lacasa *et al.,* 2010). In Spain, *P. capsici* along with *Phytophthora nicotianae* are the most destructive soil-borne pathogens of pepper crops (Nuez *et al.,* 1996; Pomar *et al.,* 2001; Andrés *et al.,* 2003; Rodríguez-Molina *et al.,* 2010).

On the other hand, *Fusarium* is a genus of filamentous fungi that contains many agronomically important plant pathogens, mycotoxin producers, and opportunistic human pathogens (Ma *et al*., 2013). In Europe, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *melonis* is one of the most significant diseases of melon and produces economically significant agricultural damage in the greenhouses and in field environments (Suárez-Estrella *et al.,* 2004; López-Mondéjar *et al.,* 2012).

2.1. *Phytophthora nicotianae*

It has been over 150 years since Anton de Bary first described *P. infestans* (1876 cited in Tucker *et al.,* 1933). Currently, 124 species have been described and, it is likely that the majority of species of *Phytophthora* have yet not been discovered (Kroon *et al.,* 2012). Lately some species that have been discovered are: *Phytophthora ramorum* (Wesses *et al.,* 2001), causing high mortality of oak trees in Californa (USA) (Rizzo *et al.,* 2002), and *Phytophthora alni*, responsible for a lethal root and collar rot of alter species in Europe (Brassier & Kirk, 2004). The species of this genus are classified within Peronosporaceae family, Peronosporales orden, Peronosporea class, and in the Oomycota division of Chromista kingdom (Index Fungorum, 2014).

We want to highlight the case of *Phytophthora nicotianae* van Breda de Haan (=*Phytophthora parasitica* Dastur (1896)), which stands out among plant pathogens since it is a threat to plant productivity on a global scale for a broad range of hosts (Erwin & Ribeiro, 1996). The host range of *P. nicotianae* includes 255 plant genera in 90 families (Cline *et al.,* 2008). *P. nicotianae* has been described as a pathogen to pepper plants in different countries such as United States, India, Tunisia and Spain (Figure 2) (Tello, 1990; Bartual *et al.,* 1991; Allagui *et al.,* 1995; Andres *et al.,* 2003; Rodriguez-Molina *et al.,* 2010; Blaya *et al.,* 2014a).

This oomycete, as well as other *Phytophthora* species, is heterothallic and has two mating types, A1 and A2. Both mating types are required to produce sexual spores, called oospores which are able to survive in the soil or in decomposing plants for several years and thus constitute highly persistent

conservation structures (Weste, 1983). Moreover, *P. nicotianae* can persist in the soil as chlamydospores in the absence of a susceptible host (Erwin & Ribeiro, 1996).

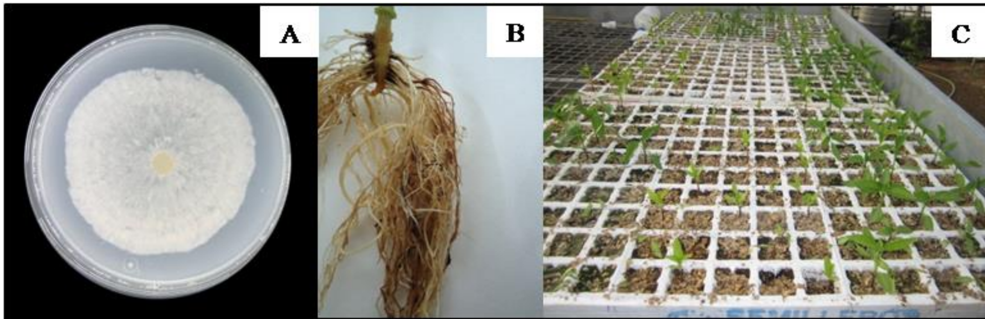

Figure 2. Phytophthora root rot. A) Mycelial growth of *Phytophthora nicotianae* in PDA; B) Phytophthora root rot symptoms (dry necrosis in root and stem); C) Effect of *Phytophthora nicotiane* in pepper seedlings at nursery level.

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As we have just mentioned above, *P. nicotianae* seems to be the current causal agent of Phytophthora root rot in pepper plants in southeast Spain (Murcia) (Guerrero *et al.,* 2012; Blaya *et al.,* 2014a). However, *P. capsici* has been considered the causal agent in this area to date. Due to the similarities of symptoms and their morphology, *P. capsici* and *P.nicotianae* may cause diagnostic confusion. Diseases caused by both*, P. nicotianae* and *P. capsici*, may initially occur in areas where water accumulates. Mature sporangia can directly germinate or, when immersed in water, release motile zoospores that travel with water in fields. Once zoospores contact the plant surface, they encyst and germinate to produce germ tubes (Hickman, 1970; Hausbeck & Lamour, 2004). The pathogen penetrates the plant through the

base of the stem, causing first necrosis in that area. At the same time, the plant becomes withered while the fungus advances through both the stem and the root, causing obstruction in the vascular system. Infected tissues turn brown to black in color and become dry, sunken, with death at the last stage of the infection (Silvar *et al.,* 2005). In contraposition to *P. capsici*, *P. nicotianae* never affects fruits or leaves (Darine *et al.,* 2007). Lately, both species have been included in a top-ten list of plant-pathogenic oomycetes based on their scientific and economic importance (Kamoun *et al*., 2014).

The control of Phytophthora root rot begins with the production of disease-free seedlings, since most of *Phytophthora* associated with root rot in the field probably originates from infested nursery stock. Thus, while preventing the introduction of the pathogen is optimal, once it is introduced, several control measures need to be used in a comprehensive management program to reduce losses from disease (Hausbeck & Lamour, 2004). Whereas crop rotation is an important foundation of disease management, the long-term survival of oospores in absence of a host limits the effectiveness of this strategy as a stand-alone tool. The oospore survival has been clearly demonstrated with a number of *Phytophthora* species, including *P. capsici* (Bowers *et al.,* 1990; Hausbeck & Lamour, 2004). Controlling excess moisture in greenhouses or in fields is another cultural control technique used since water is the single most important component to the initial infection and subsequent spread of zoospores (Hausbeck & Lamour, 2004).

The most traditionally used fungicides in agriculture target chitin and sterol synthesis, being inefficient against oomycetes since their cell walls are primarily composed of β-1,3- and β-1,6 glucanes, and of cellulose (a β-1,4-

glucane). Besides, oomycetes are unable to synthesize sterols (Attard *et al.,* 2001). Phytophthora root rot management is based on phenylamide fungicides but fungicide-tolerant strains have been detected (Veloso & Díaz, 2012). In the nursery industry, mefenoxam and propamocarb are two premier compounds used for Phytophthora disease management (Hu *et al.,* 2007). Grafting has proved to confer resistance to *P. nicotianae* and *P. capsici* in peppers (Hamdi *et al.,* 2010; Colla *et al.,* 2012). However, all these means of control (chemical control, soil fumigation, cultural practices, crop rotation) are expensive and not always effective.

2.2. *Fusarium oxysporum* f.sp. *melonis*

Fusarium oxysporum Schlechtend:Fr. is an asexual fungus distributed worldwide (Lievens *et al.,* 2008). However, only a small proportion of the total soil population has pathogenic activity (Tello & Lacasa, 1990). Pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in a large number of hosts (Nelson, 1981). In addition, over 150 *formae speciales* have been described within this species based on the host specificity. *Fusarium oxysporum* f. sp. *melonis* W.C. Snyder & H. N. Hans (FOM) causes Fusarium wilt of muskmelon (*Cucumis melo* L.) which is considered the most severe infectious disease of this cucurbit (Figure 3) (Kim *et al.,* 1993; Luongo *et al.,* 2012). This species is classified in Nectriaceae family, Hypocreales orden, and in Sordariomycetes class within Ascomycota division (Index Fungorum, 2014).

Moreover, four races (0, 1, 2 and 1,2) of this *forma specialis* have been identified based on pathogenicity to a set of differential cultivars within *Cucumis melo* (Risser *et al.,* 1976). Race 1, 2 has been subdivided further

into races 1, 2w (wilt) and 1, 2y (yellows) based on the symptoms they induce (Bouhot, 1981). While the disease was first reported in New York in 1930, and is now widespread globally, the four races of the pathogen are not uniformly distributed throughout all melon producing regions (Mirtabeli *et al.,* 2013).

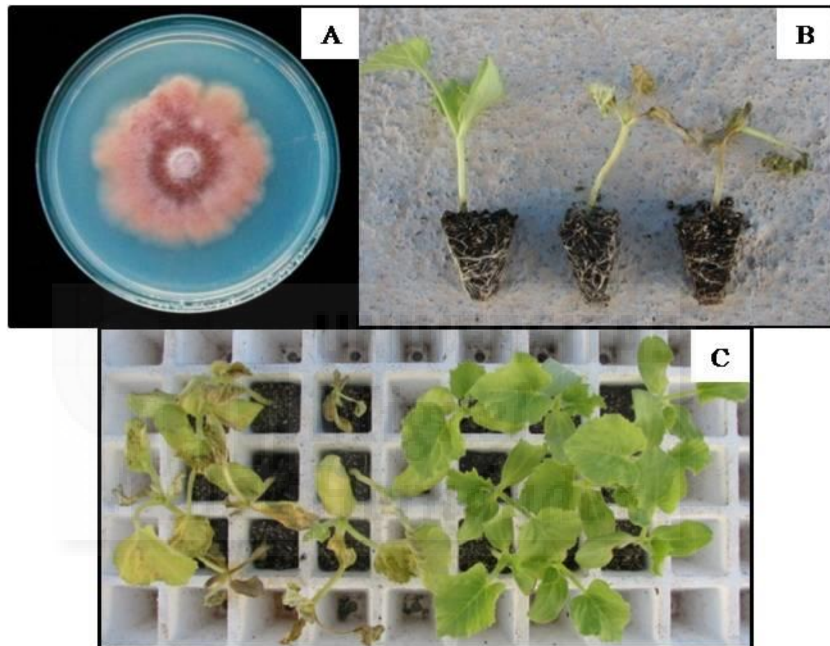

Figure 3. Fusarium wilt. A) Mycelial growth in PDA of *Fusarium oxysporum* f.sp. *melonis*; B) Detail of wilting and necrotic leaves; C) Fusarium wilt symptoms in muskmelon seedlings.

This pathogen can enter the host through root tips, primarily in the area of elongation, and is aided by wounding (Martyn & Gordon, 1996). In the first stages of the disease, the fungi develop slowly by mycelium, which passes from one xylem tube to the next through pits. Later, fungal conidia are carried by the transpiration stream in the xylem tubes. In advanced steps of the infection, the fungi spread into neighboring tissues of the xylem tubes

(Nelson, 1981). The presence of the fungi causes a reduction of the water and nutrient uptake of the infected plants. Symptoms usually appear as yellowing of leaves, wilting of branches, necrotic stems, followed by plant death. When the infection takes place close to harvest time, the quality and quantity of melon production is reduced (Martyn & Gordon, 1996) (Fig. 3b).

Fusarium wilt is very difficult to control because once introduced into a field, the fungus can survive in the soil as chlamydospores during extended periods even in the absence of the host roots or after the rotation with non-susceptible crops. *F. oxysporum* f. sp. *melonis* colonizes but does not cause disease on some non-host crops (Gordon *et al.,* 1989). The main sources of FOM inoculum include growing substrates, water and contaminated seeds (Weller *et al.,* 2008; Al-Sadi *et al.,* 2011). The conditions within greenhouse nurseries are optimal for the growth and spread of the fungus making difficult the control and eradication of this fungi (Gómez & Tello, 2000). The optimal soil temperature for penetration and infestation the host is 18-27 ºC (Martyn & Gordon 1996). Moreover, the transplantation of infected plants not exhibiting clear symptoms favors pathogen dispersal, making the elimination of *F. oxysporum* from the field environment extremely difficult (Suárez-Estrella *et al.,* 2004; Zhang *et al.,* 2005). For that, infected plants should be removed to prevent the spread of the disease (Lievens *et al.,* 2008).

Treatments of this fungus have included routine soil disinfection resulting in high economic inputs for the crop and microbiological degradation of soils (Arroyo-García *et al.,* 2003). Furthermore, after the phased out of methyl bromide, no effective curative treatments are available for controlling FOM infection (Lievens *et al.,* 2008). Acceptable levels of

disease control have been achieved with the use of resistant plant cultivars. However, breeding new cultivars for disease resistance is time consuming and does not guarantee durable resistance against new races of these pathogens (Park *et al.,* 2013).

3. Sustainable practices to control soil-borne plant disease

Soil-borne plant pathogens (fungi and oomycetes) continue to incite great losses to agricultural crops (Colla *et al.,* 2012). Different approaches may be used to prevent, mitigate or control plant diseases alone or in combination (Spadaro & Gullino, 2005). Over the past 100 years, chemical pesticides and fertilizers have been used to improve crop productivity and quality as well as to decrease crop and yield losses caused by plant pathogens and pests. However, the environmental pollution and human health problems caused by excessive use and misuse of agrochemicals, have led to considerable changes in people's attitudes towards the use of pesticides in agriculture, demanding healthy food with less chemical residues.

Nowadays, there are strict regulations on chemical pesticide use, and there is a political pressure to remove the most hazardous chemicals from the market. One of the most widely used pesticides which use was banned is methyl bromide (MeBr). Although alternatives chemical products (1,3-dicloropropene, metam sodium, dazomet and chloropicrin) were initially foreseen as possible replacement for MeBr, some of them have been already excluded in the re-evaluation of pesticides which took place under the European Directive 91/414/EEC on Plant Protection Products (Colla *et al.,* 2012; Gullino *et al.,* 2007). Consequently, the arsenal of available fumigants

has been dramatically reduced in the last years. Additionally, the European Regulation No 1107/2009 concerning the placing of plant protection products (PPPs) on the marker, and the European directive No 2009/128/EC, establishing the framework for Community action to achieve the sustainable uses of pesticides, requires, by 2014, that all professional users implement general principles for Integrated Pest Management. In this sense, non-chemical methods must be preferred for soil-borne disease management and the pesticides applied should have the least side effects on non-target organisms and the environment (Colla *et al.,* 2012).

In this context, in the last years most efforts have focused on developing alternative inputs for the application of non-chemical methods for controlling plant pests and diseases. These alternatives include biological control, cultural practices and organic amendments.

3.1. Biological control

In plant pathology, the terms “biological control” and its abbreviated synonym “biocontrol” have been applied to the use of microbial antagonists to reduce the amount of inoculum or disease producing activity of a pathogen (Cook & Baker, 1983). The organism that suppresses the pathogen is referred as the biological control agent (BCA). Biological control has become in an increasingly promising alternative to chemical control in the management of soil-borne diseases (Cook, 1993; Harman *et al.,* 2004).

Today, BCAs used in agriculture can be classified in two types. One group includes those microorganisms that are capable of controlling a large spectrum of taxonomically diverse pathogen host, including species of

Bacillus, *Pseudomonas*, *Streptomyces*, *Trichoderma*, *Clonostachys*, yeasts, etc. And the other group can counteract only one of few targeted pathogens which include biocontrol species of *Agrobacterium*, *Ampelomyces*, *Coniotrhyrium*, non-pathogenic Fusaria, atoxigenic *Aspergillus*, etc. (Woo *et al.,* 2014).

Currently, there is a limited number of biological control products available on the market, the situation varying depending on the country. In countries such as USA, Australia and New Zealand, the use of BCAs to control aerial and soil-borne plant pathogens is a widespread control method. However, in the European Union, only over fifty microorganisms have currently the status of approved in the Regulation (EC) No 117/2009, which was implemented on 14 June 2011 repealing the Directive 91/414/EEC (Table 3). The new Regulation contains the text of reference which regulates the use of plant PPPs, including chemicals as well as microbial biological control agents (MBCAs) (http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.selection).

The main differences between Europe and other countries, especially the USA, is the regulation. In EU, the placing of MBCAs is regulated the same way as the ones of chemical pesticides. However, the nature of hazards related to MBCAs and chemical pesticides are not the same, and the risks have to be assessed differently (Alabouvette *et al.,* 2011).

Table 3. Microbial control agents with status of approved under Reg. (EC) No 1107/2009 (repealing Directive 91/414/EEC).

Microbial control agent	**Strain**	**Use**
Adoxophyes orana GV	BV-0001	Insecticide
Ampelomyces quisqualis	AQ-10	Fungicide
Aureobasidium pullulans	DSM 14940, DSM 14941	Bactericide, Fungicide
Bacillus firmus	I-1582	Nematicide
Bacillus pumilus	QST 2808	Fungicide
Bacillus subtilis	QST 713	Bactericide, Fungicide
Bacillus thuringiensis subsp. *Aizawau*	ABTS-1857, GC-91	NS
Bacillus thuringiensis subsp.*Israeliensis* (serotype H-14)	AMS-52	NS
Bacillus thuringiensis subsp. *Kurstaki*	ABTS 351; PB 54; SA 11; SA 12; EG 2348	NS
Bacillus thuringiensis subsp. *Tenebrionis*	NB 176 (TM 141)	NS
Beauveria bassiana	ATCC 74040; GHA	Insecticide
Candida oleophila	0	Fungicide
Cyalia pomonella granulovirus CpGV	NS	Insecticide
Gliocadium catenulatum	J1446	Fungicide
Helicoverpa armigera nucelopolyhedrovirus	Hear NPV	Insecticide
Lecanicillium muscarium (formely *Verticillium lecanii*)	Ve6	Insecticide
Metarhizium anisopliae var. *anisopliae*	BIPESCO SIF52	Insecticide
Paecilomyces fumosoroseus Apopka	97	Insecticide
Paecilomyces fumogoroseus	Fe9901	Insecticide
Paecilomyces lilacinus	251	Nematicide
Phlebiopsis gigantean	(several strains)	Fungicide
Pseudomonas chlororaphis	MA342	Fungicide
Pseudomonas sp.	DSMZ 13134	NS
Pythium oligandrum	M1	Fungicide
Spodoptera exigua nuclear polyhedrosis virus	NS	Insecticide
Spodoptera littoralis nucleopolyhedrovirus	NS	Insecticide

Table 3 (continued)

Streptomyces K61 (formley *S. griseoviridis*)	NS	Fungicide
Streptomyces lydicus WYEC 108	NS	Bactericide, Fungicide
Trichoderma asperellum (formely *T. harzianum*)	ICCO12; T25; TV1	Fungicide
Trichoderma asperellum	T3T	Fungicide
Trichoderma atroviride (formely *T. harzianum*)	IMI 206040; T11	Fungicide
Trichoderma atroviride	I-1237	Fungicide
Trichoderma gansii (formely *T. viride*)	ICC080	Fungicide
Trichoderma hazianum	T-22; ITEM 908	NS
Trichoderma polysporum	IMI 206039	Fungicide
Verticillium allbo-atrum (formely *Verticillium dahliae*)	WC5850	Fungicide
Zucchini Yellow Mosaik Virus	Weak strain	Fungicide

NS: not specified.

Furthermore, the products not only have to satisfy toxicity tests but also demonstrate the efficacy of the preparation. The preparation should be evaluated in several experiments, in different geographic regions, and for two consecutive years (Alabouvette *et al.,* 2006).

3.1.1. *Trichoderma harzianum* as biological control agent

Fungal species of the genus *Trichoderma* have been widely used as BCAs in agriculture (Chet, 1987), *T. harzianum* being one of the most used and found very commonly in a wide variety of environments (Lorito *et al.,* 2010) (Fig. 4). The species of genus *Trichoderma* are classified within Hypocraceae family, Hypocreales order and in the Sordariomycetes class of Ascomycota division (Index Fungorum, 2014).

The potential of *Trichoderma* spp. as BCAs of plant diseases has culminated in the commercial production of several *Trichoderma* species for the protection and growth enhancement of a number of crops in the United States, India, Israel, New Zealand, and Sweden (Howell, 2003). It is considered that about 60% of all the registered biofungicides worldwide are *Trichoderma* based (Verma *et al.,* 2007).

The success of *Trichoderma* strains as BCAs, and specifically of *T. harzianum*, is due to several properties of these fungi. Their rapid growth allows them to directly **compete for space and nutrients** with phytpathogens (Hjeljord & Tronsmo, 1998). Moreover, their high efficiency in nutrient utilization and capacity to modify growing media characteristics allow them to adapt to recalcitrant organic matter (Benitez *et al.,* 2004). It is known that *T. harzianum* secrete iron chelating siderophores that limit the availability of irong for the germ-tube growth of *F. oxysporum* (Verma *et al.,* 2007). *T. harzianum* is also able to produce a high amount of antimicrobial metabolites that either impedes spore germination (**fungistasis**) or kill the cells (**antibiosis**) of pathogens. Furthermore, their ability to synthesize hydrolytic enzymes, toxic compounds and/or antibiotics could result in a direct interaction between the pathogen itself and the BCA, as in **mycoparasitism**. The possible role of chitinolytic enzymes in biocontrol was supported by the work of Lorito *et al.,* (1998), who transferred the gene encoding endochitinase from *T. harzianum* (P1) into tobacco and potato and demonstrated a high level and broad spectrum of resistance against a number of plant pathogens. *Trichoderma* BCAs have proved to be effective stimulating plant defense mechanisms and **inducing resistance** in the host

plant (Benítez *et al.,* 2004). In this sense, Yedidia *et al.,* (1999) demonstrated that inoculating roots of 7-day-old cucumber seedlings in an aseptic hydroponic system with *T. harzianum* (T-203) spores, initiated plant defense responses in both the roots and leaves of treated plants. Later, Yedidia *et al.,* (2000) showed that inoculation of cucumber roots with *T. harzianum* (T-203) induced an array of pathogenesis-related proteins, including a number of hydrolytic enzyme. Harman (2000) showed that seed treatment of corn planted in low nitrogen soil with *T. harzianum* (T-22) resulted in plants that were greener and larger in the early part of the growing season confirming the ability of these fungi to **promote plant growth.**

As it was mentioned before, one of the pitfalls in the use of BCAs, and therefore, one limitation of their commercial distribution as biopesticides, is the lack of consistence in the control of soil-borne pathogens. The combination of BCAs along with other management strategies, apart from improving the understanding of BCAs modes of action, may achieve promising results controlling plant pathogens (Pal & Gardener, 2006).

3.2. Management strategies contributing to biological control

Starting a crop from healthy seed, or healthy transplants as well as eliminating disease plants and leaning equipment before entering a new field are useful measures to prevent build-up of pathogen population (Alabouvette *et al.,* 2006). A good control of the irrigation system (timing, frequency, amount and mode of irrigation) may also improve the control of both foliar

and soil-borne diseases, above all the ones caused by *Pythium* and *Phytophthora* spp. (Katan, 2000).

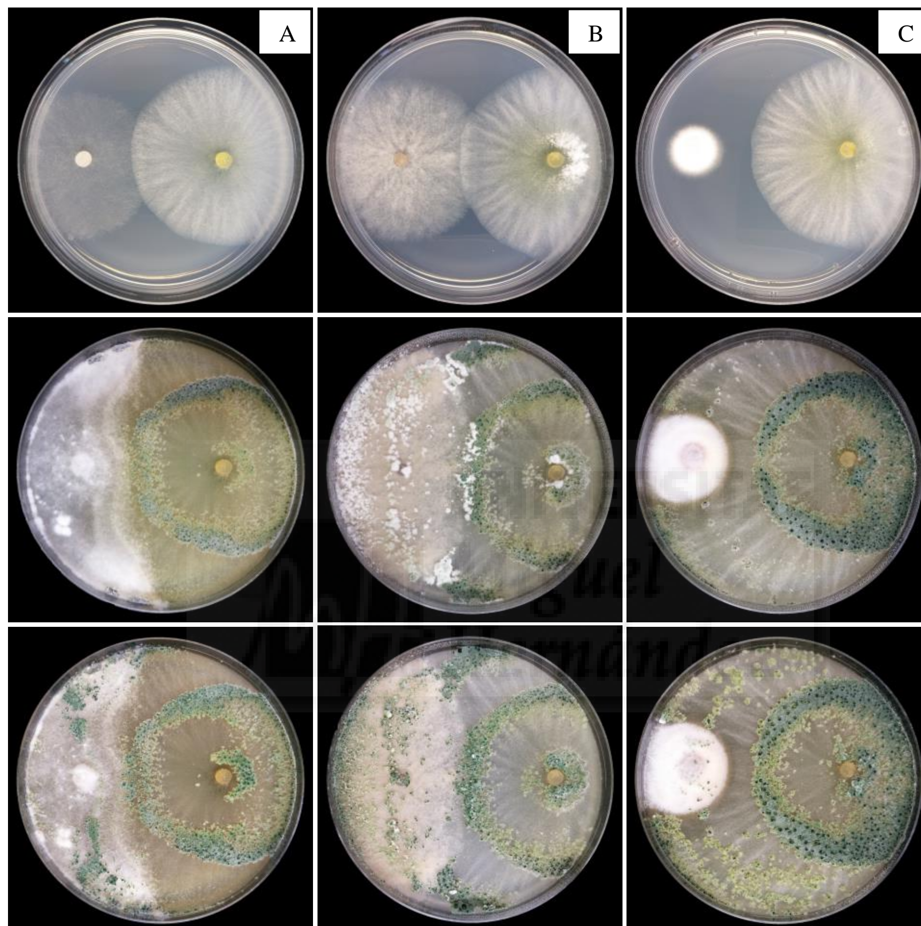

Figure 4. Dual confrontation plates with *Trichoderma harzianum* (right) against three plant pathogens (left). A) *Sclerotinia sclerotiorum*; B) *Botrytis cinerea*; C) *Fusarium oxysporum*.

Crop rotation is an effective technique for controlling plant pathogens (Cook, 1986) but today specialization in crop production and economic considerations force farmers to repeatedly grow the same plant species on the same land (Alabouvette *et al.,* 2006). Another control method

effectively used in warm and sunny areas is the **solarization** (Katan, 1996) which is based on heating the soil by solar energy. The heating is achieved by mulching the soil with transparent plastic controlling many pathogens and weeds. On the other hand, **biofumigation or biodesinfection** is based on plastic tarping of soil after amendment of fresh organic matter which results in the production of toxic metabolites and anaerobic conditions which both contribute to the inactivation or destruction of pathogenic fungi (Nuñez-Zofío *et al*. 2011, 2012). In some cases both methods, solarization and biofumigation, are combined in a technique named **biosolarization**, which improves the results obtained using both methods separately (Guerrero *et al*. 2006; Lacasa *et al*. 2010).

The use of **natural products** for the control of soil-borne pathogens is also considered an interesting alternative to synthetic fungicides due to their antimicrobial effect. Some of these products are plant and algal extracts, essential oils and chitin and its derivates (Demirci & Dolar, 2006; Xu *et al.,* 2007).

Grafting is used to reduce susceptibility against pests, root rots and wilts, and to increase yield (Rouphael *et al.,* 2010). In spite of the disadvantages associated with grafting, including the additional cost and physiological disorders due to incompatibility between rootstocks and scions, the use of resistant rootstock strongly has increased, mainly for crops such as tomato, bell pepper, and melon (Colla *et al.,* 2012). The use of **resistant cultivars** is an effective strategy since nowadays there are varieties which are resistant or at least tolerant to one or more pathogens. However, disease resistant can be overcome under good conditions for the development of the

disease. Besides, new races of the pathogen could appear (Lievens *et al.,* 2008).

Organic amendments and in particular, compost amendments have been used to improve the control of soil-borne pathogens (Bailey & Lazarovits, 2003). Composts have a high potential to be used for controlling soil-borne plant pathogens not only as amendments but also as container media. For this reason we will describe broadly the use of composts as an alternative to control soil-borne plant pathogens in the following section.

4. Composts for plant disease control

Composts are the final product of decomposition of organic matter due to the action of a succession of different microorganisms. Composts strongly differ depending on the composition and origin of organic wastes, e.g. municipal solid waste (Pascual *et al.,* 1999; Partanen *et al.,* 2010), agricultural waste (Lopez-Mondéjar *et al.,* 2010; Pane *et al.,* 2013), agroindustrial waste (Ntougias *et al.,* 2008; Blaya *et al.,* 2014b) or animal manures and slurries (Ros *et al.,* 2007; Bernal *et al.,* 2009).

The process of composting is divided into different phases: an initial phase, a thermophilic phase and the cooling or stabilization phase. The initial of the composting process takes place when the temperature rises from ambient to 40 or 45 ºC (Cook & Fahy, 1986). This initial phase is followed by the thermophilic phase, characterized by an increase of the temperature till 60-70 ºC. Most of weed seeds and pathogens are killed during this phase (Hadar & Papadopoulou, 2012). When temperature declines, the cooling

phase starts and the microorganisms re-colonize the pile (Hadar & Papadopoulou, 2012).

The need of alternative control methods for soil-borne fungal and oomycetes plant pathogens has prompted the use of composts due to their ability to suppress a wide array of plant diseases, their low cost and limited environmental effects (Hoitink & Fahy, 1986; Martin, 2003). Composts have been commonly used to control plant diseases caused by soil-borne pathogens, such as *Pythium* spp. (Pascual *et al.,* 2002), *Fusarium* spp. (Ros *et al.,* 2005) or *Phytophthora* spp. (Blaya *et al.,* 2014b) (Table 4 and Table 5). Suppressive composts provide and environment in which plant disease development is reduced, even when the pathogen is favored by the presence of a susceptible host (Hadar & Papadopoulou, 2012). However, no all composts are suppressive, this feature being dependent on the activities of extant antagonistic microorganisms, plant host, pathogen species involved and the characteristics of compost (Bonanomi *et al.,* 2006). Moreover, the ability of composts to suppress phytopathogenic agents varies, not presenting a consistency against various pathogens. Termorshuizen *et al.,* (2007) found disease suppression in 54% of all cases, no significant suppression in 42.7%, and disease enhancement in 3.3%.

Table 4. Examples of composts and compost water extracts from various organic wastes that have been used to control *P. capsici* and *P. nicotianae* in different crops.

Compost type	Pathogen	Use	Crop	Suppressive effects	References
Sewage sludge and wood chips	*Phytophthora capsici*	Soil amendment (10%)	Pepper (*Capsicum annunm*)	Reduced significantly disease severity	Lumsden *et al.*, 1983
Sewage sludge and wood chips	*P. capsici*	Amendment into steam-sterilized soil in three rates (55, 110 and 165 t ha^{-1})	Pepper	Increased Phytophthora root and crown rot	Kim *et al.*, 1997
Chitin compost (30% crab shell, 20% vermiculite, 40% rice straw, 10% rice bran) mixed with soil (compost:soil 99.9:0.1, w/w) containing chitinase producing bacteria	*P. capsici*	Soil amendment (30% of compost) in pots	Pepper	Lower root mortality of plants grown in the composts amended with the chitin source	Chae *et al.*, 2006
Six composts made of different materials: pig manure, poultry manure, sawdust, livestock waste, cow manure, dregs of oil and lees, bark, chaff, zeolite.	*P. capsici*	Root-drenched	Pepper	All the water extracts decreased the disese incidence and severity caused by *P. capsici.*	Sang *et al.*, 2010
Organic fraction of municipal waste	*P. capsici*	Soil amendment (5-10 kg m^{-2})	Pepper	High disease incidence reduction undert natural infestation conditions	Gilardi *et al.*, 2013
Artichoke sludge, chopped vineyard pruning waste and blanched artichokes (C1), (C1); garlic waste (C2) or dry olive cake (C3)	*P. capsici*	Growing media (peat:compost 1:1)	Pepper	C2 reduced the disease incidence in a 15% compared to peat; C1increased the disease incidence	Blaya *et al.*, 2014b
Aerated (ACT) and non-aerated (NCT) compost tea made of spent mushroom compost, grape marc compost, crop residue composts and vermicompost	*P. capsici*	Tea compost added as 5 mL per plant diluted 1:5 v:v in water.	Pepper	Both ACT and NCT suppressed the disease.	Marin *et al.*, 2014

Table 4 (continued)

Vermicompost of cattle manure	*Phytophthora nicotianae*	Growing media	Tomato (*Solanum lycopersicum*)	Dose-dependent suppressiveness	Szczech *et al.*, 1993
Composted municipal waste	*P. nicotianae*	Amendment of citrus soils	Citrus seedlings (*Citrus limon*)	Promote growth of citrus trees and disease decreased increasing proportions of one CMW (20%v/v)	Widmer *et al.*, 1998
Eighteen composts: green and yard waste, straw, bark, biowaste and municipal sewage.	*P. nicotianae*	Growing media (peat:compost 5:1)	Tomato	Significant disease suppressiveness (> 90%) was found in compost made of woody waste and poultry manure and the one made of woodcut, plants and horse manure	Termorshuizen *et al.*, 2006
Nine composts made of grape marc waste, spent mushroom compost (*Agaricus bisporus* cultivation), olive tree leaves	*P. nicotianae*	Growing media (peat:compost 3:1)	Tomato	All composts showed high levels of suppressiveness (applied after curing) 81-100% disease incidence decrease	Ntougias *et al.*, 2008

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Table 5. Examples of composts and compost water extracts from various organic wastes that have been used to control *F. oxysporum* in different crops.

Compost type	Pathogen	Use	Crop	Suppressive effects	References
Sewage sludge and wood chips	*Fusarium oxysporum* f.sp. *melonis* (FOM)	Soil amendment (10%)	Melon (*Cucumis melo*)	Reduced significantly disease severity	Lumsden *et al.*, 1983
Four composts: pine bark+urea (1000/1) (A); pruning waste+coffee waste (3/1) (B); pruning waste+coffee waste (4/1) (C); pruning waste (D).	FOM	Amendment in container with soil (30g compost:200g soil)	Melon	All composts reduced *F. oxysporum* incidence. Plants grown in compost C showed the lowest incidence.	Ros *et al.*, 2005
OP-SCM: cow manure+orange peels (1:1); WS-SCM: cow manure+wheat straw (1:1); TP-SCM: cow manure+dried tomato plants (2:1)	FOM	Growing media (peat:compost 1:1)	Melon	All three composts significantly suppressed disease	Yogev *et al.*, 2006
40% Citrus wastes, 20% sludge from a citrus industry, 40% green residues (C1); 60% citrus wastes + 40% green residues (C2)	FOM	Growing media (peat:compost 4:1)	Muskmelon	*F. oxysporum* incidence was diminished.	Bernal-Vicente *et al.*, 2008
40% Citrus wastes, 20% sludge from a citrus industry, 40% green residues (C1); 60% citrus wastes + 40% green residues (C2) with *T. harzianum*	FOM	Growing media (peat:compost 4:1)	Muskmelon	*Fusarium* incidence was significantly lower in C2 and its extract.	Lopez-Mondejar *et al.*, 2010
Tomato-plant residues mixed + separated cattle manure composts (1:1)	FOM	Growing media (perlite:compost 1:1)	Grafted melon	Reduction in disease severity	Yogev *et al.*, 2010
Tomato plants and cow manure composted, storaged under different conditions	FOM	Growing media (perlite:compost 1:1)	Muskmelon	All type of composts were highly suppressive	Saadi *et al.*, 2010

Table 5 (continued)

Vineyard pruning waste inoculated with *T. harzinaum* at the beginning of the composting process (C-Th 0), at the beginning of maturation process (C-Th 60) or without *T.harzianum* (C-Control)	FOM	Growing media (partial substitute of peat)	Muskmelon	C-Th60 gave the lowest pathogen incidence and disease severity.	Bernal-Vicente *et al.*, 2012
Vineyard pruning waste (GC) and GC inoculated with *T. harzianum* (GCTh)	FOM	Growing media (peat:compost 1:1)	Muskmelon	Both composts suppress the disease; GCTh the most suppressive growing media	Blaya *et al.*, 2013
Artichoke sludge, chopped vineyard pruning waste and blanched artichokes (C1); garlic waste (C2) or dry olive cake (C3)	FOM	Growing media (peat:compost 1:1)	Muskmelon	Only C3 rduced the disease incidence (15% compared to peat)	Blaya *et al.*, 2014b
Vermicompost of cattle manure	*Fusarium oxysporum* f.sp. *lycopersici* (FOL)	Growing media	Tomato (*Solanum lycopersicum*)	Dose-dependent suppressiveness	Szczech *et al.*, 1993
Pulp and paper mill residues amended or not with *Pythium oligandrum*	FOL	Growing media (peat:compost 3:1)	Tomato	Peat moss amended with compost with or without the *P. oligandrum* substancially reduced disease	Pharand *et al.*, 2002
Grape marc compost (GMC) and cork compost (CC)	FOL	Growing media	Tomato	GMC was sighly suppressive, CC was moderately suppressive	Borrero *et al.*, 2004
Cow manure+orange peels (1:1) (OP-SCM); Cow manure+wheat straw (1:1) (WS-SCM); Cow manure+dried tomato plants (2:1) (TP-SCM)	FOL	Growing media (peat:compost 1:1)	Tomato	WS-SCM and OP-SCM significantly reduced the disease severity	Yogev *et al.*, 2006
Humid olive husks+olive leaves (8%) + composted husks (25%) (C1); Humid olive husks+olive leaves (8%) + sheep manure (16%) (C2)	FOL	Growing media (peat:compost 1:1)	Tomato	C1 and C2 reduced disease by 52.2% and 56.8%.	Alfano *et al.*, 2011

Table 5 (continued)

Nine composts made of grape marc waste, spent mushroom compost (*Agaricus bisporus* cultivation), olive tree leaves	*F. oxysporum* f.sp. *radicis-lycopersici*	Growing media (peat:compost 3:1)	Tomato	Suppressivess varied widely among composts	Ntougias *et al.*, 2008
Diaiy solid compost (WDS); Dairy solid vermicompost (VDS); Vegetable waste (IBR)	*F. oxypsorum* f.sp. *radicis-cucumerinum*	Gowing media (soil:compost or soil-sand:compost1:4)	Cucumber (*Cucumis sativus*)	Potting mix with 40% WDS appears to be the most effective treatment.	Kannangara *et al.*, 2000
Cow manure+orange peels (1:1) (OP-SCM); Cow manure+wheat straw (1:1) (WS-SCM); Cow manure+dried tomato plants (2:1) (TP-SCM)	*F. oxysporum* f.sp. *radicis-cucumerinum*	Growing media (peat:compost 1:1)	Cucumber	TP-SCM was the most suppressive	Yogev *et al.*, 2006
Horticultural waste + prune (young compost) (A); Cut grass and ditch plants (old compost) (B); Organic household waste (fruit, vegetables and garde waste) (5 month-old) (C); compost C (1 year-old) (D)	*F. oxysporum* f.sp. *dianthi* (FOD)	Growing media (1:4 mixed with potting soil or potting soil-sand mixture)	Carnation (*Dianthus caryophyllus*)	All composts reduced the disease index and the presence of an antagonistic *Fusarium* improved the results obtained by composts non inoculated. Compost C was the most suppressive one.	Postma *et al.*, 2003
Grape marc compost with *T. asperellum*	*F. oxysporum* f.sp. *dianthi*	Growing media (peat:compost 1:1)	Carnation	Moderate suppressiveness	Sant *et al.*, 2010
Eighteen composts: green and yard waste, straw, bark, biowaste and municipal sewage.	*F. oxysporum* f.sp. *lini*	Growing media (peat:compost 5:1)	Flax (*Linum usitatissimum*)	Disease suppressiveness of 70% were found in compost made of horse manure (20%) and green wastes (wheat and corn straws, conifer bark) and compost made of yard waste.	Termorshuizen *et al.*, 2006

The enrichment of composts with specific strains of BCAs has been proposed to enhance their suppressive potential (Hoitink *et al.,* 1997). Good results have been achieved by introduction of species of genera *Acremonium, Chaetomium, Gliocadium, Trichoderma* and *Zygorrhynun* spp. (Hadar & Papadopoulou, 2012). The time of inoculation (after the heat peak of the composting process) and establishment of the introduced BCA at high densities are essential for the successful production of suppressive composts (Bernal-Vicente *et al.,* 2012). The use of certain *Trichoderma* spp. have been proposed due to the abilitiy of these fungi to colonize rapidly the rhizosphere, control of pathogenic and competitive microflora, and improvement of plant health and root growh (López-Mondéjar *et al.,* 2010; Bernal-Vicente *et al.,* 2012).

4.1. Factors affecting compost suppressiveness

4.1.1. Chemical and physical factors

The chemical and physical characteristics of composts are important in the suppressive effect achieved not only because they are responsible for the type and quantity of microorganisms present in composts, but also because of their effects on nutritional status of plants and pathogens (Avilés *et al.,* 2011). Particle size, nitrogen content, cellulose and lignin content, electrical conductivity, pH and inhibitors released by composts may affect the incidence of diseases caused by soil-borne plant pathogens (Hoitink & Fahy, 1986). For instance, majority of Phytophthora root rot diseases are inhibited by a low pH (Blaker & MacDonald, 1983). The low pH reduced sporangium formation, zoospore release and motility. On the other hand, high pH leads to the reduction of Fusarium wilts since pH is associated with the

availabitlity of macro- and micro-nutrients, important for growth, sporulation and virulence of *F. oxysporum* (Jones *et al.,* 1991).

4.1.2. C substrates and compost madurity

The links between specific C substrates that become available during composting and disease suppression remain elusive. One exception is the case of composts with high abundance of chitins and chitin-derived C compounds, which has been related to the potential proliferation of chitinolytic microbial agents, and therefore, the degradation of fungal pathogen cell walls. Labile C substrates, such as sugars, and high availability of celluloses arrest the suppressive activity of composts or the disease control capability of certain microbial agents (Hadar & Papadopoulou, 2012).

The degree of decomposition of the organic matter of compost has a strong effect on the rate of disease suppression (Hadar & Mandelbaum, 1986; Bonanomi *et al.,* 2010). In stable compost, easily degradable carbon sources have been used by the existing microflora, leading to a stable microbiological system. However, extremely stable composts do not support microbiological activity, so biological suppression potential is lost (Widmer *et al*., 1998). On the other hand, in fresh compost, nutrient sources have not been depleted. The high glucose concentration of this type of compost represses the synthesis of lytic enzymes involved in the parasitism of pathogens by BCAs as *Trichoderma* (Avilés *et al.,* 2011).

4.1.3. Biological factors

The sterilization of the compost usually leads to a loss of suppressiveness, suggesting a direct effect of the microbiota on disease suppression (Yogev *et al.,* 2006; Malandraki *et al.,* 2008).

The effect of suppressiveness in composts has been classified as **general suppression**, which is induced by a large metabolically active microbial community, or **specific suppression**, which is attributed to specific microbial agents that proliferate upon, or are favored by compost application, and affect pathogen growth or infection through a particular biological control mechanism, such as competition, antibiosis, parasitism, induced plant resistance or a combination of these mechanisms (Hoitink *et al.,* 1999; Avilés *et al.,* 2012; Hadar & Papadopoulou, 2012). Examples of these mechanisms of action are explained bellow.

Competition among microbial populations

Competition among microbial populations is a phenomenon that regulates population dynamics of microorganisms sharing the same ecological niche and having the same physiological requirements when resources are limited. The oomycetes *Phytophthora* ssp. and *Pythium* spp. are described as highly sensitive to microbial nutrient competition. They depend on exogenous carbon sources for germination to infect host plants (Hoitink & Boehm, 1999). Competition for nutrients and space is one of the modes of action of many BCAs such as *Trichoderma* spp. (Alabouvette *et al.,* 2009). Competition for minor elements such as iron is also frequent being one of the modes of action of fluorescent pseudomonas. The ability of *Pseudomonas*

spp. to produce siderophores limits the growth and germination of chlamydospores of pathogenic *Fusarium oxysporum* (Heydari & Pessarakli, 2010).

Antibiosis

Antagonistic process is mediated by microbes through specific or non-specific molecules such as antibiotics, metabolites, lytic agents, enzymes, volatile compounds, or other toxic substances. The activity of very well-known BCAs such as fluoresencens *Pseudomonas*, *Bacillus* spp., *Streptomyces* spp., and *Trichoderma* spp., is related to antibiosis (Alabouvette *et al.,* 2009).

Hyperparasitism

The parasitism involves specific recognition between the antagonist and its target pathogen and several types of cell wall degrading enzymes enable to parasite and to enter the hyphae of the pathogen. Four major groups of hyperparasites have been identified including hypoviruses, facultative parasites, obligate bacterial pathogens and predators (Heydari & Pessarakli, 2010). Some BCAs may exhibit predatory behavior under low nutrient levels. One example is the case of *Trichoderma*, which chitinase genes are activated when the concentration of readily available cellulose is relatively low, producing chitinases (Benhanrou & Chet, 1997).

Induction of plant resistance

The plant reacts to the contact with pathogenic or non-pathogenic organisms by elicitation of defence reactions that enhance resistance against subsequent infection by a variety of pathogens (Alabouvette *et al.,* 2006).

Induction of host defenses can be local or systemic. The Systemic Acquired Resistance (SAR) is mediated by Salicylic Acid (SA), usually produced after pathogen infection and leads to the transcription of many genes, resulting in the production of defense molecules such as phytopalexins, pathogenesis-related (PR) proteins. On the other hand, Systemic Induced resistance (SIR) is mediated by Jasmonic Acid (JA) and/or ethylene (ET), which are produced after the application of some non-pathogenic microorganisms and evolves an early recognition of the aggressor.

The induction of resistance by composts has been reported as an additional biocontrol mechanisism against both foliar and root diseases. Yogev *et al.,* (2010) demonstrated the involvement of plant-induced resistance against FOMs and *Botrytis cinerea* by compost made from tomato plant residues and cattle manure. Conversely, other authors pointed out that in the case of compost-amendment substrates, the induction of SIR in plants is considered to be a rare and variable phenonmenon (Kavroulakis *et al.,* 2005). However, induced resistance in plants has been shown as the mode of action for specific microbial agents what were originally isolated from compost media and act as BCAs. As we mentioned above, several authors reported the ability of *Trichoderma* species to induce systemic and localized resistance to an array of phytopathogens (Shoresh *et al.,* 2005; Martínez-Medina *et al.,* 2010).

5. Looking for indicators of compost suppressiveness

One of the main drawbacks of the use of composts is the lack of predictability (Hadar & Papadopoulou, 2012) which hinders their practical

use (van Elsas & Postma, 2007). The possibility of predicting suppressiveness to pathogens is a central issue for researchers (Pane *et al.,* 2011) because in spite of many efforts to find indicators of disease suppressiveness, there is still a general lack of understanding of what determines the disease-suppressive status of compost (Bonanomi *et al.,* 2010). However, several promising examples of disease suppression have been described for specific situations and diseases. Indeed, this indicates a relationship between disease suppression and specific chemical, physical and biological characteristics in the compost (van Elsas & Postma, 2007).

5.1. Chemical and physical variables

The use of indicators such as pH or CE could be useful while studying the suppressive effect of compost against certain plant diseases. Bonanomi *et al.* (2010) reported in their review a positive correlation between pH and *Fusarium* species inhition. This is in concordance with the beneficial effects of high soil pH on the control of diseases caused by *Fusarium* species as reported by Borrero *et al.* (2004). Other chemical parameters such as C, N, micro and macronutrient content have been related to suppressiveness. For instance, Kim *et al.* (2000) observed that the elements K and Mg in compost and soil amendment-treated soils were related to an increase in soil microbial activity, which may have affected the root and crown rot of bell pepper caused by *P. capsici.* Saadi et al. (2010) reported that dissolved organic carbon was correlated with several microbial properties as well as with compost suppressiveness in Fusarium wilt of melon seedlings. On the other hand, Pane *et al.* (2011) found out that the most successful parameters to

predict disease suppression of *Pythium ultimun* were O-aryl C, extractable C and C/N ratio.

It should be noted that the chemistry of the materials from which the composts is prepared is able to affect the composition of the microorganisms in composts (Castaño *et al.,* 2011). In this sense, potential of biotic suppressiveness of pathogens could be also associated to quality and bioavailability of organic matter, as previously demonstrated by solid state ^{13}C Cross Polarization Magic Angle Spinning (CPMAS) Nuclear Magnetic Resonance (NMR) spectroscopy in several reports (Boehm *et al.,* 1997; Castaño *et al.,* 2011; Pane *et al.,* 2011). NMR spectra showed to be very informative on potential of compost to suppress disease, when such property was associated to microbial community characteristics (Pane *et al.,* 2013).

5.2. Enzymatic and functional variables

Microbial activity and biomass have been correlated with disease suppression. In the case of pathogens which control has been related to the general suppression model, some promising predicting parameters could be those which measure general microbial actitivy such as Fluoroscein diacetate (FDA) hydrolysis, basal respiration, and dehydrogenase activity. FDA is the microbial actitivy estimator most commonly used to address whole microbial community and it has been proposed as a promising measure for predicting organic matter suppressiveness (Hoitink & Boehm, 1999; Hadar & Papadopoulou, 2012). However, contrasting trends between FDA hydrolysis and suppressiveness for different pathogens were found (Bonanomi *et al.,* 2010). Additionally, dehydrogenase activity has been correlated negatively with Phytophthora root rot incidence (Blaya *et al.,* 2014b) as well as

respiration was negatively correlated with disease in the *Verticillium dahiae*/eggplant system but positively in the *Rhizoctonia solani*/cauliflower system (Termorshuizen *et al.,* 2006).

Enzyme assays involved in C, P and N biogeochemical cycles such as β-glucosidase, phosphatase, protease and urease have been also used to characterize composts. Alfano *et al.* (2011) found high β-glucosidase activity in all olive-waste composts tested, which was indicatived of cellulolytic activity and thus, this activity was considered to be potentially involved in the mechanisms of hydrolysis of oomycete cell-walls. On the other hand, enzymatic activities involved in specific process such as N-acetyl-β-glucosaninidase (NAGase) or chitinase activities have been employed. High level of these activities has been correlated to the presence of chitinase-producing bacteria, which are well-characterized for its role causing lysis of pathogenic fungi (Boulter *et al.,* 2000). Blaya *et al.* (2014b) found a negative correlation between the NAGase, glucosidase and protease activities and Fusarium wilt incidence. Pane *et al.* (2011) pointed the NAGase activity as a possible parameter to predict compost suppressiveness against *R. solani*.

5.3. Microbiological parameters

As we discussed before, it is likely that the community structure of the microflora present in compost determines the degree of suppression of plant disease (van Elsas & Postma, 2007). Representatives of a range of bacterial (*Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia*, *Streptomyces*) and fungal (*Trichoderma*, *Penicillium*, *Gliocadium*, *Sporidesmium*, non-pathogenic *Fusarium* spp.) have been identified as antagonistic of one or more soil-borne plant pathogens (Avilés *et al.,* 2011). Positive correlations

have been found between these microbiological parameters and the ability of composts to suppress soil-borne pathogens (Bonanomi *et al.,* 2010).

For instance, several authors have measured the level of copiotrophoc bacteria populations, including those of *Pseudomonas* and *Bacillus* species, wich are characterized for their biocontrol effect in composts (Hardy & Ssivasithampram, 1995). Other parameters such as total culturable bacteria, total culturable fungi, actinomycetes and *Trichoderma* spp., have been reported as the main contributors to the biological compost suppressivity (Pane *et al.,* 2013). In addition, high numbers of actinomycetes have been found in composts that showed phytopathogen-suppressive effect (Craft & Nelson, 1996). Nevertheless, a prevalence of no significant correlations among their presence and suppressiveness indicated that actinomycetes are only directly involved in disease suppression of a limited number of experimental cases (Bonanomi *et al.,* 2010). All these contradictory results may show that, it was point out by Bonanomi *et al.,* (2010), the integration of different parametes may be a promising approach for identification of suppressive composts due to the many interactions of contributing factors. Further studies are required to increase the scanty data set available to be able to consider some of the above mentioned factors, realiable ones.

On the other hand, profiling of the microbial community of composts using polymerase chain reaction (PCR)-based molecular methods among others (Hadar & Papadopoulou, 2012) has been proposed as a suitable parameter to elucidate the presence of these antagonistic microorganisms. This topic is discussed in following sections due to its relevance.

6. Use of suppressive compost for growing media

Currently, the most utilized organic substrate for the preparation of potting mixes is based on peat (van der Gaag *et al.*, 2007; Carlile, 2009) because of its favourable agronomic characteristics (constant chemical and physical properties, high water retention capacity, optimal porosity and controlled pH) (Pane *et al.,* 2011). The use of composts has been suggested as a partial substitute of peat in potting mixture and as a low-cost substrate constituent in horticulture (Bernal-Vicente *et al.,* 2008; López-Mondéjar *et al.,* 2010; Moral *et al.,* 2013). Their use will benefit the potting-soil industry as the policy in several European countries is to decrease the use of peat in potting mixes due to the environmental problems derived from peat extraction (Carlile, 2009). Furthermore, peat bogs are considered an important CO_2 sink (Termorshuizen *et al.,* 2006). Since peat is hardly ever suppressive against soil-borne pathogens (Pane *et a*l., 2011), the use of composts may be advantageous by increasing the disease suppressive properties of the potting mixture (Hadar *et al.,* 2011). The combined application of peat and suppressive composts at relatively low dosages, ranging from 1% to 20% by volume, appears to be a promising perspective because it maintains the agronomic feature of peat, but simultaneously, enhances the suppressive capability of the potting mixtures (Pane *et al.*, 2011). Some authors have also used higher dosages (until 50 %) obtaining good agronomic results (Yogev *et al*., 2006; Blaya *et al*. 2013, 2014b).

It should be noted that in nurseries, prevention methods are of high relevance taking into account the role of propagating infected material in the diffusion of soil-borne plant pathogens (Spies *et al.,* 2011). Seedlings, while

growing in nurseries, are susceptible to the attack of pathogens and pests, which could reduce their growth becoming a great obstacle for their transplanted into the field (López-Mondéjar *et al.,* 2012). Nurseries are particularly exposed to the risk of emergence of disease as a result of the proximity between plants and the temperature and humid conditions. The use of composts as a growing mediun in nurseries stands out as one alternative to avoid those losses (Figure 5).

Figure 5. Pepper (left) and melon (right) seedlings growing on compost-based substrates.

On the other hand, the safe application of composts in potting media needs to fulfill certain requirements. Composts must have a high degree of stability and maturity to avoid competition for oxygen and nitrogen between microorganisms and plant roots, as well as a low salinity, and the absence of plant pathogens (Yogev *et al.,* 2006; Ntougias *et al.,* 2008). Currently, the standardization of growing media is based on the regulations established in the European Union (CEN/TC 223 [1990] on Soil Improvers and Growing Media), and in Spain, with the specific legislation about substrates (RD 865/2010) and fertilizers (RD 506/2013) (Moral *et al.,* 2013).

6.1. Agricultural and agro-industrial composts in potting media

The use of agricultural and agro-industrial composts in potting media is particularly important in the Mediterranean basin. The food industries as well as the agricultural sector in the Mediterranean area generate important amounts of organic waste (Ntougias *et al.,* 2008; Kavroulakis *et al.,* 2010; Pane *et al.,* 2011) and in particular, Spain stands out as one of the greatest horticultural producers (Table 2). Alternatives for the disposal of these organic wastes have been proposed, composting being one of the most attractive (Vargas-García *et al.,* 2010). In this sense, the recycling of agricultural and agro-industrial residues in the Mediterranean area is recommended, especially in market sensitive systems such as intensive horticulture since compost quality must be strictly guarantee (Ntougias *et al.,* 2008; Moral *et al.,* 2013; Pane *et al.,* 2013). It is a point worth mentioning that in Spain, the region of Murcia comprises the 13% of the total Spanish companies of fruit and vegetables transformation. The main activities of this industry are the production of canned and frozen products, juices, nectars and concentrates of fruits and vegetables. Large quantities of organic residues and by-products are generated from these activities, which final destination is mainly animal feed and just in some cases energy production. An average of 534082 tonnes of organic residues and by-products per year were estimated to be produced after surveying the 90% of the companies of this industry in the area of Murcia (AGROWASTE, 2011). The main residues and by-products generated and its characteristics are presented in Table 6. Moreover, a high volume of sludge (14,766 tonnes/year) is also generated as a by-product of the treatment of industrial wastewater.

7. Techniques to study soil-borne plant pathogens and the microbial communities in composts

Today, a vast number of techniques are used to improve plant disease management. We are going to focus in the techniques which regard the detection and quantification of soil-borne plant pathogens as well as the diversity of their population; and the techniques that improve our knowledge concerning the microbiota of composts.

7.1. Detection and quantification methods for soil-borne plant pathogens

Plant pathogenic fungi and oomycetes cause serious and economically important plant diseases around the world including horticultural, ornamental and fruit tree species. Rapid detection and accurate identification of plant pathogens is one of the most important strategies for controlling plant diseases as is required for taking appropriate disease management measures. An early detection of the pathogen, even before of the onset of the symptoms, is of special interest in seeds, nursery plants and propagative plant material to avoid the introduction and further spreading of new pathogens in growing areas (Lievens *et al.,* 2008; Capote *et al.,* 2012).

Different approaches, from classical methods, traditionally used in microbiology, to the molecular methods, which have gained more importance in the last decades, are available for diagnosis and detection of soil-borne fungal and oomycete pathogens in environmental samples.

Table 6. Physical and chemical characterization of different residues of food-industry in southeast Spain. Source: AGROWASTE, 2014.

	Artichoke	Dry olive cake	Lemon	Orange	Pepper	Tomato	Carrot	Peach	Broccoli	Artichoke sludge	Pepper sludge
Origin	*Cynara scolymus* L. (Asteraceae)	Olive (fruit of olea *europaea)*	*Citrus limonus*	*Citus cinensis*	*Capsicum annum*	*Lycopersicon esculentum*	*Daucus carota*	*Prunus persica*	*Brassica oleracea*	Wastewater treatment in artichoke industry	Wastewater treatment in pepper industry
pH	4.77-5.31 (5.09)	4.95-5.60 (5.23)	3.14-3.49 (3.31)	3.41-3.82 (3.65)	3.95-7.63 (5.23)	4.10-6.32 (4.88)	4.26-4.83 (4.55)	3.63-4.30 (3.97)	4.45-5.55 (5.19)	5.31-8.60 (6.52)	5.80-8.14 (6.69)
CE (1:5; 20ºC) mS/cm	4.30-6.88 (5.69)	3.10-3.70 (3.38)	2.06-2.72 (2.49)	1.92-2.21 (2.07)	2.28-10.0 (6.09)	0.39-4.83 (2.62)	0.89-1.47 (1.16)	1.45-5.04 (2.70)	5.94-7.54 (6.71)	16.4-36.9 (23.1)	11.9-66.0 (37.1)
Humidity (%)	83.7-87.4 (85.3)	59.6-69.0 (64.6)	85.3-89.1 (86.3)	82.4-85.5 (82.9)	87.1-91.3 (89.6)	86.1-94.2 (90.9)	86.1-88.2 (86.9)	75.4-88.0 (82.9)	86.0-91.3 (89.6)	69.2-90.5 (80.1)	74.1-86.6 (82.4)
Total organic matter (%)	92.8-93.9 (93.3)	93.3-95.7 (95.0)	95.4-96.5 (96.2)	95.9-97.7 (97.1)	90.7-93.1 (91.8)	86.4-95.7 (92.0)	89.8-92.1 (91.3)	93.3-97.77 (96.0)	89.5-90.8 (90.1)	69.0-93.4 (81.3)	54.4-92.8 (78.4)
N total (g/100g)	0.29-2.76 (0.93)	0.96-1.15 (1.02)	1.01-1.31 (1.09)	0.82-1.21 (0.98)	0.25-3.27 (1.03)	1.09-3.34 (1.78)	1.08-1.38 (1.25)	0.78-1.12 (0.93)	2.57-3.87 (3.25)	0.51-7.44 (3.56)	5.13-8.00 (6.84)
P (mg/Kg)	2698-3453 (2888)	1900-5280 (3773)	901-1361 (1043)	741.4-4411 (3002)	2403-5341 (3872)	4474-5606 (5040)	2045-5763 (3903)	446.7-2739 (1484)	2029-5263 (4136)	961-7379 (3689)	2139-11788 (5960)
K (%)	2.17-4.00 (2.82)	1.42-1.98 (1.67)	0.84-1.51 (1.19)	0.80-1.20 (0.93)	1.05-4.64 (2.69)	0.05-4.00 (2.46)	2.74-3.02 (2.88)	0.89-1.75 (1.31)	0.93-3.99 (3.05)	0.15-0.46 (0.34)	0.31-0.89 (0.67)
Mg (%)	0.22-0.25 (0.24)	0.04-0.17 (0.09)	0.04-0.10 (0.08)	0.05-0.13 (0.08)	0.09-0.36 (0.13)	0.002-0.26 (0.14)	0.10	0.04-0.10 (0.06)	0.25-0.29 (0.27)	BDL	BDL

Table 6 (continued)

Ca (%)	0.27-0.49 (0.35)	0.32-0.45 (0.37	0.72-1.00 (0.77)	0.41-0.66 (0.60) %	0.15-0.69 (0.40)	0.003-0.65 (0.21)	0.37-0.52 (0.45)	0.05-0.09 (0.08)	0.68-2.64 (1.20)	BDL	BDL
Na (%)	0.24-0.65 (0.44)	0.02-0.08 (0.04)	0.05-0.07 (0.05)	0.02-0.28 (0.07)	0.007-0.62 (0.19)	0.0004-0.22 (0.10)	0.48-0.54 (0.51)	0.003-0.02 (0.008	0.23-0.57 (0.34)	BDL	BDL
Mn (mg/Kg)	16.6-27.0 (20.9)	12.1-13.9 (13.0)	3.00-5.67 (4.59)	1.82-5.66 (4.09)	13.0-40.3 (18.1)	4.5-16.7 (10.8)	19.6-22.8 (21.0)	3.81-8.33 (5.89)	41.7-109.5 (59.6)	84.8-161.6 (117.8)	46.4-178.9 (100.9)
Cu (mg/Kg)	6.21-10.43 (8.53)	<5	2.45-4.71 (3.20)	1.47-3.24 (2.77)	0.68-8.86 (4.70)	0.05-8.18 (3.35)	7.12-14.7 (11.6)	0.60-4.63 (2.37)	4.54-10.50 (6.77)	16.7-99.0 (45.17)	37.5-84.7 (53.4)
Zn (mg/Kg)	18.9-27.8 (22.1)	<5	5.35-14.2 (8.79)	3.41-5.32 (5.46)	4.46-19.0 (12.2)	12.1-33.4 (23.3)	15.6-22.2 (19.7)	5.74-22.92 (9.40)	22.9-32.8 (28.3)	43-364 (236.3)	228.0-518.7 (339.8)
Cd (mg/kg)	<0.1	<0.1-0.57	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1-0.42	<0.1-0.52
Cr (mg/kg)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<1.72-99	10.5-453.7 (83.1)
Ni (mg/kg)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<1.54-79	5.90-26.8 (20.15)
Pb (mg/kg)	<0.1	<1	<0.1-0.64	<0.1	<0.1-1.31	<0.1-0.5	<0.1	<0.1	<0.1-0.14	4.9-38.19 (17.07)	4.12-11.03 (6.67)
Hg (mg/kg)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.01-1.46	0.01-0.15
Ar (mg/kg)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.01-0.52	<0.01-0.51

BDL: below detection limit.

7.1.1. Classical methods

Traditional techniques are often based on identification of disease symptoms, isolation and culturing of environmental organisms, and subsequent laboratory identification by morphology and biochemical tests (Atkins & Clark, 2004).

Identification found on observation of disease symptoms has several disadvantages since different pathogens which affect the same crops may cause similar or identical symptoms (wilt, blight, root rot) leading to mistaken diagnosis or even sometimes, specific strains are needed to distinguish among similar structures. In this sense, **microscopic techniques** have been widely used for the identification of soil-borne pathogens based on morphological characteristics.

The serial **dilution plate technique** in selective media not only allows the identification of the pathogen, but also the quantification of the colony forming units presented per gram of soil or culture substrate. The use of selective media is based on the ability of certain microorganisms to use specific substrates or their ability to grow under the presence of certain antibiotics and fungicides. For instance, the culture media Komada (Komada, 1976) has been used as a *Fusarium*-selective medium and reported to distinguish between *F. oxysporum*, *F. solani*, *F. moniliforme*, and *F. roseum* by the color of the colonies. In the case of detection of *Phytophthora* spp., different culture media are available, such as V8 juice agar, pea agar or corn meal agar amended with antibiotics against bacterial growth (penicillin, polymixin B, rifampicin, pentachloronitrobenzene (PCNB), ampicillin,

vancomycin) or with antifungal effect (nystatin and pimaricin) (Tsao & Ocana, 1969).

Sometimes isolation of phytopathogens from soils remains difficult. Baiting techniques may be used for isolation of *Phytophthora* and *Pythium* species directly from infected soils (Tsao & Ocana, 1969). Once the pure-culture is ready, the identification is based primarily on the shape of the sporangia, mycelium and in the case of sexual fungi, the morphological features of the sexual structures. Other criteria widely used to distinguish species are cardinal growth temperature, growth rate, morphological (growth) characteristics in culture, and mating behavior. However, a reliable and accurate identification within species level is often complicated due to intraspecific variation and overlapping characters, even for specialists (Ippolito *et al.,* 2002; Darine *et al.,* 2007). These techniques are time and labour-consuming, preclude the handling of large number of samples and require extensive knowledge of classical taxonomy. Besides, not all the microorganisms are considered to be cultured. It is thought that only the 1 % is able to grow under laboratory conditions (Hugenholtz, 2002).

Chemical and **physiological methods** have also been used to quantify the fungal biomass in soil. The chemical methods are based on the measure of different fungal compounds such as chitin and ergosterol (Djajakirana *et al.,* 1996; Ekblad *et al.,* 1997). The physiological ones rely on respiration rate measures after the addition of different substrates, where the CO_2 produced or the O_2 consumed is determined (Weaver *et al.,* 1994). **Immunological techniques** have been employed for detection, differentitation and quantification of fungal pathogens rapidly and are based

on recognition, by antibodies, of specific antigens either present on the surface of the pathogens or secreted by them (McCartney *et al.,* 2003). Most of them use the enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977), although new formats are available. However, the problems associated with the production of specific antibodies that may be effective (difficulty and costs) limits the use of these techniques (Narayanasamy, 2011).

7.1.2. Molecular tools

In the last decades polymerase chain reaction (PCR) has emerged as a powerful tool for the identification and study of phytopathogenic fungi and oomycetes, contributing to solve some problems related to the detection, control and containment of plant pathogens (Schena *et al.,* 2004). PCR-based detection methods are characterized for its sensitivity, selectivity, robustness, rapidity and ease of methodology (Justé *et al.,* 2008). Moreover these techniques can overcome some of the shortcoming of the traditional ones, since generally it is not necessary the culturing step of the pathogen from the infected material, reducing the diagnosis time from weeks to hours and allowing the detection and identification of non-culturable pathogens (Capote et al; 2012; Schena *et al.,* 2013). The PCR consists in the repetitive amplification (depending on the number of cycles) of a specific fragment of the genome after the extraction of DNA from the target microorganism. Ideally, DNA extraction protocol should enable to obtain a good quality DNA with a low concentration of substances inhibiting PCR reactions (Schena *et al.,* 2013). Different methodologies have been development for obtaining the nucleic acids (DNA) from plant material, fungi, oomycetes or

soil/compost (Doyle & Doyle, 1987; van Burick *et al*., 1998; Robe *et al*., 2003). Most of them are based on a first step, consisting on the physical disruption (grinding samples with liquid nitrogen or bead beater) of microbial cell walls to release nucleic acids, followed by separation of nucleic acids from the soil or tissue particles and their extraction with one or more organic solvents (mainly phenol and chloroform), then concentrated by alcohol/salt precipitation according to standard procedures (Sambrook & Russell, 2011).

The presence of natural compounds such as polysaccharides, tannins and phenolics compounds in plant tissue samples and fulvic and humic compounds in soil/compost samples, may affect PCR efficiency (Wilson *et al.,* 1997). For this reason, these compounds need to be removed during DNA extraction or dilute the samples to reduce further inhibition during the PCR reaction. Nowadays, the nucleic acid extraction protocols are almost all kit-based (commercial), so that a high variety of commercial kits either general or specifically designed for plant material, fungi/oomycetes or soil are available in the market. Examples are: Ultraclean kit (MOBIO), FastDNA Spin kit for soil (MP Biomedicals), DNeasy Plant Kit (Qiagen Sciences, USA), Nucleon Phytopure (Amersham Biosciences Europe GmbH, Germany) (Capote *et al.,* 2012). Major advantages of commercial kits are their simplicity and rapidity together with the absence of harmful chemical compounds.

The identification of appropriate target DNA regions and the subsequent development of specific PCR primers to target organisms is a crucial step in PCR assay development. A good target gene should be sufficiently variable to enable the differentiation of closely related species

but, at the same time, should not contain intraspecific variation that would jeopardize the detection of all strains (Schena *et al.,* 2013). Molecular studies with fungi and oomycetes have largely concentrated on the ribosomal RNA gene cluster. This gene cluster consists of multiple copies (up to 200 copies per haploid genome) arranged in tandem repeats comprising three ribosomal RNA subunit genes (18S small subunit, 5.8S and the 28S large subunit) separated by internally transcribed spacers (ITS1 and ITS2), and intergenic spacers (IGS), the spacer between the small and large subunit (Bridge & Spooner, 2001). The nuclear-encoded ribosomal RNA genes (rDNA) with conserved as well as variable sequences can be used to develop broad specificity PCR primers (Bruns *et al.,* 1990; White *et al*., 1990). The ITS regions of ribosomal RNA genes are the most commonly regions used for designing PCR diagnostic assays because molecular methods based on this target are potentially very sensitive (Tooley *et al.,* 2006; Schena *et al.,* 2013). After PCR amplification, molecular identification of plant pathogens is accomplished by direct sequencing and BLAST searching in GenBank or other databases (White *et al.,* 1990).

Real-time PCR (qPCR)

Not only detection but also quantification of pathogens is becoming more and more significant. Accurate quantification of DNA can be performed using real-time PCR (qPCR) (Heid *et al.,* 1996). Elimination of the required post-amplification processing steps significantly reducing the time, assay labor and risk of carryover contaminations are some of de advantages of qPCR (Schena *et al.,* 2004). Moreover, this technique allows the accurate quantification of the target pathogen, by interpolating the quantity measured

to a standard curve with known amounts of target copies (Garrido *et al.,* 2009). In addition, qPCR seems to be more sensitive than conventional PCR which is essential to detect soil-borne fungi and oomycetes that can be present at very low levels (Schena *et al.,* 2004).

The qPCR allows the monitoring of the reaction during the amplification process by the use of a fluorescent signal that increases proportionally to the number of amplicons generated. Different methodologies can be used to measure the PCR product obtained after each cycle of the PCR with the use of inespecific or specific fluorescens dyes. The **inespecific methods** are based on the use of binding dyes, such as SYBR Green (Morrison *et al.,* 1998) which is a fluorescence intercalating dye with a high affinity for double-stranded DNA. Although the no need of probe reduces costs, special attention must be paid in the formation of non-specific amplicons and dimmers, since this dye does not discriminate between the different double-stranded DNA. On the other hand, the **specific methods** are based on the use of oligonucleotides probes labelled with a donor fluorophore (reporter) covalently attached to the 5' end and an acceptor dye (quencher) attached to the 3' end. The fluourophore does not emit fluorescence in the presence of the quencher, which dissipates the energy by proximal quenching or by fluorescent resonance energy transfer (FRET). Once the primers and probe specifically hybridise to the DNA, the 5'-3' exonuclease activity of the *Taq* DNA polymerase cleaves the probe causing the liberation of the fluorophore, which start emitting fluourescence. The fluorescence detected in the qPCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Among labelled

probed, TaqMan system is one of the most used (Livak *et al.,* 1995). The TaqMan probes were designed to increase the specificity of the reaction because detection and accurate quantification require high complementarity with the target sequence. Probes may include fluorophores such as FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxi-4,5-dichloro-6-carboxyfluorescein), ROX (6-Carboxyl-X-Rhodamine), Cy5 (indo-dicarbocyanine) and quenchers such as TAMRA (6-carboxytetramethylrhodamine), BHQ (black hole quencher) and MGB (minor grove binder) (Schena *et al.,* 2004). For instance, the MGB probes that include a MGB group at the 3' end raising the *Tm* (melting temperature) of the hybrid allows the use of shorter and more specific probes. The high specificity of MGB probes make them very suitable for specific detection of fungal species based on SNPs (Massart *et al.,* 2005).

Reverse transcription PCR (RT-PCR)

One of the major drawbacks of PCR-based approaches is the inability to discern between DNA obtained from active and non-active fungal material (conidia, dead mycelia, etc.) which result in the overestimation of populations (Bridge & Spooner, 2001; Lievens *et al.,* 2006). The use of reverse transcription of RNA combined with qPCR (qRT-PCR) may solve this problem and could provide a more meaningful assessment than current DNA approaches (Atkins *et al.,* 2003; Beaulieu *et al.,* 2011).

Digital PCR (dPCR)

Digital PCR (dPCR) is an emerging technique which offers an unique approach to qPCR for measuring nucleic acids that may be particularly suited

for low-level detection (Vogelstein & Kinzler, 1999; Dube *et al.,* 2008). The dPCR is based on the principle that an absolute count of amplified targets can be achieved working on the premise that every molecule of target is successfully amplified, and therefore, this new technique should, in theory, provide the most accurate method of molecular quantification (Sanders *et al.,* 2011).

The dPCR involves performing PCR with real-time or end-point data collection in a large number of separate reaction chambers, also termed partitions. Single molecules are isolated by dilution and individually amplified by PCR; each product is then analyzed separately. This is achieved by partitioning a sample prior to PCR amplification such that at least some of the partitions contain no copies of the target sequence(s) of interest. Results are obtained by counting the number of partitions containing target sequence detected by fluorescence (regarded as positive) and the number of partitions in which there is no fluorescence (regarded as negative). Poisson statistical analysis of the numbers of positive and negative partitions yields absolute quantitation of the target sequence. This compensates of the fact that more than one copy of template may be present in some partitions (Dube *et al.,* 2008; Huggett *et al.,* 2013) (Fig. 6).

Today, dPCR instruments achieve partitioning either on chips or throught water-in-oil emulsions or droplets. The dPCR based on chips is performed in small-volumen, solid partitions that allow either real-time or end-point analysis of the individual reactions. Compared to the droplet instruments, the number or partitions that are available, typically on the order of a few thousand, is fewer.

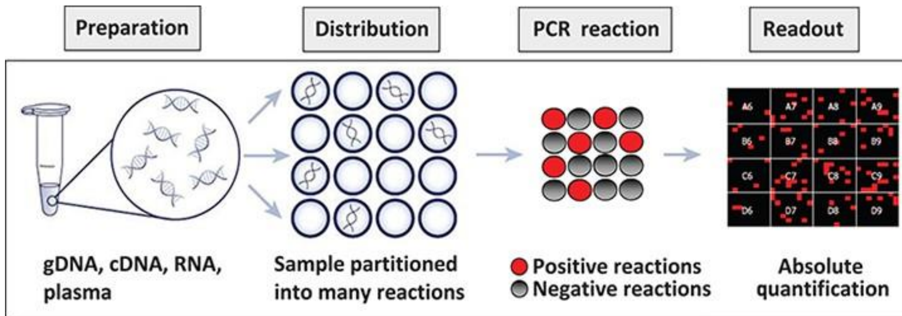

Figure 6. Absolute quantification of DNA using dPCR.

Source: www.lifetechnologies.com

Emulsion (or droplet) dPCR occurs in partitions made up of water-in-oil emulsion droplets. One of the advantages of these instruments is the great number of partitions achieved and lower running costs that most chip-based instruments. The higher number of partitions leads to an increased dynamic range, allowing analysis of a great range of sample concentrations for any given precision. However, these instruments require more technical complexity and the need for post-PCR sample manipulation (Huggett *et al.*, 2013).

The most outstanding advantage of dPCR over qPCR is to enable the absolute concentrations of DNA concentrations without external calibrators. The dPCR not only allows absolute quantification of target genes without any standards but also is considered less susceptible to PCR inhibitors present in the DNA extracts than qPCR (Hoshino & Inagaki, 2012).

The dPCR has already been used in clinical diagnosis (Schulz *et al.*, 2014; Wang *et al.*, 2014), especially for rare variant measurement, which has made of this technique a potential tool in several scenarios (e.g. the diagnosis and staging of cancer) (Vogelstein & Kinzler, 1999). However, its

applicability in plant pathology for the detection of pathogens in environmental samples (soil, composts or plants) remains uncertain.

Oligonucleotide array

Nucleic acid arrays offer the possibility to analyze a specifically selected group of microorganisms, concerning their presence or absence in a particular environmental sample, in a single experiment (Bodrossi *et al.,* 2003). In DNA arrays, DNA extracted from samples is amplified and subsequently fluorescently labeled and hybridized to the array. This technology is the most suitable technique to detect several target organisms simultaneously. This technology has been already successfully applied in diagnostics of human and animal (Lievens & Thomma, 2005) and in plant pathology, it has been successfully applied to identify DNA from pure cultures of oomycetes, nematoded and bacterial pathogens (Lévesque *et al.,* 1998; Uehara *et al.,* 1999; Fessehaie *et al.,* 2003) and from multiple tomato wilt pathogens (Lievens *et al.,* 2003*,* 2005).

7.2. Techniques to assess the population genetics of soil-borne plant pathogens

In spite of the last advances in molecular biology and the new available technology, the effective management of diseases caused by plant soil-borne pathogens is still a challenge for plant pathologists. One of the main reasons for this limited success is the lack of knowledge about the variability of the genetic structure of pathogen populations (Martin & English, 1997). Knowledge of the genetics of populations of plant pathogens may provide information about the evolutionary potential of pathogens to

overcome management strategies (Southwood *et al.,* 2012). The genetic structure of a population reflects its evolutionary history and its potential to evolve. In this sense, knowledge of the spatial distribution of genotypes within populations can proved information about their dispersal potential within fields (McDonald, 1997).

Multiples tools and techniques have been used to the study of population genetics including DNA fingerprints (RAPDs, RFLPs and AFLPs) and multilocus genotyping.

7.2.1. Fingerprinting techniques

Fingerprinting techniques have been extensively used to study the phylogenetic structure of plant pathogens populations and to differentiate strains of the same species with different host range, virulence, compatibility group or mating type (Capote *et al.,* 2013). DNA fingerprinting techniques include r**andom amplified polymorphic DNA** (RAPDs) (Williams *et al.,* 1990), **restriction fragment length polymorphism** (RFLPs) (Botstein *et al.,* 1980) and **amplified fragment length polymorphism** (AFLPs) (Vos *et al.,* 1995). The PCR-based approach of RAPD-PCR has been successfully used to identify soil-borne pathogens such as the fungal pathotypes of *P. nicotianae* that cause tobacco black shank (Zhang *et al.,* 2001) and several *forma speciales* and races of *F. oxysporum* (Lievens *et al.,* 2008). On the other hand, RFLPs in nuclear and mitochondrial genomes have been used in many studies of plant-pathogenic fungi. However, because of this technique is based on DNA-DNA hybridization is more reproducible but technically more difficult than RAPDs (McDonald, 1997). AFLPs are relatively costly and a rather complicate technical procedure (Lievens *et al.,* 2008).

Although most of these techniques have proved valuable within a particular study, results obtained with such fingerprinting tools are not always easily reproducible in different laboratories (Cooke & Lees 2004).

7.2.2. Multilocus genotypes

Genetic diversity and evolution of populations of a certain pathogen can be studied using multi-gene sequence phylogenies (Southwood *et al.,* 2012). Nucleotide sequence data offers the possibility of reconstructing patterns of descent among genotypes within a species or among populations of one or more species (Goss *et al.,* 2009). The comparison of the Single-Nucleotide Polymorphisms (SNPs) among the different isolates is ideal for evolutionary studies because they are widespread in the genome as well as easy to screen (Abott *et al.,* 2010). Closely related pathogens showing different host ranges or pathogenicity often differ in only a single to a few base pairs in target genes.

Different suitable markers have been proposed. In spite of the fact that ITS regions (Cooke *et al.,* 2000) are the most commonly sequenced for fungi and oomycetes for phylogenetic studies, these regions are usually conserved within species. In this sense, the Intergenic Spacer (IGS) DNA region has emerged as a suitable alternative to the ITS region when closely related taxa or even different strains of the same species need to be differentiated. The IGS region evolves faster and consequently, more sequence polymorphisms are present (Martin & Tooley, 2003). The wide utilization of the IGS region as target for developing specific molecular markers is primarily limited by difficulties in amplifying long fragments (approximately 2-4 kbp in fungi and 3-5 kbp in oomycetes) and the lack of effective universal primers (Capote *et*

al., 2012). Another alternative to differentiate closely related species is the use of mitochondrial genes such as the mitochondrial encoded cytochrome oxidase I (cox I) and II (cox II) and their intergenic region (mt-IGS) (Martin & Tooley, 2003; Nguyen & Seifert, 2008; Seifert *et al.,* 2007). It usually has a higher rate of evolution than nuclear DNA (White *et al.,* 1990). In the absence of sexual combination the use of mitochondrial markers, which are maternally inherited, would be useful to study clonally reproducing populations of the pathogen (Mamella *et al.,* 2011; Martin & Coffey, 2012). A general disadvantage of mitochondrial DNA is the very high AT/GC ratio which is generally more difficult to amplify and requires a higher concentration of $MgCl_2$ compared to genomic DNA.

In recent years, multi-locus approaches have been used to study the genetic diversity and evolution of different *formae speciales* of *F. oxysporum* (O'Donnell *et al.,* 1998, 2004; Southwood *et al.,* 2012) as well as different species of *Phytophthora*, namely *P. infestans* (Cárdenas *et al.,* 2011), *P. ramorum* (Goss *et al.,* 2009), *P. capsici* (Hurtado-Gonzales *et al.,* 2008) and *P. nicotianae* (Martin *et al.,* 2014).

7.2.3. Other molecular markers

Microsatellites (SSRs) are units of 1 to 4 bp long which are repeated 10 to 100 or more times in the genome of eukaryotic (this event is rare in prokaryotes). These repeats have a tendency to change in number when DNA is replicated due to DNA polymerase slippage. Size differences in the repeat length can be visualized by radiolabel or fluorescent molecules incorporated into the PCR products during amplification or by sequencing. SSRs have

been recognized as one of the most powerful alternative molecular approaches to study intraspecific variability among populations (Cooke *et al.,* 2007) and have been shown to be useful for differentiating diverse *formae specialis* of *F. oxysporum* (Leyva-Madrigal *et al.,* 2014). SSRs have been widely utilized for those species whose genome has been partially or completely sequenced since it is necessary to know the DNA sequence of the SSR flanking regions to design specific primers (Mamella *et al.,* 2012).

7.3. Methods to characterize compost microbial communities

It is well-known that microbes play key roles in the suppressive effect of composts and the appearance of some reflects the successful suppressiveness achieved against different soil-borne pathogens (Noble & Coventry, 2005). Numerous methods have been developed to more fully characterize microbial communities in soils, and specifically in composts. There are two kinds of approaches, the based on culture-based methods and the independent-culture ones.

7.3.1. Cultivation-dependent methods

Traditionally, cultivation-based methods have been used to study the microbial diversity in environmental samples. A wide array of culture media has been designed so as to maximize the recovery of diverse microbial groups (Figure 7). For instance, Suárez-Estrella *et al.* (2007; 2013a,b) used Sodium Caseinate Agar, nutrient agar and Rose-Bengal Chloramphenicol agar plates for the isolation of actinobacteria, bacteria and fungi respectively from different composts to test their potential antagonistic activity against several plant pathogens.

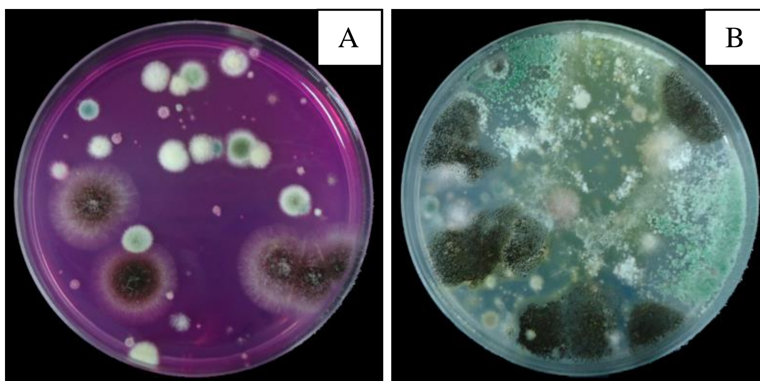

Figure 7. Different culture media used to characterize compost microbial community.A) Rose-Bengal Chlorampenicol agar plates; B) Nutrient agar plates.

Besides, a Biolog-based method, for directly analyzing the potential activity of soil microbial communities, denotes community-level physiological profiling (CLPP) (Garbeva *et al.,* 2004). Because of the inability of these methods to detect non-culturable species, which are able to be present or even been predominant in certain composts, other methods, mainly molecular ones, have become popular.

7.3.2. Cultivation-independent methods

A broad range of cultivation-independent techniques has been applied to the study of microbial communities presented in composts. These methods have been classified into two major categories: partial community analysis and whole community analysis approaches (Table 7).

7.3.2.1. Partial community approaches

Partial community analyses are based on the use of PCR-based methods to amplify total DNA/RNA extracted from an environmental sample. The 16S rDNA genes and ITS regions from bacterial and fungal

communities respectively have been widely used for the study of microbial communities (Mehta et al., 2014). These approaches include genetic fingerprinting techniques, fluorescence in situ hybridisation, clone libraries, DNA microarrays, qPCR and DNA/RNA stable isotope probing.

Microbial community fingerprinting techniques

The techniques that have been developed to fingerprint compost microbial communities include denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), single-strand conformational polymorphism (SSCP), ribosomal intergenic spacer length polymorphism (RISA) and phospholipid fatty acids analysis (PLFAs). **DGGE** is based on the separation of the PCR producs depending on their nucleotide sequences on a polyacrylamide gel containing a linear gradient of DNA denaturant (Muyzer *et al.,* 1993). In **TTGE** a temperature gradient is applied instead of the chemical denaturant. The ability to excise, reamplify and sequence particular bands in the patterns allows the identification of the microbial types or genes that underly these bands (van Elsas & Boersma, 2011). DGGE is the most widely used among the methods to study microbial communities in environmental samples, and in particular in composts (Ishii *et al.,* 2000; Garbeva *et al.,* 2004; Danon *et al.,* 2008). **ARDRA** is based on the PCR amplification of genes coding for rRNA, mostly 16S rRNA and 18S rRNA genes, and the consequent digestion with tetracutter restriction endonuclease. Restricted fragments are resolved on agarose or polyacrylamide gels to produce a specific community pattern (Vaneechoutte *et al.,* 1992). ARDRA technique has been applied to the

Table 7. An overview of some suitable methods for the assessment of partial and whole microbial communities in compost. Modified from van Elsas & Boersma (2011) and Mehta *et al*. (2014).

Method	Based on	Community level approach	Advantages	Disadvantages	Phylogenetic identification	Throughput
DGGE/TGGE	Sequence differences	Partial	Very sensitive to variations in DNA sequences; simultaneous analysis of multiple samples	Limited sequence information (< 500 bp), different fragments may have similar melting points, multiple bands per one microorganism	Yes	High
SSCP	Conformational differences	Partial	User-friendly, low cost, allow analysis of multiple samples; scan of mutations in a specific DNA region	Only well suited for small fragments (150-400 bp); high rate of reannealing of DNA strands	Yes	High
RAPD	Random amplification of genomic DNA	Partial	No knowledge of DNA sequence of targeted genome needed; easy to use	High quality DNA needed	No	High
ARDRA	Sequence differences in community DNA	Partial	Rapid, reproducible and relates to microbial diversity	Do not provide information about type of microorganisms; sometimes profiles generated difficult to resolve	No	High
TRFLP	Restriction site differences	Partial	Analysis of complex microbial communities; provides a robust index of community diversity	A limited number of bands per gel (< 100) can be resolved	Possible	High
ARISA	Differencies in intergenic spacer region length	Partial	Fast and sensitive to estimate microbial diversity; Provides a community-specific profile	A single microorganism may contribute more than one peak to the community profile	No	High
PLFA	Signature fatty acids present in different organisms that can be used to differenrtiat major taxonomic groups	Partial	Culture-independent, relatively easy and fast, inexpensive	Low sensitivy, linking PLFA to microbial communities difficult	Possible in some cases	High

Table 7 (continued)

DNA microarray	Hybridisation between complementary DNA strands	Partial	Very high throughput, direct information on sequences. Sensitive	Only chipped genes are found	Yes	High
qPCR	Amplification and detection of PCR products in real-time	Partial	Simple, reproducible, sensitive and quantitative	PCR biases and inhibition	Yes	High
FISH	Hybridisation of rRNA with fluorescently labeled probes	Partial	Quantitative, visualization of probed cells; provides insight into the metabolic state of the cells	Autoflouresence, necessity of metabolically active target cells	Yes	Low
DNA/RNA Stable isotope probing	Incorporation and metabolism by microorganisms or rare stable isotope	Partial	Concurrent examination of metabolic function and taxonomic identity	Biased incubation conditions; Problems of opportunists bluming the data	Yes	Low
Clone libraries	Sequence differences	Partial	Accurate phylogenetic identification of clone sequences	Laborious preparation of samples; time consuming; reveals only a small portion of the microbial diversity	Yes	Low
DNA-DNA hybridization	Sequence differences	Whole	Offers truly genome-wide comparison between organisms	Large quantities of DNA required; time-comsuming and labour-intensive	Yes	Low
G+C fractioning	G+C content	Whole	Reliable quantitative; allows detection of minority populations within the community	Different microorganisms can have the same G+C content; large amount of DNA (50 µg) are required	Yes	Low
Metagenomic	Direct genetic analysis of genomes	Whole	Provides access to the functional gene compostition of microbial communities; no PCR based	Massive sequencing projects still expensive; analysis of data still hard	Yes	High

DGCE/TGCE: denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis; SSCP: single strand conformation polymorphism: RAPD: random amplified polymorphic DNA: ARDRA: amplified rDNA restriction analysisTRFLP: terminal restriction fragment length polymorphism analysis; ARISA: Automated ribosomal intergenic spacer analysis; PLFA: phospholipid fatty acids; qPCR: Real time PCR; FISH: fluorescence *in situ* hybridization; G+C fractioning: Guanine plus cytosine content

analysis of the microbial community in compost (Uchiyama *et al.,* 2002). **RISA** approach involves PCR amplification of a portion of the intergenic region present between the small (16S) and large (23S) ribosomal subunits in bacteria and the transcribed spacers and the 5.8S rRNA genes (ITS1-5.8S-ITS2) in fungi (Jensen *et al.,* 1993; Ranjard *et al.,* 2001). An automated RISA (ARISA) involves the use of a fluorescence-tagged oligonucleotide primer for PCR amplification and the subsequent electrophoresis in an automated system (Ranjard *et al.,* 2001). This approach has been used successfully to assess the structure bacterial and fungal communities in compost (Schloss *et al.,* 2003; Hansgate *et al.,* 2005). Another technique that studies the DNA sequence variations present in PCR-amplified bacterial 16S and fungal 18S rDNA genes is **T-RFLP** (Liu *et al.,* 1997). This approach is based on the detection of PCR products previously labeled with the use of one 5' fluorescently labeled primer during the PCR reaction. The resulting PCR products are digested with restriction enzymes, and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer (Marsh *et al.,* 2000). Tiquia (2005) analyzed the microbial community structure and diversity in manure composts at different stages of composting using this technique. In **SSCP**, PCR products are denatured followed by electrophoretic separation of single-strand DNA fragments on a nondenaturing polyacrylamide gel. Single-stranded DNAs fold into secondary structures according to their nucleotide sequences and their physicochemical environment leading to measurable differences in mobility in the gel (Shwieger & Tebbe, 1998). SSCP has been used for genetic profiling of microbial communities including those involved during the composting process (Lee *et al.,* 1996; Peters *et al.,* 2000). **PLFA** analysis is based on the

extraction of PLFA biomarkers, which are identify by gas chromatography with flame ionization detection (GC-FID) and confirmed by mass spectroscopy (MS), if necessary. Among the profiling techniques, PLFA analysis is characterized for giving quantitative information about community structure (Ebersberger *et al.,* 2004) and has proved to be a useful tool for monitoring microbial community dynamics (Klamer & Baath, 1998). It has been used to characterize microbes in diverse systems such as marine sediments, soils, plant rhizospheres and composts (Herrmann & Shann, 1997; Bastida *et al.,* 2008) being sensitive to changes in composition and during the composting process (Amir *et al.,* 2010; Kindo *et al.,* 2012).

Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation (FISH) is a technique used to evaluate the phylogenetic identity, morphology, number, and spatial arrangements of microorganisms in different environmental samples. This technique is based on the design of fluorescently labeled 16S rRNA-oligonucleotide probes specific for the organism of interest (Hugenholtz, 2002). FISH has been used to study the influence of composts in bacterial root colonization (Iverson & Maier, 2009) as well as the spatial and temporal distribution of *Bacillus* and *Clostridium histolyticum* during composting of swine manure (Yi *et al.,* 2011).

Clone libraries

Clone libraries are useful to identify and characterize the dominant bacterial and fungal types in composts and may reveal the identity of uncultured and yet-unknown composting microorganisms (Garbeva *et al.,*

2004; Franke-Whittle *et al.,* 2009; Partanen et *al*., 2010; Blaya *et al*., 2013). Briefly, PCR-generated amplicons are ligated into a suitable vector plasmid. Later, the resulting constructs are transformed into *Escherichia coli*. After the growth of positive colonies, cloned amplicons can be isolated by plasmid extraction, and after sequencing them, the results are analyze comparing with databases such as GenBank, Ribosomal Database Project or Greengenes (van Elsas & Boerma, 2011). The clone libraries have a high resolution considering that they have to be quite large to accurately describe the microbial diversity within a compost sample (Garbeva *et al.,* 2004).

Oligonucleotide array

This technology has been successfully used in the study of microbial communities in diverse ecosystems. Some examples are the GEOChip (He *et al.,* 2007), a microarray for investigating biogeochemical, ecological and environmental processes; the PhyloChip (DeSantis *et al.,* 2007; Schatz *et al.,* 2010), designed to detect and quantify abundance of bacterial and archaeal taxa using signature probes targeting all known 16S rRNA gene sequences, the ANAEROCHIP (Franke-Whittle *et al.,* 2009), which study sludge methanogenic communities, the COMPOCHIP (Franke-Whittle *et al*., 2009b), an array which gives information about the characteristic microbiota of composts; and other arrays specifically designed to detect landfill methanotroph communities (Bodrossi *et al.,* 2003; Stralis-Pavese *et al.,* 2004), municipal wastewater microorganisms (Lee *et al.,* 2006), or disease suppressive microorganisms (Lievens *et al.,* 2007). The COMPOCHIP microarray has been used, together with other molecular techniques (DGGE

or clone libraries), to characterize the bacterial communities involved of different composts (Danon *et al.,* 2008; Fernández-Gómez *et al.,* 2012).

Real Time PCR (qPCR)

The qPCR is currently widely applied to soil/compost-extracted DNA, allowing the quantitative detection of target genes such as 16S rRNA genes or of functional genes like *amoA*, *nifH, pmoA*, or *dsrA* (van Elsas & Boerma, 2011). The qPCR may be well employed to assess to what extent, local conditions affect gene and gene expression levels. As PCR based on soil/compost DNA extracts, qPCR may be subjected to some biases.

Stable isotopes probing (SIP)

Stable isotopes probing (SIP) offers a powerful technique for identifying microorganisms that are actively involved in specific metabolic processes (Radajewski *et al.,* 2000). Different methods have been proposed such as the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA followed by fingerprinting of the active communities (Artursson *et al.,* 2005), or the incorporation of ^{13}C (stable isotope labeling) into cellular biomarkers (DNA, RNA, PFLAs) followed by separation and fingerprinting (Boschker *et al.,* 1998; Radajewski *et al*., 2000; Manefield *et al.,* 2002). Recently, Peng *et al.* (2013) used $^{13}C4$-pyrene to study the microorganisms responsible for the degradation of pyrene during composting. The coupling of molecular biological methods with stable-isotope abundance in biomarkers has provided a cultivation-independent means of linking the identity of microorganisms with their function in the environment (Radajewski *et al.,* 2000).

7.3.2.2. Whole community analysis approaches

These techniques attempt to analyze all the genetic information present in total DNA extracted from an environmental sample, in comparison to PCR-based molecular approaches that target only a single or few genes. These approaches include DNA-DNA hybridisation, Guanine-plus-citosine (G+C) content fractionation, whole genome sequencing and metagenomics.

DNA-DNA hybridisation

Whole-genome DNA–DNA hybridization (DDH) offers true genome-wide comparison between organisms. This technique measures the degree of genetic similarity between pools of DNA sequences. A value of 70% DDH was proposed as a recommended standard for bacterial species delineation (Goris *et al.,* 2007). Typically, bacterial species having 70% or greater genomic DNA similarities usually have >97% 16S rRNA gene sequence identity. Although DDH techniques have been originally developed for pure culture comparisons, they have been modified for its use in whole microbial community analysis (Rastogi & Sani, 2011).

Guanine-plus-cytosine (G+C) content fractionation

Guanine-plus-citosine (G+C) content technique is based on the fact that different prokaryotic groups differ in their guanine-plus-cytosine (G + C) content of DNA, and phylogenetically related bacterial groups only vary by 3–5% in their G + C content (Nüsslein & Tiedje, 1999). The total community DNA is physically separated by density-gradient centrifugation, into highly purified fractions, each representing a different G + C content that can be analyzed by additional molecular techniques (e.g. DGGE or ARDRA) to

better assess total community diversity (Rastogi & Sani, 2011). G + C fractionation has been widely applied in investigation of soil microbial communities to evaluate for instance, the effect of compost application on soil microbial communities (Rastogi & Sani, 2011).

Whole-microbe-genome sequencing

Exploring microbial systems through whole-genome analysis is a comprehensive and integrated approach to understand microbial ecology and function. Whole microbial genomes are sequenced using a shotgun cloning method that involves (1) extraction of DNA from pure cultures, (2) random fragmentation of obtained genomic DNA into small fragments of ~2 kb, (3) ligation and cloning of DNA fragments into plasmid vectors, and (4) bidirectional sequencing of DNA fragments. Once the sequences are obtained, they are aligned and assembled into finished sequences using specialized computer programs such as MEGAN (*ME*ta*G*enome*AN*alyzer) (Huson *et al.,* 2007). The classical shotgun sequencing was based on the Sanger sequencing method. This method is based on the use of dideoxynucleotides (ddNTPs) in addition to the normal nucleotides (dNTPs). The extension of a newly synthesized DNA strand terminates every time the corresponding ddNTP is incorporated. Moreover, one of the nucleotides or the sequencing primer should be radioactively or fluorescently labeled so that the final product can be detected. The large-scale sequencing technologies, which are discussed in the following section, are useful for whole-genome sequencing as well as metagenomics and metatranscriptomics.

Metagenomics (next-generation sequencing methods)

Metagenomics is the direct investigation of collective microbial genomes contained within an environmental sample (Riesenfeld *et al.,* 2004). The field initially started with cloning environmental DNA, being the Sanger sequencing method one of the most widely used during the last decades. It is still in use but in a small scale. Recently, few novel and powerful sequencing techniques (Next-generation sequencing methods) namely 454-based/pyrosequencing and Illumina/Solexa's Genome Analyzer sequencing, have been developed. They consist of multiparallel sequencing by synthesis, in which the pyrophosphate that is released is detected in an enzymatic cascade ending in luciferase and followed by the detection of the emitted light. Meanwhile pyrosequencing allows the production of hundreds of thousands to millions of 450-bp read in a run, Solexa platform offers a higher magnitude of reads but lower lengths (van Elsas & Boerma, 2011). In the last years, new systems namely Ion Torrent PGM, PacBio RS and MiSeq, have revolutionized the genome studies of collective microbial communties. These next generation plataforms are faster and less expensive than traditional Sanger sequencing (Metzker, 2010).

Some of the limitationss of these techniques are the high costs required for the analysis of the inmense amount of data obtained which may be limited by human capability and the ability of databases to deal with errors. On the other hand, the ultra-high throughput and lack of biases of these methods will allow the discovery of many new sequences, which is very important in the case of compost. Compost communities are extremely diverse and may contain a large microbiota represented by an enormous

number of low-abundance unique taxa, which may be involved in compost suppressiveness. This fact highlights the importance of large-scale sequencing techniques in investigating the highly diverse compost microbial communities (Lauber *et al.,* 2009; Neher *et al.,* 2013). Metagenomic investigations have been conducted in an extent variety of environments including composts (Dougherty *et al.,* 2012; DeGannes *et al.,* 2013a,b; Yeh *et al.,* 2013) and have provided access to phylogenetic and functional diversity of uncultured microorganisms (Handelsman, 2004).

7.3.2.3. Postgenomic approaches

The inability of DNA-based molecular techniques to provide information of the gene expression (functionality) as it occurs under *in situ* conditions (Wilmes & Bond 2006), has prompted the development of postgenomic approaches such as metatranscriptomics, metabolomics and metaproteomics. These new approaches reveal the link between genetic potential and functionality in microbial communities (Rastogi & Sani, 2011).

Metatranscriptomics

Metatrascriptomic (or environmental transcriptomic) involves random sequencing of microbial mRNA allowing monitoring of microbial gene expression profiles in natural environments at a particular time and place (Moran, 2009). This approach, compared to metaproteomic, has a high-resolved view of instanteous regulatory responses (Moran, 2009). The major challenge in metatranscriptomics is the fact that prokaryotic microbial mRNA transcripts are not polyA tailed, so obtaining complementary DNA (cDNA) is not easy. This results in coextraction of more abundant rRNA

molecules in the total RNA pool, which can lead to overwhelming background sequences in a largescale sequencing analysis (Rastogi & Sani, 2011).

Metabolomics

Metabolomic allows monitoring of low molecular weight metabolites produced by the extant microbial communities in certain environment conditions. The set of metabolites synthesized by an organism constitute its metabolome (Oliver *et al.,* 1998). Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002). Therefore, this approach has a high potential to elucidate changes in the levels of metabolites due to its sensibility.

Methodologically, the study of metabolome involves firstly the extraction of the metabolites and secondly, their detection. Different methods of metabolites extraction may be used. Frequently, polar organic solvents like methanol, methanol-water mixtures or ethanol are added to the sample, followed by an additional step of using non-polar solvents such as chloroform (Fienh, 2002). The metabolites detection is performed using analytical techniques such as Nuclear Magnetic Resonance (NMR) or mass spectrometry (MS). MS can be performed without previous separation of metabolites or after separation through gas chromatography (GC), High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography-mass spectrometry (UPLC).

Metaproteomics

Metaproteomics reveal information on proteins expressed by environmental microbial communities at a given point in time (Wilmes & Bond, 2006). Protein biomarkers are more reliable, compared to other cell molecules (eg. lipids or nucleic acids), and provide a clearer picture of metabolic functions than functional genes or even the corresponding mRNA transcripts of microbial communities (Wilmes & Bond, 2006; Beendorf *et al.*, 2007). Metaproteome analysis involves the extraction of total proteins from an environmental sample, separation by 1-D or 2-D electrophoresis and followed by digestion of protein spots to identify the proteins by MS or chromatography. Once the proteins are identified, it could be possible to link them with their metabolic functions (eg. involved with suppressive activities) and at the same time those to individual microbial species.

Despite all progress made in this area, one of the pitfalls of metaproteomics is the lack of available sequences in the dabatases which hinder the idenfitication of isolated proteins (Bastida *et al.*, 2009). Moreover, metaproteomics of environmental samples is a challenge regarding resolution and yield of proteins (Keiblinger *et al.*, 2012). In this respect, the preparation of protein extracts is of paramount importance and its application to soil or compost requires considerably improved protocols of protein extraction and sample preparation (Benndorf *et al.*, 2007). Until now, several authors have reported and reviewed different methods to extract proteins from environmental microbial communities including soil and water which could be used for the study of compost microbial communities as well (Benndorf *et al.*, 2007; Keiblinger *et al.*, 2012; Bastida *et al.*, 2014).

II

Chapter II. Interest and aims of study

II. INTEREST AND AIMS OF STUDY

Nowadays, there are several plant pathogens that cause serious diseases on greenhouse vegetable crops resulting in significant crop losses. *P. nicotianae* is a threat to plant productivity on a global scale for a broad range of hosts. It is the causal agent of Phytophthora root rot, which is considered the most potentially destructive disease of cultivated pepper (Pomar *et al.,* 2001). It is important to note that Spain is the first producer of pepper in Europe and the fifth in the world (FAOSTAT, 2011). On the other hand, *F. oxysporum* f.sp. *melonis* is the main causal agent of Fusarium wilt in muskmelon, which is the most severe infectious disease of this cucurbit (Luongo *et al.,* 2012). Muskmelon production and yield losses in Spain are of high relevance, since Spain is the main muskmelon producer in Europe (FAOSTAT, 2011).

Chemical pesticides have been extensively used to control these soil-borne pathogens. However, the increasing public concern about their adverse effects in the environment and human health has encouraged the research of alternative methods to control root rot and wilt diseases. Moreover, the current strategies to control both pathogens are expensive and not always effective.

To this respect, the cornerstone of the successful disease management of these two soil-borne pathogens consists of an accurate identification, early detection and a sustainable management. PCR-based assays have become powerful tools for the rapid diagnosis of plant diseases. These methods, with especial emphasis on real time PCR (qPCR), stand out among the techniques used to detect and identify soil-borne pathogens because its speed and high

sensitivity (Schena *et al.,* 2004). An early and accurate detection is of paramount importance to optimize the strategies to control plant pathogens. The detection of pathogens prior to planting will allow avoiding the use of infected soils and the transplantation of contaminated plant material as well as reducing pathogen dispersal (Bilodeau *et al.,* 2012; Sanzani *et al.,* 2013). At the same time, the development of new non-chemical strategies for disease management has focused essentially on biological control practices. Among them, the exploitation of the ability of compost to suppress diseases is regarded to have great potential (Pane *et al.,* 2011).

Composts based on agricultural and agro-industrial waste are generally free of xenobiotics and excessive heavy metal concentrations (Ntougias *et al.* 2008). Moreover, these wastes are generated in high quantities in the Mediterranean basin, composting being one of the most feasible solutions for their treatment and valorization (Kavroulakis *et al.,* 2010). In addition, it is worth mentioning the ability of some of these composts to suppress a broad range of soil-borne pathogens when used as growing media.

Currently, multiple routes that converge on the production of effective composts are available. The identification of predictor parameters that determine the suppressiveness of composts on the basis of pure compost characteristics is considered essential, which could improve the results obtained with its use and promote its commercialization (Hadar & Papadopoulou, 2012). Besides, the enrichment of composts with specific strains of biological control agents (BCAs) (e.g. *Trichoderma* spp.) has been suggested to improve the suppressive potential of composts (López-Mondéjar

et al. 2010). Among BCAs, *Trichoderma* spp. are widely used due to their ability to inhibit a wide range of plant pathogens (Verma *et al.,* 2007).

In this sense, the **main objective** of this Thesis was the development of innovative strategies to improve the efficacy of the biological control against the soil-borne pathogens *P. nicotianae* (for Phytophthora root rot control), and *F. oxysporum* f. sp. *melonis* (for Fusarium wilt control).

This main objective was developed through the following **specific objectives** or **actions**:

- To establish a TaqMan system to detect and quantify *P. nicotianae* in different environmental samples such as soil, compost and plant material and to screen its presence as well as its genetic diversity in greenhouses located in southeast Spain.
- To evaluate the use and feasibility of dPCR in comparison to qPCR as a new PCR-based technique to detect and quantify *P. nicotianae* in soil and plant samples.
- To characterize a batch of agricultural and agro-industrial composts by the study of their physical, chemical and biological properties using a broad array of techniques such as metagenomics, metabolomics, and ^{13}C-NMR, to evaluate and predict their ability to control Phytophthora root rot in pepper and Fusarium wilt in muskmelon.
- To produce *T. harzianum*-amended compost and to investigate the capability of this BCA to induce changes in the biotic and abiotic characteristics of the composts as well as to control Fusarium wilt in muskmelon compared to the non-amended compost and peat.

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III

Chapter III. Publications

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PUBLICATION 1

Characterization of *Phytophthora nicotianae* isolates in southeast Spain and their detection and quantification through a real-time TaqMan PCR

Josefa Blaya, Carmen Lacasa, Alfredo Lacasa, Victoriano Martínez, Ana B Santísima-Trinidad, Jose A Pascual, Margarita Ros

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Characterization of *Phytophthora nicotianae* isolates in southeast Spain and their detection and quantification through a real-time TaqMan PCR

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Abstract

BACKGROUND: The soil-borne pathogens *Phytophthora nicotianae* and *P. capsici* are the causal agents of root and stem rot of many plant species. Although *P. capsici* was considered the causal agent in one of the main pepper production areas of Spain to date, evidence of the presence of *P. nicotianae* was found. We aimed to survey the presence of *P. nicotianae* and study the variability in its populations in this area in order to improve the management of Tristeza disease.

RESULTS: A new specific primer and a TaqMan probe were designed based on the internal transcribed spacer regions of ribosomal DNA to detect and quantify *P. nicotianae*. Both morphological and molecular analysis showed its presence and confirmed it to be the causal agent of the *Phytophthora* disease symptoms in the studied area. The genetic characterization among *P. nicotianae* populations showed a low variability of genetic diversity among the isolates. Only isolates of the A2 mating type were detected.

CONCLUSIONS: Not only is a specific and early detection of *P. nicotianae* essential but also the study of genetic variability among isolates for the appropriate management of the disease, above all, in producing areas with favorable conditions for the advance of the disease.
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Supporting information may be found in the online version of this article.

Keywords: *Phytophthora nicotianae*; Tristeza disease; TaqMan real-time PCR; mitochondrial DNA; genetic diversity

INTRODUCTION

Sweet pepper (*Capsicum annum* L.) is one of the main horticultural crops in Murcia (southeast Spain), with over 1379 ha of total cultivated area under greenhouses (Estadística Agraria de la Región de Murcia, 2010). *Phytophthora capsici* is a soil-borne oomycete pathogen of pepper which stands out among plant pathogens because it is a threat to plant productivity on a global scale for a broad range of hosts.[1] Erwin and Ribeiro[1] showed that *P. capsici* is the main causal agent of root and crown rot (Tristeza disease) of *C. annuum* in many countries, and its presence in southeast Spain has been confirmed by Tello.[2] However, other *Phytophthora* species and other genera have been associated with this disease. *Phytophthora nicotianae* van Breda de Haan (=*Phytopthora parasitica* Dastur (1896)) has been reported as a pathogen to pepper plants in different countries such as the USA, India, Tunisia and Spain.[1,3–5] Recently, we have observed that the symptoms of Tristeza disease in pepper plants in southeast Spain (Murcia) are mainly caused by *P. nicotianae*. Nevertheless, *P. capsici* was considered the causal agent to date in this area. Due to similarities of symptoms on roots and collar, *P. capsici* and *P. nicotianae* may cause diagnostic confusion.[6]

The control of soil-borne disease caused by *Phytophthora* spp. is often difficult owing to the release into the soil of resistant structures, oospores and/or chlamydospores. Early detection and diagnosis of pathogens in plants and soil are very important to determine their transmission modes[7] and reduce crop losses. Polymerase chain reaction (PCR)-based techniques have become the primary method of identifying plant pathogens.[8,9] Real-time PCR-based techniques are faster, more sensitive and more easily automated, and do not require post-amplification procedures. The quantitative detection of plant pathogens facilitates

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the monitoring of pathogens and the study of their distribution, enabling improved disease control and minimum usage of fungicides.[9,10]

Once an epidemic has developed in a production site, it is often difficult to determine how the causal organism was introduced, how it is spread and its persistence over time.[11] Specific studies to evaluate intraspecific genetic variability and to establish the possible pathways by which the pathogen has been introduced and distributed to new areas are quite limited.[12] Different approaches have been used: random amplified polymorphic DNAs (RAPDs),[13] amplified fragment length polymorphism (AFLP),[11] microsatellites or simple sequences repeats (SSRs).[14] Recently, analyses of different intergenic regions of mitochondrial DNA were suggested to be suitable for the examination of intraspecific variation and the analysis of closely related species.[15] Intraspecific polymorphisms in mitochondrial DNA have been useful for characterizing populations by mitochondrial haplotypes for *Phytopthora infestans*,[16] *Phytophthora ramorum*[17] and *Phytophthora nicotianae*.[12] Detection and variability in *P. nicotianae* populations is an existing challenge to manage Tristeza disease, and it is critical to understand the diversity of the pathogen infecting different crops to design more efficient disease management programmes.

The aims of the present work were (i) to prove the presence of *P. nicotianae* in greenhouse pepper crops in the area of Murcia (southeast Spain) by morphological characteristics and molecular tools, (ii) to develop a real-time PCR method to detect and quantify *P. nicotianae* in pepper stem and soil samples and (iii) to characterize genetically the different populations of *P. nicotianae* isolated from the area of study.

Table 1. Oomycete and fungal isolates used in this study to evaluate the specificity of primers Nic-F1/ Nic-R4 and probe Nic-Pro

Species	Collection code[a]	Origin	Real-time PCR[b]
P. nicotianae	IMIDA Pn1	Murcia, Spain	+
P. nicotianae	IMIDA Pm61	Murcia, Spain	+
P. nicotianae	IMIDA Pm62	Murcia, Spain	+
P. nicotianae	IMIDA Pn12	Pontevedra, Spain	+
P. nicotianae	IMIDA Pn2	Badajoz, Spain	+
P. nicotianae	IMIDA Pn4	California, USA	+
P. capsici	IMIDA Pc9	Almería, Spain	−
P. cactorum	IMIDA Pc1	Almería , Spain	−
P. ramorum	IMIDA Pc2	Norway	−
P. infestans	CECT 20799	Unknown	−
P. citrophthora	CECT 2353	Germany	−
P. cryptogea A1	IMIDA Pc3	Sevilla, Spain	−
P. cryptogea A2	IMIDA Pc4	Sevilla, Spain	−
P. cinnamoni	IMIDA Pc5	Extremadura, Spain	−
Pythium spp.	CEBAS 2000	Unknown	−
Pythium ultimun	CECT 2365	Spain	−
Pythium aphanidermatum	CEBAS H52	Murcia, Spain	−
Alternaria spp.	CEBAS 1050	Murcia, Spain	−
Botrytis cinerea	CEBAS H1	Murcia, Spain	−
Fusarium oxysporum f. sp. *melonis*	CEBAS 1014	Murcia, Spain	−
F. oxysporum f. sp. *lycopersici*	CECT 2715	USA	−
Rhizoctonia solani	CECT 2815	Spain	−
Sclerotinia sclerotium	CECT 2769	Netherlands	−
Penicillium spp.	CEBAS H20	Murcia, Spain	−

[a] IMIDA, Culture Collection from Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario; CECT, Spanish Type Culture Collection; CEBAS, Culture Collection from Centro de Edafología y Biología Aplicada del Segura.
[b] +, significant amplification; −, no significant amplification. Evaluation with primers Nic-F1/Nic-R4 and probe Nic-Pro.

MATERIAL AND METHODS

Soil and pepper stems samples

Pepper plants with *Phytophthora* disease symptoms as well as soil samples adhering to the roots of those plants were collected from 12 commercial production greenhouses located in the most important pepper-producing area of the province of Murcia (southeast Spain) (supporting information, Table S1) in 2012. A total of 45 soil samples and 45 stem samples were collected. From two of the greenhouses, soil samples collected in 2011 were added to the study (Table S1).

Fungal isolates

A piece from each pepper stem collected with *Phytophthora* disease symptoms was surface disinfested with sodium hypochlorite (1%) for 5 min and placed on V8 juice agar.[1] The plates were incubated at 28 °C for 5 days in darkness for further DNA extraction and morphological characterization. Moreover, each isolate was transferred to pea agar plates (100 g L^{-1} ground peas, 100 mg L^{-1} β-sitosterol (Sigma-Aldrich, St Louis, MO, USA) and 20 g L^{-1} technical agar (Scharlau, Barcelona, Spain), adjusted to pH 5.5, autoclaved at 121 °C for 20 min and amended with 100 mg L^{-1} sterilized streptomycin) for further zoospore production.

The remaining oomycetes and fungi (*Phytophthora* spp. and other fungal species) were obtained from the collection at Instituto Murcia de Investigación y Desarrollo Agrario y Alimentario (IMIDA, La Alberca Spain), from Centro de Edafología y Biología Aplicada del Segura (CEBAS, Espinardo, Spain), University of Almeria, Centro de Investigacion La Orden-Valdesequera or purchased from Colección Española de Cultivos Tipo (CECT, Paterna, Spain) (Table 1). All isolates were stored on PDA plates at 25 °C for *Phytophthora* isolates or 4 °C for the rest of the fungi.

DNA extraction from isolates, soil and stems

A collection of isolates (12) was obtained from the survey greenhouses in 2012, two were obtained in 2011 (CC47 and CC46) and two were obtained from infected pepper plants in the northwest (CC43) and west of Spain (CC45) (Tables 1 and S1). The isolates were grown for 7 days on PDA plates. Mycelium was collected and ground in liquid nitrogen with a plastic micropestle. DNA extraction was carried out with phenol and chloroform, followed by isopropanol precipitation according to Hartl and Seiboth.[18] DNA concentration was measured with Nanodrop ND-1000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA).

Total DNA was extracted from soil samples using the Fast DNA kit for soil (Q-Biogene, Inc., Irvine, CA, USA), following the modification described by López-Mondéjar *et al.*[9] Samples were previously ground with liquid nitrogen and kept at −20 °C.

Total DNA from pepper stems was extracted according to the protocol of Doyle and Doyle.[19] Briefly, samples were ground with liquid nitrogen using a micropestle. The powder was transferred to a tube and mixed with extraction buffer (1.4 mol L^{-1} NaCl, 20 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA),

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Table 2. Primers and probe used in this study

Primer/probe	Sequence (5′–3′)	Reference
Ph2	ATACTGTGGGGACGAAAGTC	Ippolito *et al.*[23]
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*[38]
Mt2F	TGGCAGACTGTAAATTTGTTGAA	Schena and Cooke[15]
Mt5R	TTGCATGTGTTAAGCATACCG	Schena and Cooke[15]
Mt17f	AAATACTTTTTAACAAAAGGGAATTTA	Mammella *et al.*[12]
Mt12r	TGGAGTTGCTGGATCTTGAA	Mammella *et al.*[12]
CAPFW	TTTAGTTGGGGGTCTTGTACC	Silvar *et al.*[36,39]
CAPRV1	CCTCCACAACCAGCAACA	Silvar *et al.*[36,39]
Nic-F1	CCTATCAAAAAACAAGGCGAACG	Li *et al.*[7]
Nic-R4	CAGAGACTTTCGTCCCCACAGT	–
Nic-Pro	5′-6-FAM-CTTCGGCCTGATTTAGT AGT-MGBNFQ	–

100 mmol L^{-1} Tris pH 8.0, 2% cetyltrimethyl ammonium bromide (CTAB), 2% polyvinyl pyrrolidone (PVP)). Extraction with chloroform–isoamyl alcohol (24:1) was carried out. Finally, DNA was precipitated with isopropanol, washed with cold ethanol (70%) and resuspended in Tris–EDTA buffer (10 mmol L^{-1} Tris–HCl, 1 mmol L − 1 EDTA.Na$_2$) overnight. Total DNA extraction from mycelia, soil and stem samples was kept at −20 °C for further analysis.

Identification and morphological characterization of isolates

Ribosomal DNA sequencing

For each isolate internal transcribed spacer (ITS)-based identification was completed using the primers Ph2 and ITS4 (Table 2). The PCR mixture (25 μL) contained a final concentration of 1× PCR buffer, 0.2 mmol L^{-1} dNTPs mix, 0.1 mg mL^{-1} bovine serum albumin (BSA, 5 mg mL^{-1}), 20 mmol L^{-1} tetramethylammonium chloride (TMA), 0.1 μmol L^{-1} of each primer, 0.75 U Taq polymerase (1 U μL^{-1}, Biotools, Madrid, Spain) and 100 ng DNA template. The PCR temperature programme was: 94 °C for 5 min; 94 °C for 1 min, 51 °C for 1 min 30 s and 72 °C for 2 min, 35 cycles; 72 °C for 5 min. Products were separated by electrophoresis in 1× Tris–acetate–EDTA (TAE) buffer on 1% agarose gels. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Inc., Venlo, Netherlands) according to the manufacturer's instructions. PCR products were sequenced using primer Ph2 and compared with nucleotide sequences in GenBank.

Morphological characterization of isolates

All isolates were examined for taxonomic identification according to Erwin and Ribeiro.[1] Morphological characteristics of the isolates such as sporangia type, length, breadth and length/breadth ratio of sporangia were measured. To assess their mating type, each isolate was paired with known A1 and A2 strains of *P. cryptogea* (Table 1).

The ability of isolates to grow at 36 and 37 °C was evaluated by incubating V8 plates containing actively growing mycelium (grown at 25 °C for 24 h) at those temperatures for 72 h in darkness. The growth rate at 25 °C (mm d^{-1}) was evaluated by transferring PDA agar plugs (diameter 7 mm) containing actively growing mycelium into Petri dishes with PDA and incubating in darkness, on four replicates per isolate until the entire plate was covered.

To evaluate the pathogenicity of the different isolates, 2-week-old pepper (*Capsicum annuum* cv. Lamuyo) seedlings previously grown in autoclaved vermiculite were transplanted to individual 200 mL pots containing autoclaved vermiculite and were inoculated with a concentration of 10^4 zoospores mL^{-1} by adding 5 mL of the inoculum. To obtain zoospore production, pea agar plates containing the isolates grown were cut into small pieces and added to an Erlenmeyer flask containing sterile distilled water, which was incubated at 25 °C for 96 h under continuous fluorescent light conditions. Erlenmeyer flasks were chilled at 4 °C for 1 h and then incubated at 25 °C for 20 min to allow zoospore release. Zoospore suspensions were collected in sterile 50 mL centrifuge tubes, vortexed for 1 min and adjusted to 10^4 zoospores mL^{-1} by counting with a haemocytometer. Previously, zoospore suspensions were filtered through Whatman paper No. 1 to remove hyphal and sporangial debris. Three pots per isolate containing four plants each were arranged randomly inside a greenhouse. Disease incidence was determined for 43 days after inoculation (DAI). Re-isolations were made on to V8 broth culture media to confirm that the disease was caused by the inoculated pathogen. Control pots were inoculated with 5 mL distilled water.

Primers and probe design for *P. nicotianae*, specificity and sensitivity

A collection of the ITS region sequences available in the NCBI database were aligned (ClustalW) to design specific primers and a TaqMan probe for *P. nicotianae*. The primer Nic-F1 published by Li *et al.*[7] was used as a forward primer, while the reverse primer and TaqMan probe were designed *de novo* (Table 1). Real-time PCR amplifications were performed in a total volume of 25 μL using a 7500 fast real-time PCR system (Applied BioSystems, Inc., Foster City, CA, USA). The reaction mixtures contained a final concentration of 2× Premix Ex taq™ (Takara, Shiga, Japan), 0.3 μmol L^{-1} each primer, 0.2 μmol L^{-1} TaqMan probe, 0.1 mg mL^{-1} BSA (5 mg mL^{-1}, Sigma), 0.2 μL ROX reference dye II (Takara), 1 μL DNA template and sterile water. The thermal cycling conditions for amplification were an initial denaturation at 95 °C for 30 s, followed by 40 cycles each consisting of a denaturation step at 95 °C for 10 s, annealing at 60 °C for 40 s and a final step at 50 °C for 2 min. The amplification results were analysed with 7500 Fast Real-Time PCR software v2.0 (Applied BioSystems).

The sensibility of the primers NicF1-NicR4 and the probe Nic-pro were tested with a standard curve. A fragment of 108 bp from the selected ITS region of one isolate of *P. nicotianae* was cloned into vector PCR II (Invitrogen, Inc., Carlsbad, CA, USA). The transformation was made in *Escherichia coli* DH5α cells (Invitrogen) and purified with a QIAprep Miniprep Kit (Qiagen). The restriction enzyme *EcoRI* was used to confirm the presence of the inserts. The DNA concentration of the plasmid standard solution was spectrophotometrically measured using Infinite® 200 PRO (Tecan Trading AG, Männedorf, Switzerland), after Picogreen reagent staining according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA) and was related to the known molecular weight of a single plasmid molecule to calculate the number of copies according to the following equation:

$$\text{number of copies} = \frac{a \times 6.022 \cdot 10^{25}}{b \times 1 \cdot 10^{9} \times 650}$$

where a is the DNA concentration of the plasmid and b is the total length of the template. The concentration was adjusted to the number of 10^{10} ITS copies, and the standard was diluted in 10-fold steps to obtain the standard curve (supporting information, Fig. S1).

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Primer specificity was assessed using different *Phytophthora* spp. isolates and other pathogens (Table 1).

Amplification of *Phytophthora* spp. in naturally infested soils and pepper stems

Stems of pepper plants with Tristeza disease symptoms as well as the soil samples adhering to the harvest plants collected were assayed to detect the presence of *P. capsici* and *P. nicotianae*, through real-time PCR using the primers CAPFW/CAPRV1 and Nic-F1/Nic-R4 and the probe Nic-pro (Table 2) respectively.

Genetic diversity of mtDNA intergenic regions, sequence analysis and phylogenetic analysis of isolates

Two intergenic regions were amplified.[12] The first one, flanked by genes *trnY* and *rns* using primers Mt2f-Mt5r (Table 2) and the second one, flanked by genes *trnW* and *cox2*, using primers Mt17f-Mt12r (Table 2). The PCR was performed in a total volume of 50 μL reaction mixture containing 100 ng DNA template, 5× PCR buffer, 0.1 mmol L^{-1} dNTPs mix, 4 mmol L^{-1} $MgCl_2$, 0.2 μmol L^{-1} of each primer, 1.0 U Taq polymerase (1 U μL^{-1}, Biotools) and sterile water. Amplification conditions were: 94 °C, 3 min; 94 °C, 45 s; 60 °C, 30 s; 72 °C, 45 s – 35 cycles; 72 °C, 10 min. Products were separated by electrophoresis in 1× TAE buffer on 2% agarose gels and purified. Sequences were realized in both directions using amplification primers. Chromas Lite 2.01 software was used to evaluate sequences and to create consensus sequences. Sequences were aligned, analysed and edited manually using MEGA v5.[20] Sequences of primers were removed.

Polymorphisms in the sequences among isolates were noted and classified as a separate haplotype (Table 2). A haplotype network visualizing the relationship among the various haplotypes was calculated using SplitsTree v. 4.13.1[21] using the unmodified dataset with uncorrected P and a NeighbourNet network calculation.

The two regions were only analysed individually and, since no differences were observed in one of the regions, no combined data were produced. A maximum likelihood analysis was performed using MEGA v5.[20] The statistical support was determined by bootstrap values from 1000 replicates.

Statistical analysis

Testing for difference between samples was performed with the non-parametric Kruskal–Wallis test. The statistical software Statistica 10.0 (StatSoft, Inc., Tulsa, OK, USA) was used for analysis.

RESULTS

Identification and characterization of isolates

Twelve isolates obtained from the studied area located in south-east Spain were identified as *P. nicotianae* based on morphological characteristics[1] and through ribosomal DNA sequencing using *Phytophthora* species primers Ph2 and ITS4 (Table 2) and compared to other sequences deposited in GenBank (NCBI database, USA). The DNA sequences were submitted to GenBank with accession numbers KJ000327–KJ000340.

All isolates produced papillate and no caduceus sporangia. Sporangia were mainly ovoid but also globose, ellipsoid and obturbinate. The presence of chlamydospores and hyphal swellings was also evidenced (Fig. 1). Although the values of different parameters of the sporangia such as length, breadth and length/breadth ratio were typical of *P. nicotianae*, significant differences ($P \leq 0.0001$, 299 d.f.) were shown among isolates (Table 3). All the isolates were A2 mating type showing amphigynous antheridia and smooth-walled oogonia, and produced plerotic

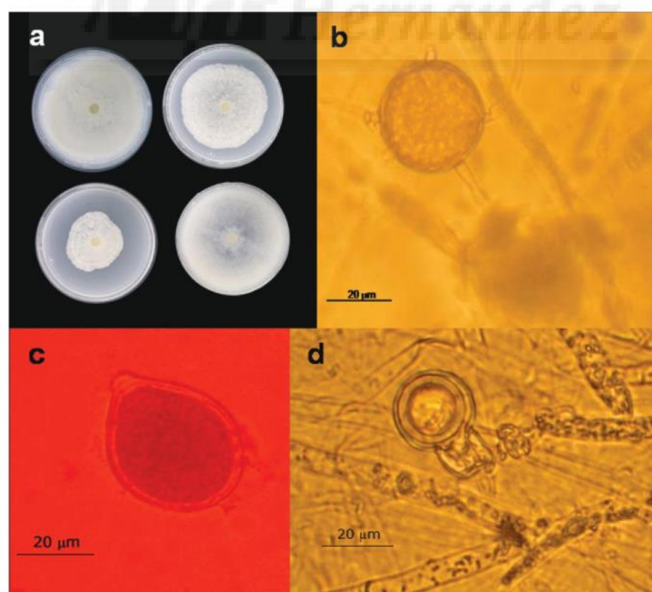

Figure 1. Morphological characteristics of *Phytophthora nicotianae*: (a) mycelial growth in PDA of different isolates; (b) chlamydiospore; (c) papillate sporangia; and (d) oospore with an amphigynus antheridum and smooth-walled oogonia.

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Table 3. Isolates of *Phytophthora nicotianae* included in the study, their sporangia length, sporangia breadth, sporangia Length/breadth ratio growth ratio, mating type and pathogenesis rate. Amplicon size, accession numbers and haplotypes are reported for the *trnY-rns* mitochondrial region sequenced in this study

Isolate	Length (μm)	Breadth (μm)	Length/ breadth ratio	Growth ratio (25 °C)	Mating type	Pathogenicity (% dead plants)	Amplicon size	Haplotype
CC2	42.91 ± 1.42	35.35 ± 1.75	1.25 ± 0.04	10.71 ± 0.33	A2	50	387	2
CC5	53.28 ± 1.51	43.34 ± 1.64	1.25 ± 0.03	8.71 ± 0.27	A2	16.67	387	2
CC8	49.39 ± 1.31	38.59 ± 0.99	1.25 ± 0.02	12.07 ± 0.00	A2	58.33	387	2
CC13	51.55 ± 1.44	41.18 ± 1.27	1.26 ± 0.02	10.22 ± 0.20	A2	25	387	2
CC15	48.53 ± 0.98	34.85 ± 0.97	1.41 ± 0.03	8.92 ± 0.38	A2	33.33	387	2
CC19	51.98 ± 1.68	40.90 ± 1.48	1.28 ± 0.02	10.56 ± 0.08	A2	41.67	386	1
CC21	52.27 ± 1.29	40.61 ± 1.17	1.30 ± 0.03	6.47 ± 0.17	A2	50	386	1
CC25	47.95 ± 1.26	37.73 ± 1.12	1.28 ± 0.02	6.13 ± 0.04	A2	75	388	3
CC28	38.74 ± 0.91	30.10 ± 0.85	1.30 ± 0.02	9.96 ± 0.10	A2	66.67	388	3
CC32	42.48 ± 1.42	31.25 ± 0.99	1.36 ± 0.03	10.88 ± 0.22	A2	58.33	387	4
CC34	48.67 ± 1.08	39.89 ± 1.06	1.23 ± 0.03	4.45 ± 0.36	A2	91.67	387	4
CC48	40.90 ± 1.44	32.69 ± 1.27	1.26 ± 0.02	10.32 ± 0.14	A2	25	388	3
CC46	–	–	–	–	A2	–	387	2
CC47	–	–	–	–	A2	–	386	1
CC43	–	–	–	–	A2	–	386	2
CC45	–	–	–	–	A2	–	386	2

Data are means ± SE.

oospores. All the isolates continued growing at 36 °C whereas growth was inhibited when the isolates where incubated at 37 °C for 24 h. Growth resumed when the incubation temperature was changed to 25 °C (data not shown). Growth rates of isolates on PDA at 25 °C also showed significant differences ($P < 0.0001$, 47 d.f.) between isolates (Table 3). Regarding pathogenicity, significant differences ($P = 0.0015$, 35 d.f.) were observed among isolates, with isolate CC34 showing the highest percentage of dead plants and isolate CC5 the lowest one (Table 3 and Fig. 2). All the isolates were capable of inducing crown and root rot and necrosis.

Specificity and sensitivity of the primers and probe for *P. nicotianae*

A BLAST search was performed to check the specificity of the set of primers Nic-F1/Nic-R4 and TaqMan probe Nic-Pro and showed 100% sequence similarity of each *P. nicotianae* in the database. Also, the set of specific primers together with the fluorescent probe amplified a 108 bp fragment from all *P. nicotianae* used in this study (Table S1). No amplification was achieved with the other species studied (Table 1).

Sensibility to *P. nicotianae* was tested through serial dilutions of the plasmid over 10 orders of magnitude, starting with an

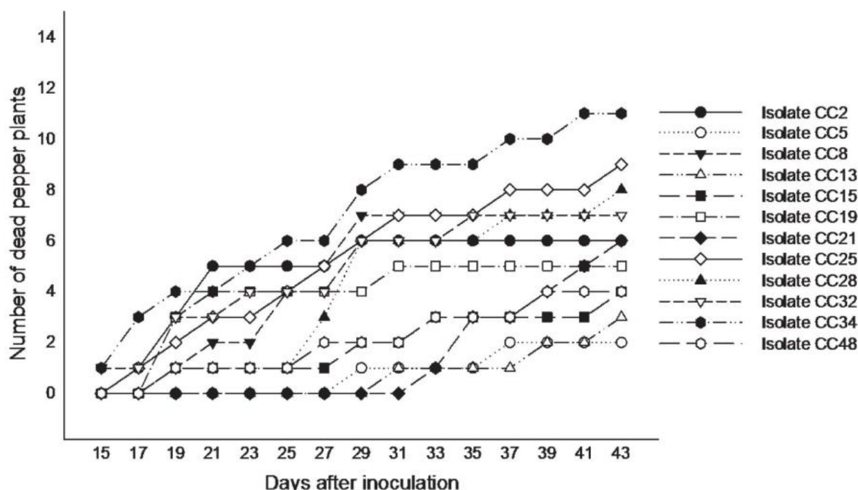

Figure 2. Disease incidence of pepper plants inoculated with 12 different isolates of *Phytophthora nicotianae*. Each plotted point is the number of dead pepper plants at different days after inoculation.

initial concentration of 228.5 ng DNA μL^{-1}, which was calculated as representing 1×10^{10} ITS copies μL^{-1}. A standard curve ranging over seven orders (from 10^7 to 10 ITS copies μL^{-1}) of magnitude was achieved with an efficiency of 98.5% (Fig. S1).

Detection of *Phytophthora* spp. in naturally infested pepper stems and soils

Stems of pepper plants with Tristeza disease symptoms and field soil samples from 12 greenhouses included in this study (Table S1) were studied for the presence of *P. nicotianae* and *P. capsici* through real-time PCR using the specific primers Nic-F1/Nic-R4 and the probe Nic-pro and the primers CAPFW/CAPRV1, respectively (Table 2). *Phyophthora nicotianae* was detected in all soil samples, while *P. capsici* was only detected in two greenhouses (GH V2 and GH K′). *Phytophthora capsici* was not found in any stem sample, while *P. nicotianae* was detected in all of the stem samples. The presence of *P. nicotianae* in soils ranged from 5.61 to 7.99 log ITS copies g^{-1} in greenhouses SM2 and VJ8, respectively, and values of *P. nicotianae* in stems ranged between 6.11 and 11.18 log ITS copies g^{-1} in greenhouses GH V2 and GH MSP2, respectively. There were significant differences in *P. nicotianae* log ITS copies g^{-1} in stem samples among the different greenhouses ($P = 0.0103$, 35 d.f.), while no significant differences were found in soil samples ($P = 0.1464$, 35 d.f.) (Fig. 3).

Characterization of polymorphic mtDNA regions

Two different primer pairs were utilized to amplify mitochondrial regions *trnY/rns* and *trnW/cox2* from a total of 16 isolates. Amplicons in *trnY/rns* exhibited variable length ranging from 386 to 388 bp, while no differences were found in *trnW/cox2* (312 bp). Sequences were deposited in GenBank with the accession numbers KJ000341–KJ000354 and KJ412203–KJ412204 for *trnY/rns* region and KJ000355–KJ000368 and KJ412201–KJ12202 for *trnW/cox2* region.

Haplotype analysis and networks

Polymorphisms in the sequences among isolates were noted and classified as a separate haplotype (Table 3). Four haplotypes were identified by analysing region *trnY/rns* (Table 3). All of the differences among haplotypes were minor since polymorphisms were caused by single-nucleotide polymorphisms (SNPs) and length mutations (indels): a total of one SNP, which was a transition, one indel of 1 bp in length and one indel of 2 bp in length.

The neighbour-joining haplotype network generated using SplitsTree v.4.13.1 generated a network with two branches that were supported by bootstrap analysis (data not shown).

Phylogenetic analysis

The region *trnY/rns* of a total of 16 isolates (12 isolates from 2012, two from 2011, one from northwest Spain and one from west Spain) (Table S1) was analysed using the phylogenetic method of maximum likelihood. A tree with two clades was observed (Fig. 4). The first clade (N1) was represented by 11 isolates divided into two mitochondrial haplotypes (H1–H2). These two haplotypes differ in 1 bp indel (a thymine) in position 172 of the studied region. The second clade comprised five isolates. This clade contained two haplotypes (H3–H4). Isolates of this clade (N2) shared a difference that distinguished them from the other clade, an SNP in position 376 of the *trnY/rns* region (Table 3 and Fig. 4). Among clade N2, haplotypes H3 and H4 differ in one thymine indel in position 173 of the analysed region. No specific associations were observed with geographic origin since two of the isolates from northwest and west Spain are included in a common haplotype (H2) with isolates from southeast Spain.

DISCUSSION

The results from this work indicate that *P. nicotianae* is the main causal agent of *Phytophthora* root rot and crown root disease on sweet pepper plants in the studied area (southeast Spain), since all the strains isolated from infected pepper stems in 2011 and 2012 were recognized after morphological and molecular analysis as *P. nicotianae*. There are a number of morphological characteristics upon which identification of *Phytophthora* species is based. According to Erwin and Ribeiro,[1] the type of sporangia, sporangium morphology (shape, size and length/breadth ratio) and heterothallic sexual reproduction found in the studied isolates are typical of *P. nicotianae*. The presence of papillated sporangia may include isolates in group I or II. However, the heterothallism of all the isolates with amphigynous antheridia allows the inclusion

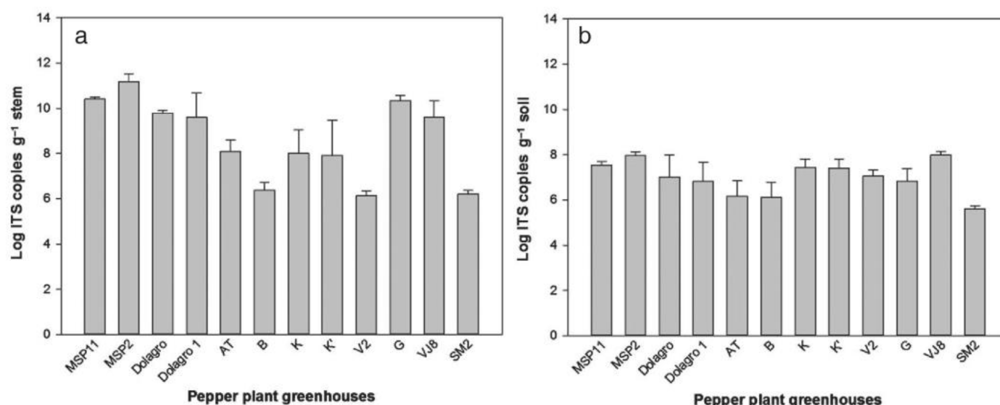

Figure 3. Quantification of *Phytophthora nicotianae* in naturally infested pepper stems (a) and soils (b) in the different greenhouses studied. Bars indicate the standard error of three replicates, $n = 3$.

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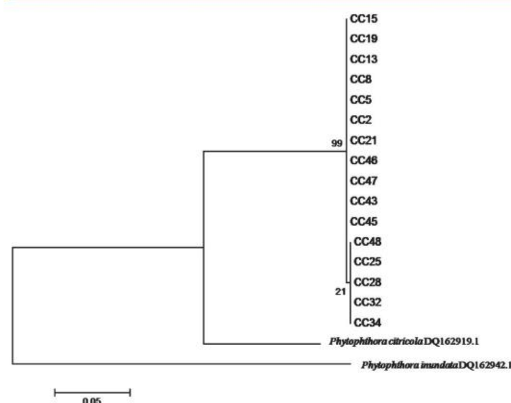

Figure 4. Phylogenetic relationships between *Phytophthora nicotianae* isolates based on the sequences of the mitochondrial intergenic region trnY-rns. Sequences of two *Phytophthora* spp. – *P. inundata* and *P. citricola* – were included as outgroup. The tree was built using the maximum likelihood algorithm based on the Tamura–Nei model. Numbers on node represent the statistical support (1000 bootstrap replicates), and only the nodes with values greater than 50% are shown. Scale bar indicates nucleotide substitution per site.

of the isolates among group II. The caducity of sporangia excludes *P. capsici*. The lenght/breadth ratio of the sporangia showed values lower than 1.4, which is also typical for *P. nicotianae*, as well as the presence of chlamydospores and hyphal swellings. These results are notably different from those reported in previous studies in the same area, where *P. capsici* was considered the causal agent.[2]

PCR-based studies have emerged as a powerful tool for the identification and the consequent detection of microbial pathogens in environmental samples, owing to their sensitivity, selectivity, robustness, rapidity and ease of methodology. Identification of *P. nicotianae* as the principal causal agent of Tristeza disease instead of *P. capsici* has several implications for developing disease management strategies. Although many of the greenhouses employ chemical treatments (both chloropicrin and 1,3-dichloropropene) or biosolarization, it seems these strategies are not enough to control *Phytophthora* spp. The capacity of the method to amplify pathogen DNA from infected but symptomless plants suggests that it could be useful for detection at nursery level (growing media or seedlings).

Diagnostic PCR methods and primers have been devised for *P.* nicotianae.[7,22–29] However, in most cases it remains difficult to establish that those primers are specific.[7] On the other hand, real-time PCR technique is faster and has higher sensitivity than other methods such as PCR. Sequencing of the ITS region was a successful approach to differentiate *P. nicotianae* from other species and to obtain a specific set of primers and probe to detect and quantify *P. nicotianae.* Although this region is not always sufficiently diverse to allow the separation of closely related taxa, we successfully differentiated *P. nicotianae* from other *Phytophthora* species. We have used the forward primer (NicF1) designed for a simultaneous detection of *P. nicotianae* and *P. cactorum* by Li *et al.*,[7] and designed a reverse primer (NicR4) and a Taq-Man probe (Nic-pro) to detect and quantify *P. nicotianae* through real-time PCR. The use of primers designed in the ITS region allows the detection of low quantities of target DNA, due to the presence of multiple different copies of rRNA spacer regions in the fungus genome (414 ± 12 copies per haploid genome in *P. infestans*[30]), increasing the specificity of real-time PCR.[31] One of the major issues in pathogen detection in environmental samples (soil, roots and stems) is the ability of the primers to detect the pathogen in a complex DNA environmental sample with all the microbial community present. The specific set of primers and probe could permit detection of the target from stem and soil samples.

Although *P. capsici* was detected in two soil samples in two different greenhouses, it is unlikely to be the causal agent of Tristeza disease since *P. nicotianae* was the species detected in the plant samples. In the case of *P. capsici*, clonal reproduction appears to be limited to single fields in a growing season, while oospores appear to play a key role in survival from year to year.[32] On the other hand, *P. nicotianae* can persist in the soil as chlamydospores in the absence of a susceptible host.[1]

A better understanding of *P. nicotianae* isolate variability and dynamics is helpful for designing more effective strategies for managing *Phytophthora* diseases. It is important to determine how the pathogen has been introduced and how it is spreading. Tracking specific haplotypes or a genetically unique individual in space is one promising strategy.[32] *Phytophthora nicotianae* is a heterothallic species, increasing the potential for sexual out-crossing and so providing the pathogen with advantages via increased genetic diversity. However, it seems that, in the studied area, *P. nicotianae* reproduced primarily asexually, since in all of the pepper greenhouses examined only the A2 mating type was found, which could suggest that the pathogen survives mainly as chlamydospores. Similar results were shown by Rodríguez-Molina *et al.*[5] in Caceres, Spain.

In the absence of sexual combination the use of mitochondrial markers, which are maternally inherited, would be useful for studying clonally reproducing populations of the pathogen.[12,33] The analysis of mitochondrial intergenic regions characterized by intraspecific variation in DNA sequences was used in this work to evaluate mitochondrial haplotypes and their phylogenetic relationships from the isolates obtained from the studied area. The few intraspecific variations were observed due to single-nucleotide polymorphisms. The four haplotypes detected by individually analysing region *trnY/rns* could suggest that the studied isolates were asexually reproduced through propagules (hyphal fragments, sporangia, chlamydospores, zoospores), which could lead to a lack of diversity among the isolates.[12] Nevertheless, many studies have demonstrated that, despite the theoretical advantages of sexual recombination, a clonal lineage of *P. nicotianae* could dominate a population, as happened with a succession of clonal lineages of *P. infestans* in many potato production regions.[34] Few haplotypes differed only in the number of bases in a homopolymeric thymine region (haplotypes with eight, nine and ten thymine bases). The isolates recovered from greenhouses GH B and GH K (CC19 and CC21 respectively) are included in the same haplotype (H1), which is the one with eight thymine bases. These greenhouses could be the source of inocula among the different greenhouses. The other haplotypes may be generated by slippage events during DNA replication through dinucleotide and trinucleotide expansion. Schlötterer and Tautz[35] showed that this event is a source of length polymorphisms in sequence stretches in human populations.

Near both greenhouses GH B and GH K there is a citrus production field which may be infected by *P. nicotianae*. It is also important to mention the ability of *P. nicotianae* to infect different hosts.[1] Infected irrigation water might be an example of how *P. nicotianae* may be spread among production facilities. Isolates recovered

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from greenhouse GH K (CC47 and CC21) over two years of sampling (2011 and 2012 respectively) had an identical sequence as well as isolates CC46 and CC15 among them, recovered from greenhouse AT over two years (2011 and 2012 respectively). These data indicate the potential of this pathogen to survive in spite of the use of chemical treatments such as mixtures of chloropicrin and 1,3-dichloropropene, which are used in greenhouses GH K and GH AT. *Phytophthora nicotianae* is well known for producing abundant chlamydospores in infected tissue, which could have contributed to the survival of this clonal lineage from 2011 to 2012.[11]

Phylogenetic groups or mitochondrial haplotypes were not correlated with phenotypic traits such as growth rate, sporangia length, breadth or length/breadth ratio, mating type or pathogenicity. This is in accordance with previous reports which used both biological and molecular approaches with *Phytophthora* isolates.[12,36]

Losses can be severe especially in greenhouses in which many plant species are simultaneously and repeatedly cultivated with limited crop rotation. In such conditions multiple generations of the pathogen can occur and different genotypes, including opposite mating types, can come in contact, thereby increasing the success of oospore production and evolutionary divergence.[12] Furthermore, it has been suggested that the phenomenon of out-crossing could occur between individuals of different species. In fact, genetic variants were created in the laboratory by fusion of *P. capsici* and *P. nicotianae* zoospores.[8,37] This fact should be taken into account in the studied area since both species coexist, at least in two greenhouses. The diagnostic method used in this study to detect *P. capsici* had a high sensitivity.[36]

The correct identification of *P. nicotianae* and *P. capsici* will be of special importance for diagnostic laboratories and for growers, especially if we consider that the persistence of the two species is different, as well as the variability among species. Moreover, the use of a TaqMan probe will be essential for detection of the pathogen in the early stages, even before the onset of the disease symptoms in plants as well as permitting determination of the critical levels at which disease takes place.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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PUBLICATION 2

Molecular methods (digital PCR and real-time PCR) for quantification of low DNA copy of *Phytophthora nicotianae* on environmental samples

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Pesticide Management Science: Under review

Molecular methods (digital PCR and real-time PCR) for the quantification of low copy DNA of *Phytophthora nicotianae* in environmental samples

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ABSTRACT

BACKGROUND: Currently, real-time polymerase chain reaction (qPCR) is the technique used most to quantify pathogen presence. Digital PCR (dPCR) is a new technique with the potential to have a substantial impact on plant pathology research due to its reproducibility, sensitivity and low susceptibility to inhibitors. In this study, we evaluated the feasibility of using dPCR and qPCR to quantify *Phytophthora nicotianae* in several background matrices, including host tissues (stems and roots) and soil samples

RESULTS: In spite of the low dynamic range of dPCR (3 logs compared to 7 for qPCR), this technique proved to be very precise, this precision being applicable at very low copy numbers. The dPCR was able to detect accurately the pathogen in all type of samples in a broad concentration range. Moreover, dPCR seems to be less susceptible to inhibitors than qPCR in plant samples. Linear regression analysis showed a high correlation between the results obtained with the two techniques in soil, stems and root samples with R^2=0.873, 0.999 and 0.995 respectively.

CONCLUSIONS: These results suggest that dPCR is a promising alternative for quantifying soil-borne pathogens in environmental samples, even in early stages of the disease.

Keywords: digital PCR, real-time quantitative PCR, *Phytophthora nicotianae*, inhibition, environmental samples.

1. Introduction

Currently, most plant materials for propagation are produced in just few large nurseries and then are distributed to other nurseries situated in other regions or countries. This trade increases the risk that plants infected with *Phytophthora* are introduced into new areas where the indigenous flora can become the victim of these potential new pathogens.[1] Specifically, the trade of potted ornamentals and fruit tree species in nurseries represents one of the most efficient dissemination pathways of *P. nicotianae*.[2-4] Nowadays, there are no effective means to control *Phytophthora* diseases, but the technologies to detect and identify these plant

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pathogens are rapidly improving. Molecular diagnostics for faster and more precise identification of species could improve the detection of *Phytophthora* spp. and provide instrumental support for integrated disease management strategies to control diseases caused by *Phytophthora* spp.[1]

Real-time PCR, also known as quantitative PCR (qPCR), is widely used to detect and quantify pathogens as well as biological control agents in environmental samples. [5-8] The high level of sensitivity of the qPCR-based assays used to detect the pathogen in pre-symptomatic infections[9] and to quantify pathogens throughout the entire disease cycle makes these methods stand out from the traditional ones.[8] For qPCR the construction of standard curves using known concentrations of DNA standards as the template for estimating the copy number of the target genes is required. In qPCR, the quantification is based on the assumption that standard and environmental DNAs are PCR-amplified with the same efficiency. However, the presence of inhibitory substances in environmental samples, such as humic acids, fulvic acids and polysaccharides, decreases the efficiency of PCR.[10]

Digital PCR (dPCR) is a new technology that allows absolute quantification of target genes without any standards.[10] This method is based on the partitioning of a single sample into 20,000 much smaller, segregated reactions. A standard PCR reaction can then be employed to amplify the target (s) in each partition which can be individually counted by the associated target dependant fluorescence signal as positive or negative. The simple readout of partitions as a binary code of ones (positive) and zeros (negative) represents the “digital” aspect of the technique. Because the presence of a target in a given partition is a random event, the associated data fit a Poisson distribution.[11,12] This compensates the fact that more than one copy of a template may be present in some partitions.[13,14] This technique is particularly suited to the low-level detection of DNA even in a highly concentrated complex background, and it works on the premise that every target molecule is successfully amplified. [13,15-17] Consequently dPCR should, in theory, provide the most accurate method of molecular quantification.

In clinical diagnosis dPCR has been widely used and is a potential tool in several scenarios (eg. the diagnosis and staging of cancer).[13,18,19] However, the use of dPCR in plant pathology is still in its infancy. Our study aims to evaluate the use of dPCR as a suitable method to detect and quantify *Phytophthora nicotianae* in infected pepper plants and soils, in comparison to qPCR.

2. Experimental methods

2.1. Sample collection process and DNA extraction

Tests were carried out utilizing soils collected from fields where plants infected with *P. nicotianae* had been observed in the preceding years. Seventeen soil samples adhering to roots of pepper plants with Phytophthora root rot symptoms were collected, sieved to remove large, non-soil materials and stored at -20 ºC until processing. The texture of all the soils was classified as sandy loam. The pH was in the range 7.5-8.3 and the organic matter content (%) between 0.8 and 2.2. Total DNA was extracted from the soil samples using the FastDNA® SPIN kit for soil (Q-Biogene, Carlsbad, CA, USA), following the modification described by López-Mondéjar et al.[6] Prior to the extraction, the samples were ground with liquid nitrogen and kept at -20 ºC. Moreover, from the same field, plants with symptoms of Phytophthora root rot were collected and nine of them were included in the present work.

Also, the capability of dPCR to detect *P. nicotianae* in asymptomatic plants was assessed using artificially infected plants obtained in an *in vivo* experiment. This assay was carried out under greenhouse nursery conditions. Pepper plants were sown and grown on autoclaved peat (15 g) and

inoculated with 1.5 mL of *P. nicotianae* (~ 10^3 cfu mL^{-1}) according to Blaya et al.[20] A total of nine stems and 18 roots were collected from non-symptomatic plants three days after inoculation. All the tissue samples were kept at -20 ºC until processing. Total DNA from pepper stems and roots was extracted according to the protocol of Doyle and Doyle.[21] The DNA concentration was estimated by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a NanoDrop® ND-1000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The purity was determined by calculating the ratio of absorbance at 260 nm and absorbance at 280 nm (A_{260}/A_{280}) and the ratio at 260 nm and 230 nm (A_{260}/A_{230}).

A pure culture of a strain of *P. nicotianae* from the CEBAS collection (CEBAS MSP11) was utilized in our study to validate the sensitivity of the dPCR. The isolate was grown for 15 days on a PDA plate at 25 ºC in darkness. Mycelia were collected in a tube and ground in liquid nitrogen with a plastic micropestle. The DNA was extracted using a phenol and chloroform protocol, followed by isopropanol precipitation according to Hartl and Seiboth,[22] and was kept at -20ºC until processing.

2.2. Digital PCR

Digital PCR amplifications were performed using the QuantStudio® 3D (Applied Biosystems, Waltham, MA, USA) in a total volume of 16.5 μL. The reaction mixtures contained a final concentration of 1x QuantStudio 3D Digital PCR Master Mix (Applied Biosystems), 0.3 μM each of the Nic-F1 and Nic-R4 primers,[23] 0.2 μM TaqMan probe Nic-Pro,[23] 5 μL DNA template and sterile water. The thermal cycling conditions for amplification were an initial denaturation at 95 ºC for 10 min, followed by 40 cycles each consisting of a step at 60 ºC for 2 min and another at 98 ºC for 30 s, and a final step at 60 ºC for 2 min. The amplification results were analyzed with QuantStudio® 3D AnalysisSuite™ Cloud Software (Applied Biosystems) to count the number of positive chambers (*H*) out of the total number of chambers (*C*), from which the Poisson distribution was used to estimate the average number of molecules per chamber (λ) so that $\lambda = -\ln(1 - H/C)$. No template controls (NTC) were performed by adding water in place of template. The QuantStudio® 3D dPCR system was used according to the manufacturer's recommendations. To achieve the confidence variance (CV) of 5% recommended by the manufacturer, more than one chip was performed for the same sample when it was required. Also, some samples required further dilutions than the ones needed to overcome the inhibition, to avoid saturation of partitions.

2.3. Real-time PCR

Real-time PCR amplifications were performed using a 7500 Fast Real-Time PCR system (Applied Biosystems), in a total volume of 25 μL. The reaction mixtures contained a final concentration of 1x TaqMan Universal Master Mix II no UNG (Applied Biosystems), 0.3 μM each of the Nic-F1 and Nic-R4 primers,[23] 0.2 μM TaqMan probe Nic-Pro,[23] 0.1 mg mL^{-1} BSA, 5 μL DNA template and sterile water. In some cases and to improve the detection limit of low-level-copy samples, qPCRs were performed in a total volume of 50 μL containing the same final concentrations of all reagents as mentioned above, but adding 10 μL of DNA template. Samples were run in triplicate. The thermal cycling conditions for amplification were an initial denaturation at 95 ºC for 10 min, followed by 40 cycles each consisting of a denaturation step at 95 ºC for 10 s, annealing at 60 ºC for 40 s, and a final step at 50 ºC for 2 min. The amplification results were analyzed with 7500 Fast Real-time PCR software v2.0 (Applied Biosystems).

2.4. Standard curve and qPCR efficiency

The DNA used for the standard curve was extracted from mycelia of *P. nicotianae* as described before. The isolate was amplified using Nic-F1, Nic-R4 and the probe Nic-Probe[23] and was cloned and transformed as indicated in Blaya et al.[23] The DNA concentration of the plasmid standard solution was determined using the Quan-iT™ PicoGreen® dsDNA kit (Invitrogen, Carlsbad, CA, USA) and was related to the known molecular weight of a single plasmid molecule to calculate the number of copies. The standard curve was generated by seven-fold dilutions of the plasmid adjusted to 10^{10} ITS copies. In every qPCR, at least five of these dilutions with three replicates each were included in the plate to interpolate the amplification results to the absolute quantity of target in the sample. The slope of the standard curve was used to determine the PCR efficiency ($E=10^{-1/slope}-1$).[24]

2.5. Control of inhibitory substances

An Internal Positive Control (IPC) (Applied Biosystems) was included in a representative collection of samples of each type in the dPCR and qPCR to indicate the presence or absence of PCR inhibitory substances in the extracts. The IPC reagents were used according to the manufacturer's instructions. The IPC was detected using a VIC-labeled probe (Applied Biosystems) and the target was detected using a FAM-labeled probe resulting in a duplex PCR assay. The PCR inhibition was determined by comparing IPC Ct (Cycle threshold or Ct is the PCR cycle in which fluorescence exceeds the threshold) values of negative controls without a template (ddH_2O) with IPC Ct values of samples. For samples with partial inhibition, the IPC Ct value was delayed in the case of qPCR, and the concentration of copies μL^{-1} measured in VIC was decreased in the case of dPCR.

2.6. Statistical analysis

A linear regression analysis (y=ax + b) was conducted with data from the dPCR and qPCR measurements. The standard deviation (% SD) of the qPCR measurements (n=3) was calculated.

3. Results

3.1. Quantification of *P. nicotianae* on pure culture

Both qPCR and dPCR were assessed for their ability to detect *P. nicotianae* DNA extracted from a pure culture. A serial dilution of 10-fold (from 10^{-1} to 10^{-8}) of the target DNA extracted (70 ng μL^{-1}) was quantified using dPCR and qPCR.

The dPCR proved to be a very precise technique (expressed numerically as very low standard deviations), this precision being applicable at very low copy numbers (Table 1; Fig. 1). The number of partitions measured with dPCR in each sample is shown in Table 1. In all samples more than 18000 partitions were measured from the total of 20000 contained in the chips used. The number of molecules per partition (λ) is shown in Table 1.

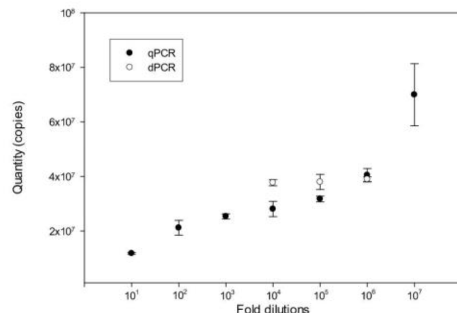

Fig. 1. Quantification (ITS copies) of a pure culture of *P. nicotianae* through several dilutions with digital PCR (dPCR) (white circles) and real time PCR (qPCR) (black circles). The standard deviation of three technical replicates is represented as the error bar.

Table 1. Digital PCR amplification results of *P. nicotianae* gDNA (70 ng μL^{-1}) extracted from a pure culture.

Dilution	Number of chips	Copies μL^{-1}	λ	Precision (%)	Number of positive partitions	Number of partitions measured
10^4	3	3895	3.37	2.39	16414	16999
		3734	3.18	2.23	16813	17593
		3679	3.23	2.26	17639	18365
10^5	3	348	0.30	3.06	4295	16533
		395	0.34	2.86	4883	16884
		397	0.34	2.89	4789	16486
10^6	3	38.4	0.032	8.44	585	17910
		40.0	0.035	8.48	580	17050
		38.6	0.034	8.78	542	16510

λ is another factor considered essential to determine the precision of the estimation, with $\lambda = -\ln(1 - k/n)$, where k is the number of positive partitions and n, the number of partitions.

The dynamic range was 3 logs for dPCR and 7 logs for qPCR (Fig. 1). The dPCR was more sensitive in the 10^4-fold and 10^5-fold dilutions but less sensitive in the 10^6-fold dilution. The dynamic range of qPCR was more variable than that of dPCR (Fig. 1).

3.2. Evaluation of DNA purity

Two methods were chosen to extract DNA from soil and plant samples. Whereas a commercial kit, FastDNA® Spin kit, was chosen for soil samples, the CTAB method was used to extract DNA from plant samples (stems and roots). Both methods were able to extract high quantities of DNA from the different samples. In, particular the CTAB method yielded high DNA template quantity (Table 2). The efficiency of both extraction methods was evaluated by the amount and quality of DNA extracted from samples using spectrophotometric measurements namely, ratios A_{260}/A_{280} and A_{260}/A_{230}. Values between 1.8 and 2.0 for A_{260}/A_{280} indicate low levels of contamination by protein and aromatic substances, and values above 2 for A_{260}/A_{230} indicate the absence of PCR inhibitors such as polysaccharides, salts, lipids, and phenolic compounds.[25] The A_{260}/A_{280} values show that both methods provided good DNA quality for all samples. Only two soil samples had values slightly below 1.8 (Table 2), which may indicate the presence of proteins. The values of A_{260}/A_{230} in the soil samples were very low (Table 2). The CTAB method gave higher A_{260}/A_{230} values in roots than in stems. In stems, half of the samples showed A_{260}/A_{230} values lower than 2 (Table 2).

3.3. Verification of the absence of inhibitory substances

We assessed the presence of inhibitors in the DNA extracts using IPC, for both qPCR and dPCR. We observed that a 10-fold dilution of the DNA extracts was enough to remove all the inhibitory effects in dPCR despite the type of sample, since the number of IPC copies μL^{-1} quantified did not decrease. Conversely, in qPCR, 20-fold dilutions of DNA extracts from stems and roots were required to remove the inhibition, whereas in DNA extracts from soils, 10-fold was enough to remove all the inhibition. Taking into account these results, 20-fold dilution of DNA extracts from stems

Table 2. Spectrophotometric quantification with NanoDrop® ND-1000 of soil, stem and root samples. DNA yield and purity.

Soils				Stems				Roots			
Sample code	DNA yield (ng g^{-1})	A_{260}/A_{280}	A_{260}/A_{230}	Sample code	DNA yield (ng g^{-1})	A_{260}/A_{280}	A_{260}/A_{230}	Sample code	DNA yield (ng g^{-1})	A_{260}/A_{280}	A_{260}/A_{230}
S1	253	1.84	0.11	St1	9.4	2.13	1.86	R1	27.8	2.07	2.27
S2	698	1.81	0.33	St2	1.5	1.98	2.04	R2	31.0	2.04	2.12
S3	442	1.81	0.19	St3	5.1	2.13	2.05	R3	6.6	1.98	1.75
S4	310	1.92	0.14	St4	8.7	1.99	1.53	R4	16.8	1.96	1.93
S5	185	1.84	0.09	St5	8.3	1.98	2.03	R5	11.0	1.98	2.07
S6	162	1.88	0.07	St6	8.6	2.05	2.03	R6	23.9	2.08	2.18
S7	256	1.91	0.11	St7	7.4	2.10	1.97	R7	18.8	2.09	2.30
S8	590	1.84	0.29	St8	6.1	2.03	2.02	R8	11.7	1.98	2.00
S9	315	1.80	0.14	St9	6.0	2.00	2.08	R9	10.8	2.00	2.04
S10	481	1.89	0.20	St10	27.9	2.10	2.24	R10	9.9	2.03	2.13
S11	404	1.84	0.18	St11	5.9	1.99	1.95	R11	8.0	2.04	2.22
S12	325	1.80	0.14	St12	16.7	2.05	2.08	R12	5.3	2.09	2.19
S13	250	1.88	0.11	St13	6.3	2.01	1.79	R13	15.3	2.04	2.15
S14	381	1.80	0.18	St14	6.1	2.08	1.97	R14	14.0	2.04	1.97
S15	294	1.74	0.13	St15	3.0	1.81	1.81	R15	14.5	2.04	1.97
S16	448	1.64	0.19	St16	8.7	2.07	2.28	R16	8.8	1.98	2.03
S17	335	1.91	0.15	St17	5.7	2.15	1.23	R17	6.4	2.03	1.91
				St18	2.5	2.10	1.25	R18	6.9	2.02	1.90

and roots and 10-fold for soil samples were used in qPCR while only 10-fold dilution was used in dPCR. Taking into account these results, 20-fold dilutions of DNA extracts from stems and roots and 10-fold dilutions of soil samples were used in qPCR while only 10-fold dilutions were used in dPCR.

3.4. Detection and quantification of *P. nicotianae* in environmental samples

To compare the sensitivity and specificity of dPCR and qPCR performed on environmental samples, 17 samples of soils and 18 stems and roots were collected, DNA-extracted, and the abundance of *P. nicotianae* measured in the proper dilution. The analysis of qPCR data can be highly subjective, since Ct values can be altered by changes to baselines and threshold levels.[26] We took into account all the considerations reported by Nolan et al.[26] to be able to generate reliable data among which the standard curve slopes in all PCRs were between -3.2 and -3.5 and the reproducibility of replicates had an $R^2 > 0.98$. In the case of dPCR, the copies μL^{-1} obtained and the dilution used as well as the number of chips used are shown in Table 3. More than one chip was used in some samples to obtain the desired precision, CV < 5% (Table 3). In some samples, more than one 10-fold dilution was required to fulfill the technical requirements of dPCR.

The dPCR was also able to quantify *P. nicotianae* in environmental samples. As far as soil samples are concerned, dPCR was able to quantify concentrations (copies μL^{-1}) from 3000 copies μL^{-1} to 100 copies μL^{-1}, approximately (Table 3). For some samples, more than one chip was required to obtain the desired precision (CV < 5%) (Table 3). The pathogen concentration in some soil samples (S13, S14, S15, S16, S17) was considered to be below the detection limit due to CV values

> 5 % with dPCR, while this was the case for just one soil sample (S17) with qPCR. Only one soil sample (S18) could not be detected, neither by qPCR nor by dPCR (Table 3)

In the case of DNA extracts from plant samples (stem and roots), dPCR was able to quantify samples within a broad concentration range (1.44×10^7-143 copies μL^{-1}). Also, some samples required more than one chip and others needed some dilutions to avoid saturation of the partitions (Table 3) and get CV values < 5 %. Only four stem samples (St7, St8, St9, St10) and four root samples (R15, R16, R17, R18) had concentration values below the detection limits (CV < 5 %).

Linear regression showed quantitative agreements between both methods regardless of the type of sample, achieving R^2=0.873, 0.999, and 0.995 in soils, stems, and roots respectively. The ratio between the copies μL^{-1} detected by dPCR and qPCR in the different samples is shown in Table 3. The dPCR provided higher estimates than qPCR in half the soil samples, most of the stem samples, and all the root samples (Table 3).

4. Discussion

The main goal of this study was to analyze the capability of digital PCR to detect and quantify *P. nicotianae* accurately. While comparing the sensitivity of the two techniques in the quantification of a pure culture of *P. nicotianae*, we have observed that dPCR is less versatile than qPCR since it only allows fairly precise measurement over a dynamic range of 3 orders of magnitude versus the 7 orders of qPCR. The dynamic range of dPCR is limited by the number of partitions available.[14] In other words, the precision becomes poor when the mean number of molecules per partition is very low and as the number of positive partitions approaches saturation. In our study, the lower dilutions (< 10^4) were completely saturated and it was not possible to quantify them. On the other hand, quantification of dilutions greater than 10^6 was not possible either since λ was too low and therefore the SD was too high. It should be noted that in some cases it is possible to achieve the desired precision by increasing the number of technical replicates, and hence, the total number of reactions analyzed.[12] In some environmental samples, more than one chip was necessary to achieve a CV < 5%.[14] However, when λ was really low, even the use of a high number of chips did not improve its CV, and the pathogen concentration of these samples was reported to be below detection limit (Table 3). It is considered that dPCR is most precise at an optimal concentration of approximately 1.59 molecules per partition.[27] In spite of this, dPCR was able to yield very precise measurements throughout its dynamic range (Fig. 1). Nevertheless, it is of practical importance to get a rough estimate of the concentration of our target of interest in order to make appropriate dilutions. Otherwise, too many partitions will contain multiple copies.[28] In this study, for example most of the root samples required serial dilutions. Poisson correction can compensate the occurrence of multiple target molecules per partition to a limit extent, enabling the precise calculation of concentrations -even under conditions of limiting dilution.[11]

While the precision of dPCR decreased as DNA concentration decreased (from the 10^6 dilution of the pure culture onwards), qPCR seems not to have been affected by the DNA concentration within the dynamic range. It is worth mentioning that the precision of dPCR was higher compared with qPCR as measured by the mean SD. This has been reported also in other studies.[29]

Special emphasis was placed on the use of dPCR and its comparison with qPCR in soil and plant samples. When comparing the ability of both techniques to quantify target genes in environmental samples, we should take into account that the quantitative data generated in qPCR are only as accurate as the standards used.[29]

Table 3. Quantification of DNA extracts from soil, stem and root samples using dPCR and qPCR.

Soil sample code	dPCR					qPCR		dPCR/qPCR ratio
	Copies μL^{-1}	SD[a]	Number chips	Dilution factor	CV[b]	qPCR	SD	
S1	1064	-	1	0.1	4.89	898	5.37	1.18
S2	1330	-	1	0.1	4.39	1361	298	0.98
S3	2418	-	1	0.1	3.39	1986	201	1.22
S4	1160	-	1	0.1	4.68	999	56.5	1.16
S5	3022	-	1	0.1	3.04	3636	509	0.83
S6	1222	-	1	0.1	4.68	2204	177	0.55
S7	496	33.6	2	0.1	5.06	397	19.5	1.25
S8	546	12.5	2	0.1	4.95	294	17.9	1.85
S9	758	4.2	2	0.1	4.17	848	61.3	0.89
S10	295	21.4	3	0.1	5.39	292	29.0	1.01
S11	195	15.2	4	0.1	5.23	243	12.2	0.80
S12	BDL[c]	-	1	0.1	-	215.5	10.9	0.50
S13	BDL	-	1	0.1	-	23.4[e]	5.16	-
S14	BDL	-	1	0.1	-	79.0[e]	0.33	-
S15	BDL	-	1	0.1	-	14.1[e]		-
S16	BDL	-	1	0.1	-	BDL	-	-
S17	ND[d]	-	1	0.1	-	ND	-	-
Stem sample code	**dPCR**					**qPCR**		**dPCR/qPCR ratio**
	Copies μL^{-1}	SD	Number chips	Dilution factor	CV	qPCR	SD	
St1	$1.19\cdot10^{5}$	-	1	0.01	1.89	$1.68\cdot10^{5}$	$2.90\cdot10^{4}$	0.71
St2	$2.75\cdot10^{5}$	-	1	0.001	3.13	$2.32\cdot10^{5}$	$2.38\cdot10^{4}$	1.18
St3	$5.02\cdot10^{2}$	-	1	0.1	2.54	$3.90\cdot10^{2}$	123.5	1.29
St4	$3.98\cdot10^{4}$	-	1	0.01	2.29	$2.92\cdot10^{4}$	814	1.36
St5	$2.76\cdot10^{5}$	-	1	0.001		$7.07\cdot10^{5}$	$4.25\cdot10^{5}$	0.39
St6	BDL	-	1	0.1	-	BDL[e]	-	-
St7	BDL	-	1	0.1	-	31.4[e]	10.2	-
St8	BDL	-	1	0.1	-	21.2[e]	7.50	-
St9	BDL	-	1	0.1	-	BDL[e]	-	-
St10	$1.44\cdot10^{7}$	-	1	0.00001	4.38	$1.90\cdot10^{7}$		0.79
St11	$3.75\cdot10^{5}$	-	1	0.01	1.98	$5.26\cdot10^{5}$	$5.49\cdot10^{4}$	1.4
St12	$4.30\cdot10^{3}$	-	1	0.1	2.69	$3.34\cdot10^{3}$	191	1.29
St13	$3.70\cdot10^{4}$	-	1	0.01	2.93	$2.58\cdot10^{4}$	$1.26\cdot10^{3}$	1.44
St14	$4.19\cdot10^{4}$	-	1	0.01	2.82	$2.98\cdot10^{4}$	$3.31\cdot10^{3}$	1.41
St15	$6.29\cdot10^{3}$	-	1	0.1	2.35	$3.83\cdot10^{3}$	614	1.60
St15	161	50.4	4	0.1	5.40	BDL[e]		-
St17	143	39.4	4	0.1	5.85	BDL[e]		-
St18	213	20.1	4	0.1	5.33	90.61	1.56	2.38
Root sample code	**dPCR**					**qPCR**		**dPCR/qPCR ratio**
	Copies μL^{-1}	SD	Number chips	Dilution factor	CV	qPCR	SD	
R1	$5.41\cdot10^{5}$	-	1	0.001	2.43	$1.72\cdot10^{5}$	$2.73\cdot10^{4}$	3.1
R2	$9.83\cdot10^{3}$	-	1	0.1	2.1	$4.83\cdot10^{3}$	548	2.0
R3	$8.69\cdot10^{3}$	-	1	0.1	2.04	$2.99\cdot10^{3}$	71	2.9
R4	$4.08\cdot10^{5}$	-	1	0.001	2.81	$3.09\cdot10^{5}$	$6.36\cdot10^{4}$	1.32
R5	$2.43\cdot10^{5}$	-	1	0.001	3.921	$2.08\cdot10^{5}$	2842	1.17
R6	$2.52\cdot10^{5}$	-	1	0.001	3.39	$1.93\cdot10^{5}$	$1.41\cdot10^{4}$	1.31
R7	$7.32\cdot10^{4}$	-	1	0.01	2.68	$4.79\cdot10^{4}$	1234	1.53
R8	$3.96\cdot10^{4}$	-	1	0.1	2.80	$2.28\cdot10^{4}$	2064	1.7
R9	$5.37\cdot10^{4}$	-	1	0.01	2.48	$3.81\cdot10^{4}$	4140	1.4
R10	$5.57\cdot10^{4}$	-	1	0.01	3.11	$4.15\cdot10^{4}$	9685	1.34
R11	$3.54\cdot10^{4}$	-	1	0.01	2.85	$2.36\cdot10^{4}$	1623	1.5
R12	$1.26\cdot10^{4}$	-	1	0.01	4.53	7089	457	1.78
R13	$1.29\cdot10^{4}$	-	1	0.01	4.26	8895	1460	1.62
R14	BDL		-	0.1	-	BDL[e]	-	-
R15	BDL	-	1	0.1	-	BDL[e]	-	-
R16	BDL	-	1	0.1	-	BDL[e]	-	-
R17	BDL	-	1	0.1	-	BDL[e]	-	-
R18	BDL	-	1	0.1	-	BDL[e]	-	-

[a] SD: standard deviation. [b] CV: confidence variance. [c] BDL: below detection limit. [d] ND: no detected. [e] qPCR was performed in 50 μL reaction to increase the detection limit of low-level-copy samples.

For *P. nicotianae* specifically, and other pathogens in general, DNA standards are usually obtained using dilutions of cloned *P. nicotianae* DNA fragments in recombinant plasmids, or dilutions of genomic DNA extracted from pure cultures of *P. nicotianae*. We chose a fluoresce-based method to quantify the plasmid of our standard curve, since it is able to measure the fluorescence intensity of nucleic acids in the presence of a NA-binding dye such as Picogreen, which excites preferentially when bound to double-stranded DNA (Yang et al. 2014).[29] This approach requires the construction of standard curves to extrapolate the fluoresce measurement into a DNA concentration. Indeed, the use of this approach to quantify DNA in reference standards for qPCR has been suggested.[29]

Some, or all, environmental samples may contain inhibitors that are not present in the nucleic acid samples used to construct the calibration curve, leading to an underestimation of the DNA levels in test samples.[26] On the other hand, dPCR allows absolute quantification without any standards and, indeed, it is considered to be less susceptible to PCR inhibitors present in DNA extracts.[10] Successful DNA amplification is of paramount importance for the detection and quantification of pathogen DNA in environmental samples, which depend on both the ability of DNA extraction methods to obtain good quality DNA and the ability of the DNA amplification method used for its quantification. The inhibiting agents may interact with the DNA polymerase or bind primers to the template, thereby reducing the efficiency and/or reproducibility of the PCR which may contribute to inaccurate PCR results.[30-32] The A_{260}/A_{230} values in the soil samples were below 2, showing that some constituents of the soil organic matter may have been co-extracted with the DNA.[30,33,34] We observed that FastDNA® SPIN kit extracts from soils required at least a 10-fold dilution to overcome inhibition in qPCR since amplification of IPC was uninhibited with these dilutions. Dineen et al.[34] reported that no dilution was needed to remove inhibition from soils with sandy or sandy clay texture when using the FastDNA® SPIN kit, whereas a 100-fold dilution was required in the case of sandy loam soil. In the present study, soil DNA was extracted from a soil of sandy loam texture, which could explain the inhibition found in these samples. In dPCR, we observed that a dilution factor ≥10-fold prevented PCR inhibition in soil samples. This extraction kit has proven to be a good method to extract high DNA yields from soils with different textures, in comparison to other commercial kits available.[34] However, the quality of the DNA extracts, considering the A_{260}/A_{230} ratio, was quite low. The ability to remove PCR inhibitors should be considered along with DNA yield in the selection of an extraction method.[34] Although dilution was an effective way to eliminate inhibition, it also reduced the amount of template DNA, which could compromise the ability of both techniques to accurately detect and quantify such samples[10] principally, in those samples with low-level target gene concentrations.

The CTAB method used in this study has been widely used to extract DNA from leaves, seeds, and processed food.[35] In our study, it was useful since it gave both high DNA yield and DNA purity, the latter being slightly higher in roots than in stems. A higher content of polysaccharides, which are difficult to separate from DNA, in stems may be the reason for this difference.[37] In spite of the high A_{260}/A_{230} ratios obtained with these extraction methods, the test performed with the IPC to determine the suitability of the extracted DNA for qPCR,[32] showed that 20-fold dilutions were required to remove the inhibition in plant samples in qPCR while 10-fold dilutions were enough to remove the inhibition in dPCR. According to published reports, CTAB-extracted DNA needs further purification to be used for real-time PCR.[32] However, in previous studies, it has been suggested that in spite of the fact that DNA extracted using the CTAB method may possess some substances

inhibitory for qPCR, dilutions were enough to remove all the inhibition.[25]

The dPCR had a broad range of detection in environmental samples, being able to detect accurately low-level copies in different matrices such as soils, stems, and roots. In previous studies, the ability of dPCR to obtain accurate copy numbers of target DNA in environments containing low microbial biomass and high organic matter was demonstrated.[10] However, when the pathogen was present at low levels (around 100 copies in soils, 144 copies in stems, and 166 in roots), dPCR was not able to quantify it accurately, since the precision of the measurements was below the recommended one. In spite of these limitations, dPCR seems to be more resilient to differences in sample quality since, it as it was speculated by Hindson et al.[11] this technique is more tolerant of PCR inhibitors by virtue of being an end-point approach. Unlike qPCR, in which the quantification cycle depends on variable features such as the instrument, fluorescent reporter dye, and assay efficiency, dPCR relies on a simple count of the number of successful amplification reactions.[14] It is worth mentioning the high pathogen concentrations found in some roots in spite of being collected only a few days after inoculation.

One of the advantages of qPCR compared to dPCR is the possibility to increase the volume of the PCR and thus the amount of DNA added, lowering the detection limit of this technique. However, the volume of reaction of the chips used is limited to 16.5 μL, and the amount of DNA to 6.3 μL. For this reason, qPCR was able to detect some samples which dPCR was not able to quantify accurately. Nevertheless, we observed that in all root samples and most stem samples, dPCR estimated more copies μL^{-1} than qPCR. Moreover, since this type of sample was less susceptible to inhibition, lower dilutions were required, reducing the possibility of obtaining concentrations below the detection limit in samples with low-level copies (by not detecting any copy or because of the low precision obtained). A pairwise analysis of the ratios between the techniques (dPCR and qPCR) revealed that they compared well although there were some discrepancies. These results are in agreement with previous studies comparing the two technologies.[16, 29,38]

It should be noted that some authors have suggested that copy numbers from qPCR and dPCR are not directly comparable due to the fact that qPCR quantifies the copy number of ITS genes whereas dPCR quantifies the number of molecules.[10] This may explain why, in some cases qPCR was able to quantify higher copies μL^{-1} in spite of being less sensitive than dPCR.

In the case of the soil sample in which *P. nicotianae* could not be detected, neither with dPCR nor with qPCR, the pathogen may either have been absent or present at concentrations below the detection limit of both techniques**.** With IPC being unaffected, we avoided the inclusion of false negative results.

5. Conclusions

The dPCR proved to be a very precise technique for the identification of *P. nicotianae*, this precision being applicable at very low copy number in soils, stems, and roots. We highly recommend the use of DNA extracts from roots to evaluate the presence of the pathogen since *P. nicotianae* was able to establish itself well in this matrix within the first hours after infection. Rapid and accurate detection of *Phytophthora* spp. could improve the integrated disease management strategies to control and avoid the spread of the diseases caused by this pathogen.

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PUBLICATION 3

Identification of predictor parameters to determine agro-industrial compost suppressiveness against *Fusarium oxysporum* and *Phytophthora capsici* diseases in muskmelon and pepper seedlings

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Identification of predictor parameters to determine agro-industrial compost suppressiveness against *Fusarium oxysporum* and *Phytophthora capsici* diseases in muskmelon and pepper seedlings

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Abstract

BACKGROUND: The lack of reliable prediction tools for evaluation of the level and specificity of compost suppressiveness limits its application. In our study, different chemical, biological and microbiological parameters were used to evaluate their potential use as a predictor parameter for the suppressive effect of composts against *Fusarium oxysporum* f. sp. *melonis* (FOM) and *Phytophthora capsici* (*P. capsici*) in muskmelon and pepper seedlings respectively. Composts were obtained from artichoke sludge, chopped vineyard pruning waste and various agro-industrial wastes (C1: blanched artichokes; C2: garlic waste; C3: dry olive cake).

RESULTS: Compost C3 proved to offer the highest level of resistance against FOM, and compost C2 the highest level of resistance against *P. capsici*. Analysis of phospholipid fatty acids isolated from compost revealed that the three composts showed different microbial community structures. Protease, NAGase and chitinase activities were significantly higher in compost C3, as was dehydrogenase activity in compost C2.

CONCLUSION: The use of specific parameters such as general (dehydrogenase activity) and specific enzymatic activities (protease, NAGase and chitinase activities) may be useful to predict compost suppressiveness against both pathogens. The selection of raw materials for agro-industrial composts is important in controlling *Fusarium* wilt and *Phytophthora* root rot.
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Keywords: suppressive compost; agro-industrial wastes; *Fusarium oxysporum*; *Phytophthora capsici*

INTRODUCTION

Currently, there are several devastating soil-borne pathogens of worldwide distribution that infect solanaceous and cucurbitaceous hosts, causing multiple diseases at the nursery level.[1] Fusarium wilt of muskmelon (*Cucumis melo* L.), caused by *F. oxysporum* Schlechtend.: Fr. f. sp. *melonis* WC Snyder & HN Hans (FOM), is considered the most severe infectious disease of this cucurbit.[2] It occurs through most of the Mediterranean countries, with impacts in both the greenhouse nursery and field environments.[2–4] It is very difficult to control Fusarium wilt because the fungus survives in the soil as chlamydospores during extended periods even in the absence of the host roots.[5] Currently, no effective curative treatments are available for FOM infection.[6] Phytophthora root rot caused by *Phytophthora capsici* Leonian is also one of the most potentially destructive diseases affecting cultivated peppers (*Capsicum annuum*) in many countries.[7] *Phytophthora capsici* has been considered the main collar and root rot pathogen in Europe.[8–11] Phytophthora root rot management is based on phenylamide fungicides but fungicide-tolerant strains have been detected. Alternatives methods of control for both pathogens are increasingly being investigated, being the biological control methods ones of the most promising.[12]

The food industries in the Mediterranean area generate important amounts of organic wastes that can be recoverable as compost,[13,14] owing to their characteristically high level of organic matter, nutrients and low heavy metal content. The use of these composts as organic substrate at the nursery level has not been widely studied, and could contribute to reducing the non-renewable use of peat, which is the most utilized organic substrate for the preparation of potting mix.[14] Using these composts could also help to reduce the use of chemical fungicides owing to

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Table 1. Chemical characteristics of composts and peat (values on dry weight basis)

Parameter	Compost C1	Compost C2	Compost C3	Peat
pH	6.45 ± 0.01c	7.02 ± 0.03a	6.88 ± 0.08b	5.52 ± 0.03d
EC (dS m^{-1})	11.74 ± 0.15a	11.42 ± 0.29a	5.47 ± 0.29b	2.04 ± 0.04c
TOC^z (g kg^{-1})	312.75 ± 2.05b	316.6 ± 3.82b	368.23 ± 0.46a	147.72 ± 8.13c
Total N (g kg^{-1})	35.20 ± 0.08b	36.70 ± 0.09a	30.48 ± 0.02c	3.40 ± 0.02d
Total P (g kg^{-1})	5.30 ± 0.1a	5.60 ± 0.01a	3.90 ± 0.02b	0.30 ± 0.001c
Total K (g kg^{-1})	18.80 ± 0.30a	16.60 ± 0.07a	11.40 ± 0.03b	0.60 ± 0.003c
C/N	8.90 ± 0.18c	8.63 ± 0.07c	12.08 ± 0.06b	44.04 ± 1.31a
Total Cd (mg kg^{-1})	BDL	BDL	BDL	BDL
Total Cr (mg kg^{-1})	15.21 ± 2.57a	16.92 ± 0.07a	14.50 ± 0.48a	0.61 ± 0.01b
Total Cu (mg kg^{-1})	30.70 ± 1.84a	30.54 ± 2.06a	27.05 ± 2.38a	10.32 ± 2.66b
Total Fe (g kg^{-1})	1.54 ± 0.32ab	1.85 ± 0.07a	1.39 ± 0.13b	0.41 ± 0.01c
Total Ni (mg kg^{-1})	8.81 ± 1.30b	10.43 ± 0.28a	6.82 ± 0.01c	BLD
Total Pb (mg kg^{-1})	3.74 ± 1.50a	1.58 ± 0.52a	2.30 ± 0.16ab	1.16 ± 0.01b
Total Zn (mg kg^{-1})	144.67 ± 23.55a	141.72 ± 7.59a	106.88 ± 1.85b	5.70 ± 0.46c

Abbreviations: EC, electrical conductivity; TOC, total organic carbon; BDL, below detection limits. Data are means ± standard error of three replicates. For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($P \leq 0.05$).

their bio-pesticide effect, whereas peat is hardly ever suppressive against soil-borne pathogens.[15,16] Accordingly, the current policy in several European countries is to reduce peat use by substituting it with other organic sources.[17] In several studies, agro-industrial composts have shown a certain ability to suppress soil-borne pathogens.[13,15,18] Different mechanisms in this suppression have been described, attributed to either biotic or abiotic characteristics of the compost. Some of them are competition for carbon and nutrients, antibiosis, mycoparasitism, improved plant nutrition[19,20] and activation of disease-resistant genes in plants controlled by signaling pathways involving hormones, such as salicylic acid (SA), jasmonic acid (JA) and abcisic acid (ABA).[21] This latter mechanism has become one of the most promising strategies for controlling plant pathogens by using composts.[22]

Composts that are suppressive to some pathogens and conducive to others cannot be extensively applied unless their effects on diseases can be accurately predicted.[16] Correlations of disease suppression with chemical, physical and microbiological variables have been used to predict the suppressive capabilities of composts. According to Bonanomi *et al.*,[16] the most useful parameters are FDA activity, substrate respiration, microbial biomass, total culturable bacteria, fluorescent *Pseudomonads* and *Trichoderma* population. However, there are scarce studies using the phospholipid fatty acids profile (PLFAs) as biomarkers of the structure microbial community, and some specific enzymatic activities such as β-glucosidase, *N*-acetyl-β-glucosaminidase (NAGase) and chitinase activities as reliable disease predictors against FOM and *P. capsici.*

The main objective of this study was to identify possible predictor parameters that determine the suppressiveness of three composts obtained from artichoke sludge, chopped vineyard pruning waste and different agro-industrial waste against *F. oxysporum* f. sp. *melonis* in melon and *P. capsici* in pepper seedlings. The analyzed parameters were related to microbial diversity (PLFAs); microbial activity (general and specific enzymatic activities, such as dehydrogenase, β-glucosidase, NAGase and chitinase activities); and chemical parameters. Plant hormones, such as SA, JA and ABA were also analyzed to identify any effect of the composts on plant physiology.

MATERIALS AND METHODS

Composts

Three agro-industrial composts were made from artichoke sludge (AS, 150 g kg^{-1}), chopped vineyard pruning waste (VPW, 500 g kg^{-1}) and various agro-industrial wastes (expressed as dry weight), as follows: compost 1 (C1): AS + VPW + blanched artichoke (350 g kg^{-1}); compost 2 (C2): AS + VPW + garlic waste (350 g kg^{-1}); and compost 3 (C3): AS + VPW + dry olive cake (DOC, 350 g kg^{-1}). The AS and agro-industrial wastes were obtained from a fruit and vegetal processing factory (Murcia, Spain) and from a oil olive production factory (Albacete, Spain). They were collected and transported to the experimental farm of CSIC in Santomera (Murcia, Spain) where the composting process was made. Composts were produced in open-air piles of 10 m^3 over 3 months. The moisture content was initially set at 40–50% and was maintained by watering. Piles were periodically turned to ensure aeration and temperature evolution was monitored periodically (data not shown). The chemical characteristics of the composts are shown in Table 1. Once the composting process was finished, composts were milled and passed through a 1 cm sieve. Three samples of each compost pile were taken by mixing nine subsamples from random sites on each pile. Samples were stored at 4 °C for further analysis. Before starting the greenhouse nursery experiment, compost and peat were analyzed through chemical analysis, enzymatic activities assays and PLFA analysis.

Fungal strains

The pathogen FOM was isolated from infected muskmelon plants from a greenhouse nursery. Conidia were recovered as described by Blaya *et al.*[23] The pathogen *P. capsici* was isolated from pepper plants showing disease symptoms. The inoculum of *P. capsici* was produced by transferring one agar plug (5 mm) of 7-day-old mycelia on pea agar medium (100 g L^{-1} ground peas, 100 mg L^{-1} β-sitosterol and 20 g L^{-1} technical agar, adjusted to pH 5.5), autoclaved at 121 °C for 20 min and amended with 100 mg L^{-1} sterilized streptomycin. The culture was maintained at 28 °C for 7 days. The mycelia was recovered from the content of two Petri dishes and mixed with 100 mL sterile distilled water using a blender.

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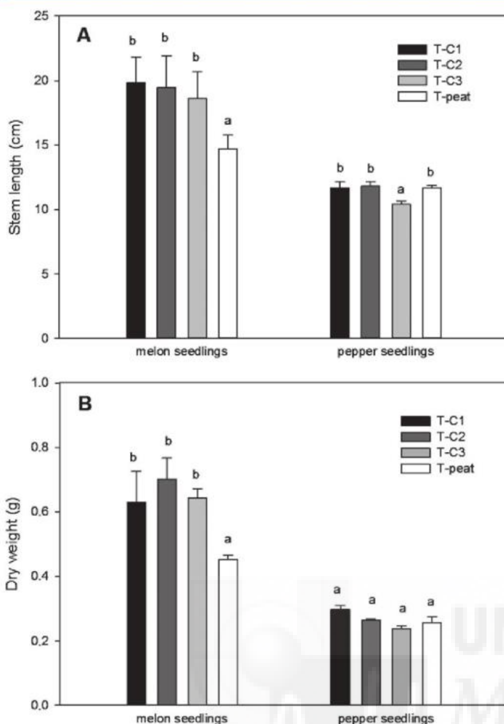

Figure 1. (A) Stem length (cm) and (B) dry weight (g) of aerial parts of melon and pepper seedlings grown in different treatments: compost C1, compost C2, compost C3 and peat. Bars indicate the standard error of six replicates. For each parameter, data values followed by the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

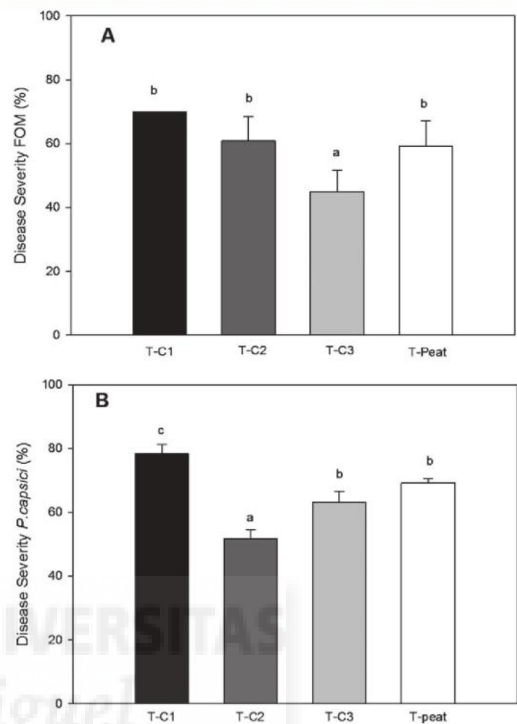

Figure 2. (A) Disease severity in melon and (B) pepper seedlings. Bars indicate the standard error of six replicates. For each parameter, data values followed by the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

Greenhouse nursery experiment

The treatments used as a growing medium for cultivating muskmelon and pepper seedlings were 1:1 (w/w) mixture of compost and black peat: compost C1 (T-C1), compost C2 (T-C2) and compost C3 (T-C3) treatments. Black peat alone was used as a control treatment (T-peat). Muskmelon (*Cucumis melo* L. cv. Giotto) and pepper (*Capsicum annuum* cv. Lamuyo) seeds were sown on the different growing media assayed as treatments, with a cover of vermiculite, in polystyrene trays, one seed per well (5 cm diameter well). Seeds were germinated in a growth chamber at 28 ± 1 °C and 90–95% relative humidity. After seeds germinated, the different trays (each one considered as one replicate) were randomly distributed in a polyethylene-covered greenhouse nursery under natural daylight conditions. Once the first true leaf appeared (15 and 30 days after sowing in muskmelon and pepper seedlings, respectively), six replicates with 10 plants per replicate were inoculated either with 2 mL of a FOM conidial suspension to achieve a final concentration of 10^4 conidia g^{-1} substrate or with 2 mL *P. capsici* mycelium to achieve a final concentration of 10^3 cfu g^{-1} substrate. A total of 60 plants of muskmelon and 60 of pepper (six replicates with 10 plants per replicate) were inoculated with 2 mL distilled water and used as the control.

Five seedlings per replicate were harvested 30 days after inoculation and both the dry weight and height of the aerial parts of seedlings were measured to determine whether composts are a viable alternative for peat as organic growing media for cultivating muskmelon and pepper plants under greenhouse conditions.

The rest of the seedlings (five plants per replicate) were used for hormone determinations. The plants were frozen in liquid nitrogen immediately after harvest and lyophilized, but only the aerial parts were used. The levels of SA, ABA and JA hormones were determined following the method reported by Flors *et al.*[24] The analyses were carried out using a Waters Alliance 2695 HPLC system (Milford, MA, USA). The chromatographic system was interfaced to a Quatro LC mass spectrometer (Micromass; http://www.mciromass.co.uk).

Just before seedlings were harvested, the ability to suppress diseases was assayed for the three compost treatments and peat. Disease severity for muskmelon seedlings was evaluated as follows: 1, healthy plants; 2, yellowing; 3, stem wilting; 4, dead plants. For pepper seedlings, disease severity was rated according to the following scale: 1, healthy plants; 2, wilting; 3, necrotic stem; 4, dead plants.

Chemical analysis of composts and peat

The pH and electrical conductivity (EC) of composts and peat were measured in a 1:10 (w/v) water-soluble extract in a conductivimeter and pH meter. Total organic carbon (TOC) was determined by the method of Yeomans and Bremmer[25] and total

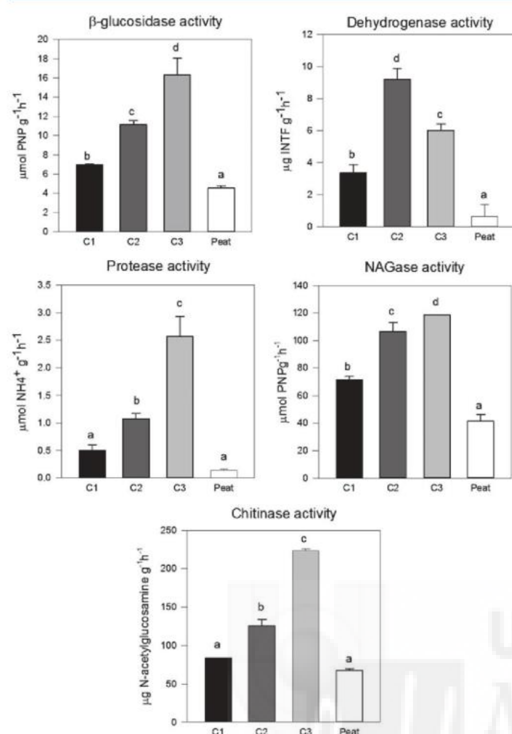

Figure 3. Enzymatic activities of β-glucosidase, dehydrogenase, protease, NAGase and chitinase activities in composts and peat. Bars indicate the standard error of three replicates. For each parameter, data values followed by the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

organic nitrogen by the Kjeldahl method, as modified by Bremner and Mulvaney.[26] Nutrients and heavy metals were determined after nitric–perchloric digestion by inductively coupled plasma–optical emission spectrometry (ICP-OES; ICAP 6500 DUO THERMO, Thermo Fisher Scientific). The analyses were performed in triplicate.

PLFA analysis

Phospholipids were determined in 2 g composts and peat according to Bastida *et al.*[27] The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω9c and cy19:0 were chosen to represent bacterial biomass (bacterial PLFA), and 18:2ω6 was taken to indicate the fungal biomass (fungal PLFA). The ratio of bacterial PLFA to fungal PLFA (bacterial PLFA/fungal PLFA) represents the ratio between bacterial and fungal biomass. The Gram-positive ($Gram^+$) specific fatty acids i15:0, a15:0, i16:0 and i17:0 and the Gram-negative ($Gram^-$) specific fatty acids cy17:0, 18:1ω9c and cy19:0 were taken as a measure of the ratio between $Gram^+$ and $Gram^-$ bacterial biomass ($Gram^+$:$Gram^-$). The fatty acids 15:1, 16;1ω7, 15:1ω6, 16:1ω5, 17:1, 18:1ω9c, 18:1ω7 and 18:1ω9t represent monounsaturated fatty acid, whereas the fatty acids 14:0, i15:0, a15:0, 15:0, i16:0, 16:0, i17:0, cy17:0, 17:0, 18:0, 20:0, 22:0 and 24:0 represent saturated fatty acids. The ratio of monounsaturated PLFAs to saturated PLFAs is expressed as monounsaturated/saturated. All the results are given in nmol g^{-1}. The analyses were performed in triplicate.

Enzymatic activities

Dehydrogenase activity was measured by the method reported by Garcia *et al.*[28] and β-glucosidase activity was determined following the method reported by Eivazi and Tabatabai.[29] Protease activity, on the other hand, was measured by the method described by Bonmati *et al.*[30] *N*-Acetyl-β-glucosaminidase (NAGase) activity was measured according to Parham and Deng.[31] Finally, chitinase activity was determined by measuring the quantity of *N*-acetylglucosamine formed by enzymatic hydrolysis.[32] The analyses were performed in triplicate.

Statistical analysis

The chemical properties and PLFAs of composts and peat as well as disease severity and hormone profiles were subjected to one-way analysis of variance (ANOVA). When the *F*-statistic was significant, Tukey's post hoc test ($P \leq 0.05$) was used to separate means. Factor analysis was performed using the relative abundances of all identified PLFAs. The extraction of the factors was carried out using the method of principal components. The factorial solutions were rotated using the Varimax normalized method. A Pearson correlation was made between all the dates, separating both pathosystems. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Greenhouse nursery experiment

The length of muskmelon and pepper seedlings was significantly affected ($P < 0.001$ and $P = 0.03$ respectively) by treatment (Fig. 1A). For muskmelon seedlings, the compost treatments showed significantly greater stem length than the peat treatments (T-peat), while for pepper seedlings the smallest plant length appeared in T-C3 treatment. The rest of the treatments showed no significant differences with respect to T-peat.

The weight of muskmelon seedlings was also affected ($P = 0.008$) by treatment. Compost treatments showed higher plant weight than T-peat, with no significant differences between the different compost treatments. However, no significant differences were found in the case of pepper seedlings (Fig. 1B).

Plant disease severity caused by FOM and *P. capsici* was significantly affected ($P = 0.002$, $P < 0.001$ respectively) by treatment (Fig. 2). Compost treatments showed different suppressive behavior depending on the pathosystem. Muskmelon plants grown in T-C3 treatment showed significantly lower disease severity when infected with FOM, while pepper plants grown in T-C2 treatment showed significantly lower disease severity when infected with *P. capsici*. However, T-C1 treatment did not show any suppressive effect, and in the case of FOM showed no significant difference compared to T-peat. Regarding *P. capsici*, plant disease severity was even higher in T-C1 treatment than in peat.

Chemical parameters

Both pH and EC showed significant differences ($P < 0.001$) according to the different treatments (Table 1). Composts showed significantly higher pH (6.45–7.02) than peat (5.52), with compost C2 presenting the highest value. Composts also showed significantly higher EC values than peat. Composts C1 and C2 showed the highest EC values (11.74 and 11.42 dS m^{-1}), which were up to two times

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Table 2. Bacterial, fungal, Gram-positive, Gram-negative, saturated, monounsaturated PLFA concentration and Shannon index in composts and peat

Parameter	Compost C1	Compost C2	Compost C3	Peat
Fungal PLFA (mol g^{-1})	0.50 ± 0.23a	0.69 ± 0.01a	0.66 ± 0.01a	0.00 ± 0.00b
Bacterial PLFA (mol g^{-1})	3.4 ± 0.44a	4.0 ± 0.03a	3.1 ± 0.07a	0.21 ± 0.19b
Bacteria Gram-positive (mol g^{-1})	1.0 ± 0.04a	0.92 ± 0.02a	1.1 ± 0.04a	0.00 ± 0.00b
Bacteria Gram-negative (mol g^{-1})	2.4 ± 0.40a	3.1 ± 0.64a	2.0 ± 0.03a	0.21 ± 0.19b
Total saturated PLFA (mol g^{-1})	9.0 ± 0.44a	10.4 ± 2.6a	9.4 ± 0.18a	2.2 ± 0.18b
Total monounsaturated PLFA (mol g^{-1})	1.4 ± 0.28a	2.2 ± 0.11a	1.9 ± 0.08a	0.29 ± 0.05b
Bacteria/fungi ratio	6.8 ± 0.87a	5.8 ± 0.92ab	4.7 ± 0.11b	0.00 ± 0.00c
$Gram^{+}$:$Gram^{-}$ ratio	0.43 ± 0.05b	0.31 ± 0.06c	0.5 ± 0.01a	0.00 ± 0.00d
Monosaturated:saturated ratio	0.15 ± 0.02b	0.22 ± 0.04a	0.2 ± 0.01ab	0.13 ± 0.03b

Abbreviations: PLFA, phospholipid fatty acid profile. Data are means of three replicates. For each parameter, data values followed by the same letter are not significantly different according to Tukey's post hoc test ($P \leq 0.05$).

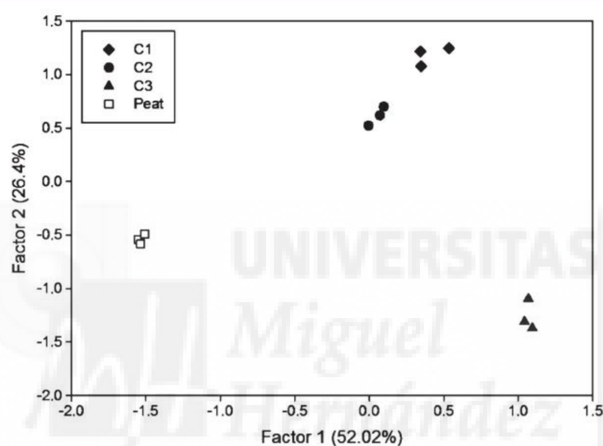

Figure 4. Distribution of compost C1, compost C2, compost C3 and peat after factor analysis using all individual PLFAs analyzed.

higher than those of compost C3 and up to five times higher than those of peat. Composts also showed higher macronutrient content than peat. C1 and C2 showed the highest values for P and K, and compost C2 the highest value for N (Table 1).

Microbial parameters

Enzymatic activities

All the enzymatic activities measured were significantly different among treatments ($P < 0.001$). For all composts, β-glucosidase, dehydrogenase and NAGase activities showed significantly higher values than for peat. Protease and chitinase activities, on the other hand, were only significantly higher in composts C2 and C3 than in peat (Fig. 3). C3 showed the highest value of enzymatic activities overall, with the exception of dehydrogenase activity, which was higher in compost C2 (Fig. 3). In contrast, compost C1, compared with the rest of composts, showed the lowest values for all enzymatic activities and even had similar values to peat for two activities (protease and chitinase) (Fig. 3).

PLFA analysis

The levels of bacterial PLFAs ($P = 0.001$) and bacteria Gram-positive ($P = 0.001$) and bacteria Gram-negative^{-} PLFAs ($P = 0.005$) were significantly affected by treatment, and in all cases were significantly higher in composts than in peat (Table 2). The $Gram^{+}$:$Gram^{-}$ PLFA ratio was significantly different ($P < 0.001$) in each compost following the order C3 > C1 > C2, whereas the bacterial:fungal PLFA ratio was significantly different following the order C1 > C2 > C3. The monounsaturated:saturated ratio was significantly lower in compost C1 and peat compared to composts C2 and C3, which showed the highest ratios (Table 2).

The principal component analysis of PLFAs isolated from compost revealed that the three composts showed different microbial community structures. Multivariate factor analysis performed with the relative abundances of the PLFAs identified two main factors (Fig. 4): Factor 1, explaining 52.02% of the variance, and Factor 2, 26.4%. For Factor 1, the post hoc test established four significantly different ($P \leq 0.05$) groups (peat and composts C1, C2 and C3), where fatty acids 18:0, i15:0 and 18:2ω6 had the highest statistical factor weights (Table 3).

Hormone measurements

In this study, we analyzed changes in the hormonal profile of muskmelon and pepper seedlings associated with *F. oxysporum* and *P. capsici* infection, respectively, and with the different treatments. Results showed that inoculation of both pathogens

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Table 3. Factor loading matrix of each PLFA after varimax rotation

	Load factor of each variable	
Parameter	Factor 1	Factor 2
18:0	0.986	0.14
i15:0	0.973	0.16
18:3ω6	0.971	−0.34
i16:0	0.967	−0.05
18:1ω9c	0.967	−0.09
i17:0	0.967	0.11
18:2ω6c	0.937	0.13
15:0	0.885	−0.44
cy17:0	0.846	0.49
16:0	0.828	−0.46
17:0	0.817	−0.52
12:0	0.766	−0.42
17:1	0.737	−0.07
14:1	0.653	0.01
22:0	0.476	−0.76
20:1	0.402	0.87
14:0	0.387	0.82
15:1	0.325	0.80
20:0	0.324	0.81
22:6ωc	0.323	0.804
Cy19:0	0.187	0.93
13:0	−0.80	0.58

strongly modified the defense-related hormones measured by SA, ABA and JA (Table 4).

Muskmelon seedlings

In the absence of pathogen, higher shoot SA levels were observed in muskmelon seedlings grown in T-C1 and T-C2 treatments. Opposite results were found in the case of JA, where T-C3 treatment and T-peat seedlings showed the highest levels. No differences in ABA levels were found among treatments ($P > 0.934$). The presence of FOM mediated significantly higher SA and ABA concentrations in all treatments, whereas it only mediated a higher JA concentration in peat. The peat treatment (T-peat) showed significantly higher values than the compost treatments. No significant differences in any hormone concentration were found among composts (Table 4).

Pepper seedlings

In absence of pathogen, shoot SA and ABA concentrations in pepper seedlings were significantly higher in T-peat than those found in the different compost treatments, with no observed differences between composts. Shoot JA concentration was only detected in pepper seedlings in T-C2 treatment. In the presence of the pathogen, for SA, the T-peat treatment showed significantly higher concentrations than the compost treatments. For shoot ABA concentrations, T-C1 treatment showed the highest concentration, with up to a twofold increase. JA was only detected in T-C3 treatment and T-peat.

Correlation analysis

Correlation analysis showed that there were negative and significant correlation coefficients between disease severity and β-glucosidase, protease, NAGase and chitinase activities in muskmelon seedlings (Table 5). In the case of pepper seedlings, negative and significant correlation coefficients were found between disease severity and general microbial activity such as dehydrogenase activity (Table 5). It is important to note the negative correlation coefficient between disease severity and JA in pepper seedlings grown on peat and the positive correlation coefficient between disease severity and ABA in infected pepper seedlings (Table 5).

DISCUSSION

Studies under greenhouse nursery conditions are quite important to obtain healthy seedlings and avoid possible crop losses in the field due to pests and diseases. All composts assayed were found to be good organic substrates as an alternative to reduce the use

Table 4. Measures of different hormone concentrations (in nanograms per gram fresh weight-FW-) in muskmelon and pepper seedlings

		Muskmelon seedlings		Pepper seedlings	
Parameter	Treatments	Without *F. oxysporum*	With *F. oxysporum*	Without *P. capsici*	With *P. capsici*
SA ($ng\ g^{-1}$ FW)	T-C1	1481 ± 63.4a	4667 ± 209b	782 ± 47.5b	953 ± 24.7ab
	T-C2	1326 ± 16.3a	7364 ± 1120b	646 ± 101b	932 ± 30.6ab
	T-C3	854 ± 139b	3032 ± 158b	677 ± 58.3b	779 ± 72.3b
	T-Peat	715 ± 78.0b	55309 ± 3582a	1894 ± 85.9a	1112 ± 139a
ABA ($ng\ g^{-1}$ FW)	T-C1	313 ± 77.2a	353 ± 23.8b	386 ± 11.8b	884 ± 20.5a
	T-C2	320 ± 77.7a	422 ± 17.4b	350 ± 5.8b	587 ± 51.6b
	T-C3	296 ± 20.9a	448 ± 3.3b	379 ± 32.6b	473 ± 62.2c
	T-Peat	324 ± 37.5a	731 ± 116a	552 ± 40.1a	506 ± 26.7bc
JA ($ng\ g^{-1}$ FW)	T-C1	16.8 ± 1.9b	21.4 ± 0.33b	BDL	BDL
	T-C2	19.7 ± 5.1b	14.6 ± 14.5b	125 ± 0.40	BDL
	T-C3	33.2 ± 5.5a	29.7 ± 9.2b	BDL	10.3 ± 2.4
	T-Peat	35.6 ± 6.9a	240 ± 2.0a	BDL	11.7 ± 3.5

Abbreviations: SA, salicylic acid; ABA, abscisic acid; JA, jasmonic acid; BDL, below detection limit. Data are means ± standard error of three replicates. For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($P \leq 0.05$).

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Table 5. Correlation between disease severity and different biochemical parameters and hormone profiles

Parameter	Correlation coefficient in melon plants	Correlation coefficient in pepper plants
Glucosidase activity	−0.790**	NS
Dehydrogenase activity	NS	−0.762**
Protease activity	−0.801**	NS
NAGase activity	−0.631*	NS
Chitinase activity	−0.904**	NS
JA in control plants	NS	−0.808**
ABA in infected plants	NS	0.588*

NS, not significant; JA, jasmonic acid; ABA, abscisic acid.
Asterisks indicate significant differences at $^{*}P \leq 0.05$ $^{**}P \leq 0.001$.

of peat. Stem length and dry weight of muskmelon and pepper seedlings in composts were similar to or even higher than in peat, which could be due to the higher macro- and micronutrient content of composts. EC and pH have an important influence on seedling quality. Our composts showed a range of pH values (6.45–7.0) at which most greenhouse-grown species display better growth because the availability of nutrients is greater.[33,34] The ECs of the composts assayed had high values (5.47–11.74 dS m^{-1}). Our results agree with those of other authors who have observed positive growth results in plants obtained using composts with initial EC values higher than 8 dS m^{-1}.[33,35]

The ability of certain composts to suppress soil-borne pathogens tends to be variable among different composts and pathogen species. It has been observed that materials that suppress one pathogen are often ineffective against or conducive to another pathogen.[14,36] The structure of soil microbial communities is of primary interest when studying soil suppressiveness. Changes in microbial community composition have been widely related to suppressive organic amendments and farming practices.[37] Jindo *et al.*[38] used the different relative abundances of phospholipid fatty acids as an indicator of changes in the microbial community structure in different composts. The microbial community structure of compost is an important factor for their suppressiveness and it can be influenced by pH.[39] Compost C1 not only showed a significantly lower monounsaturated PLFA:saturated PLFA ratio compared to other composts and the lowest pH values, but also showed higher disease severity for both pathogens compared to the rest of the composts and even higher disease severity for *P. capsici* than peat. The monounsaturated:saturated ratio has been used by some authors to indicate C availability in soils.[27] The lowest monounsaturated PLFA:saturated PLFA ratio shown in compost C1 and peat indicated the low biodegradability of the carbonated substrates, which may lead to a lower microbial activity, as was demonstrated showing the lowest levels of dehydrogenase activity. Ntougias *et al.*[40] showed that the ability of composts to suppress *F. oxysporum* could be attributed to the shift of microbial communities to actinomycetes, which may be involved in the mechanisms of disease suppression because of their ability to produce chitinases and antibiotics.[41] In this sense, compost C3 showed the highest levels of protease, NAGase and chitinase-specific activities and the lowest Fusarium disease severity, indicating a potentially high level of colonization of chitinolytic enzyme-producing microorganisms.[42] Although disease suppression may depend on a few species that represent only a small proportion of the total microbial biomass[43] and may not be detected by PLFAs, the $Gram^{+}$:$Gram^{-}$ ratio indicated the preferential development of Gram-positive bacteria in compost C3, which may be interpreted as a shift towards an enriched actinomycete community.

Phytophthora species are often considered highly sensitive to microbial competition and are recognized for their general suppressiveness.[40] The higher monounsaturated:saturated ratio in compost C2 could be related to the significantly high levels of dehydrogenase activity[27] and hence the lower disease severity observed in pepper seedling in compost T-C2. Similar results have been reported in previous studies regarding a positive correlation between compost microbial activity and suppression of Pythium and Phytopthora root rots.[44,45] Although it seems that *a priori* little change may be induced in compost characteristics as a consequence of using only one different material, the PLFA technique shows significant differences in the microbial community structure of the different composts. The composition of bacterial and fungal communities seems to be affected by the chemistry of the parent organic material from which the compost is prepared[19] and so that, the disease suppression achieved by composts.[46] In our study the most suppressive compost for FOM, the compost with dry olive cake (compost C3) – material that contains a high percentage of lignin (20%) – could sustain sufficient microbial activity over time, fed by slow degradation of its compounds. Lignin decomposes even more slowly than cellulosic and less biodegradable substances.[19] In the case of compost C2 – the most suppressive compost against *P. capsici* – a different raw material used was garlic, which has been recognized as a substance with a broad-spectrum antimicrobial activity against many genera of bacteria and fungi.[47] It may exert control not only in *P. capsici*[48] but also in microorganisms which colonize the compost during the maturation phase of the composting process. As a result, the microbial activity of this compost may be inhibited until low levels of active components of garlic remain in the compost. Thus the concentrations of recalcitrant (lignin from dry olive cake) and antimicrobial (active components of garlic) compounds could define the longevity of the suppressive effect of compost C3 and C2, respectively. Several authors have proposed that induced resistance is one of the mechanisms that drive the phenomenon of disease suppression by composts[22] due to their rich microbial community. Several microorganisms have been described as inducing either systemic acquired resistance (SAR) or induced systemic resistance (ISR) in plants against a wide range of pathogens.[49,50] SAR is a response to pathogen infection that renders plants more resistant to subsequent infection. It is characterized by SA accumulation both at the initial point of infection and throughout the plant, and involves the induction of certain pathogenesis-related (PR) genes.[51] Shoot SA concentrations found in diseased muskmelon seedlings could suggest that SA plays a greater role in *F. oxysporum*–melon seedling interactions than it does in pepper seedlings. Mauch-Mani and Slusarenko[52] showed that *F. oxysporum* induced SAR and PR proteins in *Arabidopsis*. The plant hormone ABA has mostly been considered to act as a negative regulator of disease resistance, as it can interfere with signal transduction pathways that are controlled by other defense-related plant hormones such as SA, JA or ethylene (ET).[24] In our assay, pathogen infection modified the balance of the above-mentioned defense-related hormones by increasing mainly shoot SA and ABA concentrations. The high disease severity observed in infected pepper seedlings fits with the higher ABA concentration in the presence of the pathogen compared to infected muskmelon seedlings.

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Induced systemic resistance (ISR) is triggered by the inoculation of the plant with certain beneficial microorganisms; the plant is stimulated to respond more quickly and intensely when it is attacked by a pathogen. ISR is dependent on JA and ET.[53,54] The significantly higher shoot JA concentration found in the absence of the pathogen in muskmelon and pepper seedlings in T-C3 and T-C2 treatments respectively could have led to a specific stimulus in the plants, enhancing their ability to resist pathogen attacks. Despite the activation of the defense pathway in muskmelon and pepper seedlings as a consequence of FOM or *P. capsici* attacks, our results show a susceptible plant-pathogen interaction. Martínez-Medina et al.[55] observed similar results when neither *T. harzianum* nor *G. intraradices* were present in peat inoculated with *F. oxysporum*. Zhang et al.[56] suggested that the microflora in composts had an effect on PR proteins in both Arabidopsis and cucumber plants, although much of that activation resulted from infection by the pathogen. It has been observed that hormonal imbalance and activation of inappropriate defense responses may result from the ability of certain pathogens to manipulate the defense-related regulatory network of plants.[57]

CONCLUSIONS

It can be concluded that biological and microbiological parameters such as specific enzymatic activities (protease, NAGase, chitinase) in the case of Fusarium wilt caused by FOM and general enzymatic activities (dehydrogenase activity) in the case of Phytophthora root rot caused by *P. capsici*, could be used as potential indicators of compost suppressiveness. These parameters may be useful to evaluate the level and specificity of the suppression effect, representing a step toward an accurate prediction of compost suppressiveness.

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PUBLICATION 4

Insights into the suppressiveness of composts against *Phytophthora nicotianae* achieved using omics

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Insights into the suppressiveness of composts against *Phytophthora nicotianae* achieved using omics

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ABSTRACT

Phytophthora root rot is an economically important disease in pepper crops. The use of suppressive composts has become an alternative method for its control due to the lack of effective control measures. Although attempts have been made to reveal the relationship between microorganisms and compost suppressiveness, little is known about the species and their function in the microbial community associated with disease suppression. We report the metagenome and metabolome of different agro-industrial composts and peat, as well as their ability to control *Phytophthora nicotianae* under in vivo conditions. Insights into the microbial community structure and the metabolic variation associated with compost suppressiveness were obtained. The presence of well-known antifungal microbes such as *Streptomyces* and *Fusarium*, was not enough to control Phytophthora root rot for all composts. The composition and degree of stabilization of the composts and peat, revealed by ^{13}C-NMR spectroscopy, influenced the microbial activity and thus the disease suppression potential. Composts COM-A and COM-B, which contained high percentages of vineyard pruning waste and pepper waste as starting materials, were the most suppressive. Furthermore, the outcome PCA of the metabolic composition of both composts, was grouped into a cluster, completely separated from the non-suppressive composts. Further studies of compost metabolome may contribute to the identification of the sources of variability and shed light on the contribution of microorganisms to compost suppressiveness. To conclude, we propose an integration of different parameters (e.g. microbial activity and organic matter composition or metagenomics and metabolomics) as a promising approach for the identification of suppressive composts.

1. Introduction

Sweet pepper (*Capsicum annuum* L.) is one of the main horticultural crops in Murcia (southeast Spain), with over 1334 ha of total cultivated area in greenhouses.[1] Here, *Phytophthora nicotianae* (*P. nicotianae*) has been reported as the main causal agent of Phytophthora root rot in pepper plants[2]. Management of this disease is based on phenylamide fungicides but fungicide-tolerant strains have been detected.[3]In this sense, alternative methods of control are being studied, and the use of composts is one of the most promising.[2]

The food industries in the Mediterranean area generate important amounts of organic wastes that can be recovered after composting, an ancient practice by which by-products or organic residues are degraded through the activities of successive groups of microorganisms. Composts can be used in growing media as a partial substitute for peat - to produce high quality seedlings and thus

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increase the guarantee of crop success after transplanting.[4,5,6] Furthermore, composts have been proven to suppress a wide variety of soil-borne plant pathogens under nursery and field conditions.[5,7,8,9] Unfortunately, the suppressiveness of composts is inconsistent and some studies even reported an increase of disease incidence.[10,11] The understanding of the complexity and mechanisms behind disease suppression is critical for its maximization. In this sense, the quality of compost organic matter is important with respect to the efficacy of the suppression and the regulation and maintenance of microbial communities in composts.[12] Shifts in the chemical composition of compost organic matter can be characterized by ^{13}C- Nuclear Magnetic Resonance (^{13}C-NMR) spectroscopy,[13] which provides direct information on the structural and conformational characteristics of humic carbon back bones.[14] However, its ecological significance should be elucidated in relation to the microbial communities inhabiting the organic matter,[15] that can be studied through molecular analysis – such as fingerprinting techniques and sequencing.[16,17,18,19] High-throughput sequencing (e.g. 454-pyrosequencing or Ion- torrent) is a powerful alternative for the identification at a greater depth of the microbial community composition and diversity. Today, there are few studies available regarding the metagenomics of composts and most of them only include either bacterial or fungal communities.[20,21]

It should be noted that genomic information itself is not enough for understanding the biological processes that take place within compost. Several studies pointed out the central role played by metabolites in cellular activities and the mileage that could be gained by monitoring at the level of the metabolome.[22] Metabolites (low-molecular-weight compounds such as amino acids, sugars, and lipids) play significant roles in the microbial regulation of the central and secondary metabolism. They participate in the resulting signal transduction pathways and regulate metabolic routes at different levels: genetic, transcriptional, translational, or at the level of the proteins.[22] Not only can they contribute to external signals as indicators of the environmental conditions or by sensing such signals, but also they vary in response to a variety of stimuli (e.g. nutritional deficit, external stressors, or disease).[23] Currently, the implementation of metabolomics for environmental monitoring (e.g. soil, compost, water) is still at an early stage, mostly applied as a screening tool to assess the potential toxic effect of pollutants.[24]

The aim of this work was - for the first time, to the best of our knowledge - to study jointly the quality of compost organic matter, its metagenome and its metabolome. A detailed evaluation of the differences in suppressive composts, in comparison to non-suppressive or conducive composts, could offer a deeper insight into the mechanisms involved in the suppression of *P. nicotianae* in pepper crops and help to obtain reliable parameters for the prediction of suppressive parameters.

2. Methods

2.1. The compost assayed and their analysis

Four agro-industrial composts were made from different wastes (expressed as dry weight) as follows: Compost A (COM-A): pepper sludge (125 g kg^{-1}), pepper wastes (125 g kg^{-1}), and vineyard pruning wastes (750 g kg^{-1}); Compost B (COM-B): pepper wastes (170 g kg^{-1}), artichoke wastes (160 g kg^{-1}) and vineyard pruning wastes (680 g kg^{-1}); Compost C (COM-C): pepper sludge (190 g kg^{-1}), pepper wastes (20 g kg^{-1}), garlic wastes (20 g kg^{-1}), carrot wastes (340 g kg^{-1}), almond shells (40 g kg^{-1}) and vineyard pruning wastes (380 g kg^{-1}); Compost D (COM-D): artichoke sludge (150 g kg^{-1}), artichoke wastes (344 g kg^{-1}), vineyard pruning wastes (500 g kg^{-1}), and compost (86 g kg^{-1}).

The composts were produced in open-air piles of 200 kg, bioxidative phase lasting 75 days and maduration 42 days. The moisture content was initially set at 40-50 % and was

maintained by watering. The piles were turned periodically to ensure aeration, and the temperature evolution was monitored periodically (data not shown). Once the composting process was finished, the composts were milled and passed through a 1 cm sieve. Three samples of each compost pile were taken by mixing nine sub-samples from random sites within each pile. The samples were stored at -20 ºC and 4 ºC for subsequent analysis.

The pH and electrical conductivity (EC) of the composts and peat (P) were measured in a 1:10 (w/v) water-soluble extract in a conductivimeter and pH meter, respectively. The total organic carbon and nitrogen were measured with an Elemental Analyzer (LECO TruSpec C/N) and nutrients by ICP-OES (ICAP 6500 DUO). Physical properties were measured following Bustamante et al.[4] Dehydrogenase activity was measured by the method of Garcia et al.[25] The physical, physico-chemical, and biological characteristics of the composts and peat are shown in Table 1.

2.2. Organic matter analysis by ^{13}C-NMR

The organic matter composition of the four composts and one peat was estimated by spectral intensity integration over regions with chemical shift characteristics of different organic carbon functional groups. The CPMAS ^{13}C-NMR experiments were performed in a Bruker Advance DRX500 operating at 125.75 MHz for ^{13}C. The samples were packed into a 4-mm-diameter cylindrical zirconia rotor with Kel-F end-caps and spun at 10000 ± 100 Hz. A conventional CPMAS pulse sequence[26] was used, with a 1.0-ms contact time. Between 2000 and 5000 scans were accumulated, with a pulse delay of 1.5 s. The line broadening was adjusted to 50 Hz. Spectral distributions (the distribution of total signal intensity among various chemical shift ranges) were calculated by integrating the signal intensity, expressed as a percentage, in five chemical shift regions: 0-45 (aliphatic structures), 45-60 (methoxy groups), 60-110 (polysaccharides structures region), 110-160 (aromatic structures) and 160-210 (carboxyl, carbonyl, amide C).[27] The alkyl/O-alkyl ratio was also calculated.[13]

Table 1. Physical, physico-chemical and biological properties of the composts and peat.

	COM-A	COM-B	COM-C	COM-D	Peat
pH	8.5 c	8.9d	8.8d	6.2b	5.5a
EC (mS cm^{-1})	1.9ab	2.6c	1.8a	3.8d	2.0b
Total C (g kg^{-1})	43d	32b	38d	27a	48e
Total N (g kg^{-1})	25d	22b	21c	24c	13a
P (g kg^{-1})	3.7c	4.0c	0.40b	5.6d	0.3a
K (g kg^{-1})	23d	27e	15.3b	16.6c	0.6a
AD (g cm^{3})	0.46b	0.52b	0.52b	0.39a	0.38a
TPS (%)	72ab	69a	69a	76c	75bc
WHC (mL L^{-1})	441a	469a	423a	467a	547b
Air content (%)	27.6b	22.0a	27.2b	29.4b	20.5a
Dehydrogenase activity (mg INT g^{-1})	27.2c	37.0d	9.4b	3.2a	0.32a

EC, electrical conductivity; AD: apparent density; TPS: total pore space; WHC: water holding capacity. Data are mean of three replicates. For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($p \leq 0.05$).

2.3. DNA extraction, sequencing, and analysis

Total DNA was extracted using the FastDNA® Spin Kit for soil (Q-Biogene, Carlsbad, CA, USA), following the manufacturer´s instructions. The DNA concentrations of samples were determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., DE, USA); the samples were then stored at -20 ºC until required. For the molecular analysis of bacterial communities, the 16S rRNA gene was amplified using primers P1, P2, P3, and P4 (Table 2) and for the fungal community, the ITS1 and ITS2 region of the fungal rRNA gene was amplified using the ITS5/ITS2 and ITS3/ITS4 primers (Table 2). Each sample was amplified in triplicate; the amplicons were purified using the QIAquick PCR Purification Kit (250) (Qiagen, Hilden, Germany) and composited together at equimolar concentration prior to sequencing. For PCR amplification, each 25 µl PCR mix contained the following reagents: 1X KAPA2G Fast HotStart ReadyMix2 (2X) (Kapa Biosystems, Boston, MA, USA), 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 5 µl of DNA.

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Table 2. Primers used for sequencing.

Name	Size (*E. coli*)	Forward	Reverse
P1	112	AGAGTTTGATCMTGGCTCAG	TTACTCACCCGTICGCCRCT
P2	196	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG
P3	105	ACGCGARGAACCTTACC	ACGAGCTGACGACARCCATG
P4	353	AGAGTTTGATCMTGGCTCAG	CYIACTGCTGCCTCCCGTAG
Name	**Size (*S. himantioides*)**	**Forward**	**Reverse**
ITS5-ITS2	306	GGAAGTAAAAGTCGTAACAAGG	GCTGCGTTCTTCATCGATGC
ITS3-ITS4	385	GCATCGATGAAGAACGCAGC	TCCTCCGCTTATTGATATGC

The PCRs for primers P1, P2, P3, and P4 were performed using the following conditions: 15 cycles of denaturation at 90 ºC for 30 s, amplification with a temperature gradient of 70 ºC-50 ºC for 30 s, and a final extension of 72 ºC for 30 s. Additionally, samples were held for 30 cycles of denaturation at 94 ºC for 45 s, amplification at 50 ºC for 45 s, and a final extension of 72 ºC for 45 s. The PCRs for primer V6 and the ITS region had an initial denaturation step at 95 ºC for 3 min, followed by 25-40 cycles of denaturation at 95 ºC for 15 s, amplification at 60 ºC for 15 s, extension at 72 ºC for 15 s, and a final extension of 72 ºC for 1 min.

A library was created using the Ion Plus Fragment Library Kit, and barcodes were added by the Ion Xpress ™ Barcode Adapters 1-96 Kit. The template preparation was performed with the Ion OneTouch ™ 2 System and the Ion PGM ™ Template Kit OT2 400. Finally, the platform sequenced the samples using Ion Torrent PGM (Life Technologies, Carlsbad, CA, USA) with the Sequencing Kit Ion PGM 400 in chips Ion 318 Chip kit and Ion 314 Chip kit.

The data analysis was performed using the software packages QIIME v1.8.0. and USEARCH v7.0.1090. Sequences shorter than 60 bp and/or Q mean quality score below 25 were removed. Primers and barcodes were removed and a chimera filter was used. The remaining high quality sequences were grouped in operational taxonomic units (OTUs) following the Open Reference method; sequences were clustered against the GreenGenes v13_8, for the bacterial community, and against UNITE/QIIME 12_11 ITS, for the fungal community, using the *unclust* method with 97 % similarity. Sequences not matching the database were subsequently clustered de *novo*. A representative set of OTUs was generated and then the taxonomy of each of the OTUs was assigned using the same database.

2.4. Metabolite extraction and analysis

Metabolite extraction was performed using a water extraction (1:10, compost to deionized water) of six replicates of the composts and peat. The mixtures were shaken for two hours, after which the supernatant was passed through a 0.2-mm filter. The supernatant was analyzed using an Agilent 1290 Infinity UPLC system coupled to a 6550 Accurate Mass quadrupole TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany) using an electrospray interface with jet stream technology. Separation was achieved on a reverse phase Poroshell 120 EC-C18 column (3X100 mm, 2.7 µm: Agilent) operating at 30 ºC. The mobile phases were water:formic acid (99.9:0.1 v/v; phase A) and acetonitrile:formic acid (99.9: 0.1 v/v; phase B). An isocratic flow of 95% phase A and 5% phase B was maintained for 3 min. The flow rate was set constant at 0.4 mL/min and the injection volume was 3 µL. The optimal conditions of the electrospray interface were as follows: gas temperature 280 ºC, drying gas 9 L/min, nebulizer 45 psi, sheath gas temperature 400 ºC, sheath gas flow 12L/min. Spectra were acquired in single MS mode with m/z range of 100-1100, negative polarity, and an acquisition rate of 1.5 spectra/s. Internal mass calibration, by simultaneous acquisition of reference ions and mass drift compensation, was used to obtain low mass errors.

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Data were processed using the Mass Hunter Qualitative Analysis Software (version B.06.00, Agilent Technologies). After that, a peak grouping was carried out, following a script, by R software. Statistical analysis was carried out with Metaboanalysis 2.5 software. A multivariant analysis of mass compounds by principal component analysis was used. For heatmap clustering of samples and mass compounds the squared Euclidean distance and ward linkage were utilized.

2.5. Suppressiveness bioassay

The pathogen *P. nicotianae* was isolated from pepper plants with disease symptoms. The inoculum of *P. nicotianae* was produced by transferring one agar plug (5 mm) of 7-day-old mycelia on pea agar medium (100 g L^{-1} ground peas, 100 mg L^{-1} β-sitosterol, and 20 g L^{-1} technical agar, adjusted to pH 5.5), autoclaved at 121 ºC for 20 min and amended with 100 mg L^{-1} sterilized streptomycin. The culture was maintained at 28 ºC for 7 days. The mycelia were recovered from the content of two Petri dishes and mixed with 100 mL of sterile distilled water, using a blender.

The composts were mixed with a commercial peat (50/50 v/v) to obtain different treatments: TCOM-A, TCOM-B, TCOM-C, TCOM-D, and TPeat (100% peat, the control). Seeds of pepper (*Capsicum annuum* cv. *Lamuyo*) were sown in trays of 150 pots, with one seed per pot and, a covering of vermiculite. Six replicates of each treatment were established randomly, each replicate consisting of 10 seeds. Germination was carried out in a germination chamber at 28 ± 1 ºC. Once the seeds had germinated, the trays were placed in a growth chamber under daylight conditions. Four replicates of each treatment were inoculated with 2 mL of *P. nicotianae* (~10^3 cfu g^{-1} substrate) after the first true leaf appeared. The suppressive effect of the different treatments was determined by measuring the disease incidence (number of diseased plants) 23 days after inoculation.

2.6. Statistical analysis

The physical, physico-chemical, and biological characteristics of the composts and peat, as well as the results from the suppressiveness bioassay, were subjected to one-way analysis of variance (ANOVA). When the F-statistic was significant, Tukey's post hoc test ($p \leq 0.05$) was used to separate means. Pearson correlations were made between the results from suppressiveness bioassay, the bacterial and fungal communities and the organic matter composition. The statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Physical, physico-chemical, and biological analyses

The main physical, physico-chemical, and biological characteristics of the composts and peat are shown in Table 1. Both pH and EC showed significant differences depending on the substrate (F=745; $p<0.001$; F=236; $p<0.001$, respectively). Peat had the lowest pH whereas, among the composts, COM-B, COM-C, and COM-A showed the highest pH values. Compost COM-C had the highest EC value and CCOM-B the lowest. As far as physical characteristics are concerned, Peat (P) and COM-D showed the lowest D_A values. All substrates showed an air capacity higher than 20 %, being significantly higher in composts than in peat; compost COM-A had the highest value (Table 1).

Dehydrogenase activity differed significantly among treatments (F=139; $p<0.001$), composts COM-A and COM-B showing the highest levels, and peat the lowest.

3.2. The suppressive effect of different growing media

The disease incidence of *P. nicotianae* on pepper 23 days after inoculation differed significantly among treatments (F=10.039; *p=0.001*) and indicated that treatment TPEAT

(100% peat) was the most conducive growing medium, followed by T-COM-D and T-COM-C, which only reduced the disease incidence by 13 % and 23 %, respectively, compared to TPEAT (Figure 1). Treatment TCOM-A was the growing medium most suppressive of *P. nicotianae*, with a reduction of 60 % compared to TPEAT, followed by TCOM-B, with a reduction of 50 % (Figure 1). Pepper plants in non-infested growth media did not show any symptoms of Phytophthora root rot.

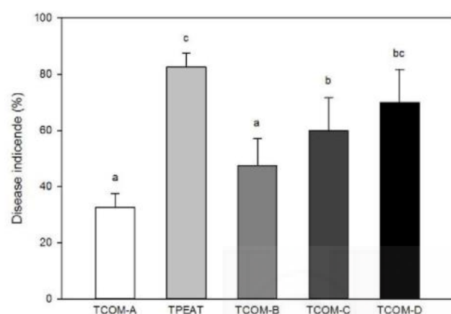

Fig. 1. Disease incidence in pepper seedlings caused by *P. nicotianae*.

3.3. Composition of the organic fractions in the compost and peat

The relative integration values for the five specific organic carbon regions from the composts and peat are shown in Figure 2a. Significant differences were observed among fractions (F=265.22 $p<0.05$). The fraction 0-45 ppm, corresponding to the aliphatic fraction ascribed to lipids, waxes, terpenoids, cutins, and suberins, and the fraction 60-110 ppm, corresponding to the carbohydrate region (polysaccharides, amino acids, amino sugars, lignin substitutes, and others),[13] showed higher relative abundances than the rest of fractions, namely 45-60 ppm (methoxy groups), 110-160 ppm (aromatic C structures) and 160-210 ppm (carboxyl and ester group) (Figure 2A).

Composts COM-A and COM-B showed lower relative abundances in the aliphatic and carbohydrate structure regions ($p<0.05$) compared to composts COM-C and COM-D and peat (P), whereas peat showed the highest relative abundance in both regions (Fig. 2a). The relative abundance in the aromatic C structure region (110-160 ppm) followed the trend: COM-B>COM-D>P>COM-A>COM-C, while for carboxyl and ester groups (160-210 ppm) it was COM-B>COM-A>COM-C>COM-D>P (Fig 2a). The alkyl/O-alkyl ratio followed the trend COM-C>P>COM-A=COM-D>COM-B (F=320 $p<0.05$) (Figure 2B).

3.4. Metabolomes of the different composts and peat

A principal component analysis (PCA) was applied to construct and validate a statistical model, to find differences among the

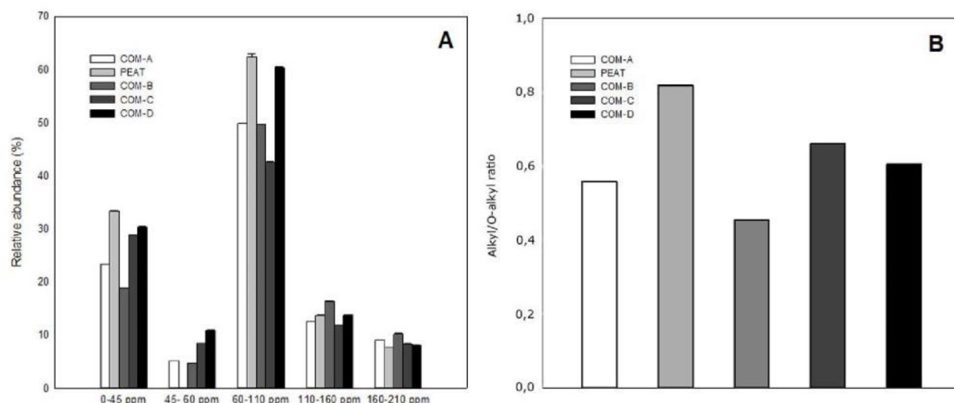

Fig. 2 Chemical composition of the composts and peat revealed by ^{13}C-NMR; **A.** Distribution of organic carbon functional groups. **B**. Alkyl/O-alkyl ratio values.

metabolomes of the composts and peat. The two relevant axes explained 88.6 % of the variance (PC1 60.7 % and PC2 27.9 %) (Fig. 3). According to Factor 1, multivariate analysis showed three different clusters – peat being from one cluster consisting of composts COM-A and COM-B and from another composed of composts COM-C and COM-D (Fig. 3). Differences among the composts and peat with regard to their suppression of *P. nicotianae* became evident in the heat map generated with the data of 54 mass compounds which were found frequently across the profiles (Fig. 4). Several mass compounds received a high loading score in Factor 1 and contributed the most to the separation of peat from the composts (175; 97; 247.8; 278.9; 179.9; 216.9; 374.8; 232.9; 330.8; 194.9; 336.9; 352.8; 218.9; 164.9) (Fig. 4). Meanwhile, other mass compounds (184.9; 260.9; 300.9; 238.9; 254.9; 316.9; 262.9; 310.9; 186.9; 278.9; 234.9; 312.9) contributed to the separation of suppressive from conducive composts.

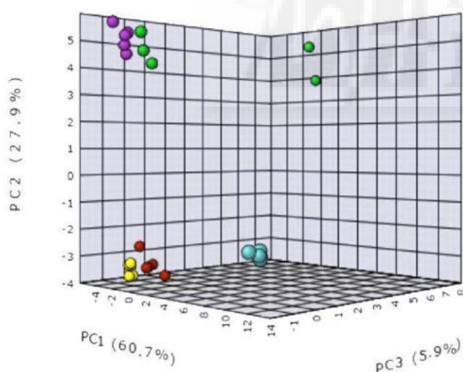

Fig. 3. Principal component analysis according to the metabolome obtained from the composts COM-A (green balls), COM-B (pink balls), COM-C (red balls), COM-D (yellow balls), and peat (blue balls), n=6.

3.5. Fungal and bacterial communities of different the composts and peat

Of 317802686 reads, we obtained a total of 1695818 reads after quality filtering, 397657 from 16S rRNA and 163334 from fungal ITS gene sequence across all samples. The OTU clustering and taxonomic assignment, performed using these sequences, yoield, 22803 and 3239 individual OTUs from16S rRNA and fungal ITS genes, respectively.

Fungal community composition

The classified sequences for the composts and peat were affiliated to three fungal phyla. The most abundant phylum was Ascomycota, accounting for 47 % of all sequence reads, followed by Basidiomycota (2.03 %) and Zygomycota (0.06 %). The percentage of sequences was classified as other fungi was 5.9 %, while the percentages assigned to unidentified fungi or not assigned to any fungal phylum were 32 % and 5.9 %, respectively. Examination of the taxonomic structure at the order level (Fig. 5) showed that, within the phylum Ascomycota, the most abundant orders were Sordariales, Hypocreales, and Microascales.

Composts COM-A and COM-B showed higher relative abundances of Ascomycota (63.14 and 67.38 %, respectively), within this phylum, COM-A had the highest relative abundance of Sordariales and COM-B, the highest abundance of Hypocreales (Fig. 5). On the other hand, compost COM-C showed a high abundance of Saccharomycetales and compost COM-D of Microascales, while these orders were almost inexistent in the other composts and peat (Fig. 5). Peat showed a high relative abundance of Ascomycota, followed by Basidiomycota (Fig. 5).

At the genus level, the most abundant classified genera (>1 %) for each compost and peat are shown in Table 3. The genera with the highest relative abundances in the composts were *Zopfiella, Fusarium, Haematonectria, Galactomyces, Doratomyces, Geomyces, Coprinellus, and Thermomyces.*

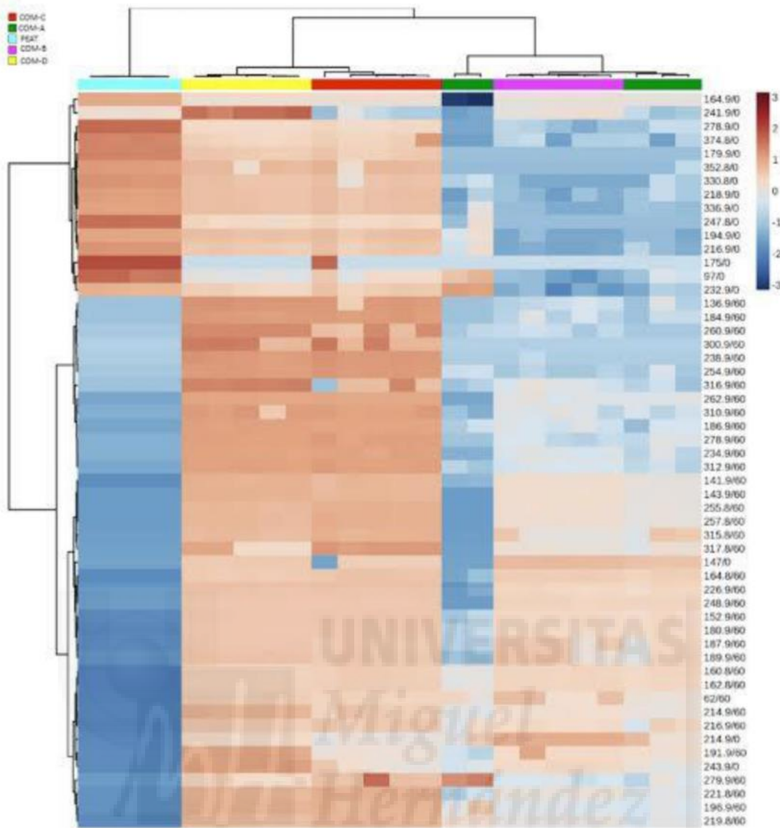

Fig. 4. A heatmap illustrating the 54 metabolites that differ among the composts and peat. Colors indicate relative quantity of each metabolite.

Bacterial community composition

The classified sequences were affiliated with 19 bacterial phyla, and the remaining ones were unassigned. The dominant phyla, found in all composts and peat, were the Proteobacteria (39.89 % of total sequence reads), Actinobacteria (30.53 %), Bacteroidetes (12.97 %), Chloroflexi (6.25 %), and - to a lesser extent - Firmicutes (4.87 %), Gemmatimonadetes (1.97 %), Acidobacteria, (1.07 %) and TM7 and TM6 (<0.41 %).

Composts COM-A and COM-B showed higher relative abundances of Proteobacteria, mainly due to the high abundance of Alphaproteobacteria and, in the case of compost COM-B, also because of Gammaproteobacteria. Composts COM-B, COM-C and COM-D showed the highest relative abundance of Actinobacteria (Fig. 6). A higher relative abundance of Bacteriodetes was found in COM-A, COM-B, and peat. Chloroflexi was presented to a high degree in compost COM-B as was Gemmatinomidetes in compost COM-D (Fig. 6).

The most abundant classified genera (>1%) for each compost and peat are shown in Table 4. The most frequent genera include: *Microbacterium*, *Mycobacterium*, *Streptomyces*, *Devosia*, and *Rhodoplanes*.

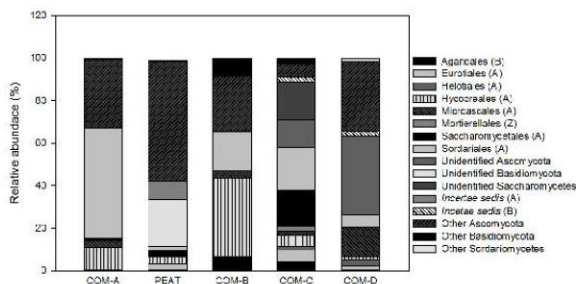

Fig. 5. Relative abundances of the fungal order identified in the composts and peat. (A) Ascomycota; (B) Basidiomycota; (Z) Zygomycota.

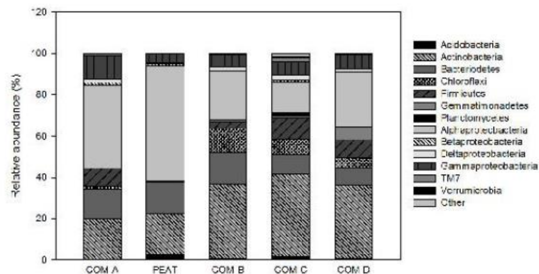

Fig. 6. Relative abundances of the bacterial phyla and sub-phyla identified in the compost and peat.

4. Discussion

Suppressive composts, similar to suppressive soils, are examples of natural biological control of diseases the result of a three-way interaction between the microorganisms in the compost (composition, diversity, functions, and activities), plant pathogen, and plant.[28] Besides, the induction of plant defense mechanisms could be an important component of compost suppresiveness.[29] Deeper understanding of the process involved in microbial ecology could provide directions for manipulations of the microbial community, leading to reproducible suppressive composts. This study provides a unique insight into compost organic matter, by solid state ^{13}C-NMR, bacterial and fungal communities, by high-throughput sequencing, and the metabolites produced by the microbial consortia of composts, in order to elucidate differences among growing media that are conducive to or suppressive of *P. nicotianae.*

In our study, all the composts and peat had high organic carbon contents, although not all of them stimulated the microbial activity. This means that not only the amount but also the nature and composition of the organic matter are important in the development a heterotrophic microbial community.[30] The NMR spectra are be very informative on the potential of composts to suppress diseases, when this property is associated with the microbial community characteristics.[31] We observed that the relative abundances of carbohydrate structures (60-110 ppm) and aliphatic structures (0-45ppm) were positively correlated with Phytophthora root rot incidence (r=0.519; $p<0.05$ and r=0.804; $p=0.005$, respectively). The transformation into simpler alkyl chains of some of the breakdown products of the polysaccharides, which were present in some of the materials used to make the composts, may have led to the high levels of the aliphatic structure regions observed. Another reason could be the presence of highly aliphatic biopolymers, in the cuticles of higher plants, that are highly resistant to decomposition.[32,33] Peat and compost COM-D showed higher relative abundances in the polysaccharide structure region than the other composts. Despite these high values, both growing media showed the lowest microbial activity. Similar results were reported by Castaño et al.[27] Probably, a large amount of cellulose is physically protected by lignin encrustation, protecting it from degradation,[34] therefore, this cellulose does not contribute to the maintenance of the microorganisms involved in suppressive phenomena, accounting for the decline in microbial activity[30]. On the other hand, the most suppressive composts, COM-A and COM-B, which have two common raw materials, namely, pepper sludge (12-17%) and a high content of vineyard pruning wastes (68-75%) - showed a lower relative abundance of carbohydrates. Similar results were observed by other authors[35,36] who considered

Table 3. Most abundant fungal genera identified (>1% relative abundance) in the composts and peat.

Phylum	Genus	COM-A	PEAT	COM-B	COM-C	COM-D
Ascomycota	*Zopfiella*	14.7	0.06	0.16	8.87	0.01
Ascomycota	*Fusarium*	4.40	0.00	20.2	3.32	0.30
Ascomycota	*Pseudallescheria*	0.00	0.00	0.00	0.18	9.87
Ascomycota	*Haematonectria*	1.68	0.00	9.12	0.44	0.05
Ascomycota	*Doratomyces*	1.29	0.02	0.09	0.16	2.64
Ascomycota	*Galactomyces*	0.75	0.00	0.24	12.81	0.00
Basidiomycota	*Myriococcum*	0.09	0.00	0.00	2.22	2.62
Ascomycota	*Geomyces*	0.00	8.64	0.00	0.00	0.03
Basidiomycota	*Coprinellus*	0.01	0.00	5.41	1.97	0.00
Ascomycota	*Scytalidium*	0.00	0.00	0.00	1.88	2.30
Ascomycota	*Thermomyces*	0.08	0.00	0.01	4.38	1.10
Ascomycota	*Scedosporium*	0.06	0.00	2.42	1.35	0.71
Ascomycota	*Chaetomium*	0.28	0.00	0.44	1.58	0.03
Ascomycota	*Penicillium*	0.01	2.16	0.01	0.02	0.54
Ascomycota	*Cephalotheca*	0.00	1.50	0.00	0.00	0.76
Ascomycota	*Aspergillus*	0.02	0.00	0.01	1.12	0.73
Ascomycota	*Candida*	0.00	2.72	0.00	0.23	0.01
Ascomycota	*Pichia*	0.00	0.00	0.01	3.37	0.00
Ascomycota	Hypocrea	0.00	1.83	0.00	0.02	0.02

that the low bio-availability of carbohydrates is crucial for suppression of *Pythium* and *Rhizoctonia solani*. It is plausible that composts COM-A and COM-B followed a general suppression model, as suggested by the dehydrogenase activity - which was associated significantly with suppressiveness. Blaya et al.[6] also found a positive correlation between compost microbial activity and suppression of *P. capsici*. Pane et al.[31] observed a positive correlation between ^{13}C-NMR spectral areas typical of phenolic and methoxyl C, resonating in the 145-165 and 46-60 ppm ranges, respectively, and a reduction in damping off caused by *R. solani* and *S. minor*. However, we did not find differences among composts in those NMR spectral areas. The alkyl/O-alkyl ratio is considered as a sensitive index of the stabilization and humification of organic matter (Pane et al.

Table. 4. Most abundant bacteria genera identified (>1 % relative abundance) in the composts and peat.

Class	Order	Family	Genus	COM-A	PEAT	COM-B	COM-C	COM-D
Actinobacteria	Actinomycetales	Microbacteriaceae	*Agrococcus*	1.10	0.00	1.08	0.23	0.20
Actinobacteria	Actinomycetales	Microbacteriaceae	*Microbacterium*	3.81	0.03	2.72	2.23	0.15
Actinobacteria	Actinomycetales	Mycobacteriaceae	*Mycobacterium*	0.53	2.86	0.51	0.40	0.48
Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	3.63	6.38	5.11	2.23	2.72
Actinobacteria	Actinomycetales	Streptosporangiaceae	*Nonomuraea*	0.05	0.01	0.27	1.15	3.02
Actinobacteria	Actinomycetales	Thermomonosporaceae	*Actinomadura*	0.06	0.84	0.38	0.93	1.70
Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	0.00	0.00	0.11	0.00	1.75
Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Olivibacter*	1.17	0.00	0.13	0.00	0.00
Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	1.05	0.00	0.11	0.02	0.00
Saprospirae	Saprospirales	Chitinophagaceae;	*Niastella*	0.00	1.82	0.00	0.00	0.00
Bacilli	Bacillales	Bacillaceae	*Bacillus*	1.87	0.00	0.58	1.45	0.56
Bacilli	Bacillales	Planococcaceae	*Ureibacillus*	1.62	0.00	0.20	0.84	0.74
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Devosia*	4.43	1.04	0.63	0.62	2.28
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	1.19	0.04	0.41	1.20	1.75
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Pedomicrobium*	1.86	0.02	1.25	0.92	0.90
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Rhodoplanes*	2.51	5.83	1.97	1.66	4.16
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*	2.30	0.62	1.19	0.32	2.17
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	1.74	0.02	0.07	0.01	0.03
Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Dokdonella*	0.10	2.52	0.03	0.04	0.21
Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteimonas*	1.94	0.00	0.44	0.19	0.04

2011).[15] Hadar and Mandelbaum[37] found that the degree of decomposition of compost has a strong effect on the rate of disease suppression. Composts that are excessively stabilized do not support microbial activity, so disease suppression is lost.[38] Our results show that peat and compost COM-C were more stabilized than the other compost showing the highest alkyl/O-alkyl ratios as well as higher pathogen incidence.

Although the physical properties *per se* are not considered enough to explain the observed suppression of Phytophthora root rot by some types of compost,[28] it has been suggested that the air capacity in composts is an important feature in those diseases where free water is vital in the asexual multiplication of fungi[12,39] In the present study, in spite of the fact that all composts showed a high air content (>20 %), not all of them were able to suppress Phytophthora root rot. Nevertheless, high air capacity is a good feature of growing media, since it improves plant growth. In previous studies, Phytophthora root rot in rhododendrons was most prevalent in growth media with an air capacity lower than 15 %, whereas suppressive tree bark composts usually have an air capacity of over 25 %.[28]

Due to the relationship between the increase in microbial activity and the reduction of pathogen incidence, an interesting point tackled in this study was the investigation of the bacterial and fungal community composition - in order to fully understand the community involved in the disease suppression by composts. Phylum analysis of the fungi revealed that the most common phyla in the composts and peat were Ascomycota, as reported by other authors[40,41] and, to a lesser extent, Basidiomycota.[40] The Basidiomycota phylum does not seem to be as abundant in composts as in soils, as reported by Lim et al.[42] Fungal populations have been reported as the main contributors to the biological suppressiveness of compost.[43] The incidence of *P. nicotianae* was negatively correlated with the Ascomycota phylum (r=-0.953 p=0.012). Within this phylum, the two orders recorded most frequently were Sordariales and Hypocreales, the latter being especially rich in fungal antagonists and parasites.[44] Probably, the incorporation of vineyard pruning wastes at different rates into our composts (30-75%) led to the development of fungi associated with hardwood compost, as reported by Neher et al.[45] Sordariales and Hypocreales were identified as the most dynamic taxa associated with the suppressive composts: COM-A and COM-B, respectively. The most abundant genera were *Zopfiella* and *Fusarium* in compost COM-A, and *Fusarium* and *Haematonectria* in compost COM-B. The genus *Zopfiella* has been reported to produce metabolites active against several species of fungi, bacteria, and yeast, such as *Botrytis cinerea*, *Phytophthora infestans*, or *Pythium ultimum*.[46,47] Also, *Fusarium* includes non-pathogenic isolates of *F. oxysporum*, identified as biocontrol agents.[48] Compost COM-C showed a high presence of different microorganisms associated with antifungal activity - such as *Zopfiella, Fusarium*, or *Chaetomium*, well-recognized mycoparasites whose antibiotic activities have been previously studied.[49] We also observed in this compost high relative abundance of *Galactomyces*, that occurs in compost and has the ability to produce cellulolytic enzymes.[50] Hydrolytic enzymes play an important role in the pathogenicity of plants by facilitating fungal penetration through the host cell wall.[51] By contrast, compost COM-D showed a very low relative abundance of these fungi, while *Pseudallescheria* was the genus represented most. Within this genus, *P. boydii* is the most well-known species, since it is a fungal human pathogen that is widespread in soils and produces a fungistatic substance strongly inhibitory to phytopathogens.[52] The fungal community of peat was characterized by the presence of *Geomyces*, a genus of filamentous fungi in the family Myxotrichaceae, known to be phychrophilic and often the most common fungal group found in cold and low-nutrient environments.[53] Relatively high abundances of *Penicillium* and *Hypocrea* were also observed in peat. In spite of the presence of these latter

microbes, which have been shown to control soil-borne plant pathogens,[54,55] peat was conducive to Phytophthora root rot. Similar results have been reported before, the presence of these microorganisms being related to non-suppressive soils.[56]

The dominant bacteria in the composts and peat were Proteobacteria (Alpha- and Gamma-), Actinobacteria, and Bacteroidetes. Similar results were reported in several studies[21,17,45] for different composts at the cured stage. Although no correlations were found between the bacterial communities and Phytophthora root rot incidence in the present study, we observed the presence of some species which may be related to disease suppression. Hadar and Papadopoulou[57] pointed out that the presence of Gamma-proteobacteria, Firmicutes, and Actinobacteria was an indicator of disease suppression. The most suppressive composts, COM-A and COM-B, had high relative abundances of *Streptomyces* spp., although the conducive composts COM-C and COM-D as well as peat also showed important abundances of this genus. These results suggest that although these microbes may be involved in disease suppression, they are not necessarily the key factor in disease control.[58] On the other hand, the cell-wall degrading enzymes of *Streptomyces* spp. are produced during logarithmic growth, and especially when the nutrient supply is limiting.[59] Thus, the characteristics of composts COM-A and COM-B may favor the growth of this genus more than the other growing media. *Bacillus* spp. have also been reported to be biocontrol agents against bacterial and fungal diseases,[60,61] mostly as a source of antibiotics[62] and they had a high relative abundance in compost COM-A.

In this work, we introduced the study of the compost metabolome in conjunction with the study of the compost microbiome, since the presence of certain metabolites could be used to understand the ability of a compost to control soil-borne pathogens, specifically *P. nicotianae*. Metabolites are similar in the majority of species; thus, a fully annotated genome is not required for analysis, and analytical methods are transferable between species.[63] Compost/soil metabolomics attempt to capture the complexity of metabolic networks via the comprehensive characterization of the small-molecule metabolites (e.g. amino acids, sugars, and lipids) in biological systems.[23] Moreover, the unification of metabolomics and metagenomics could be used to provide a high-throughput solution to link microbial taxonomy and their function.

The outcome PCA obtained with the metabolomes of the composts and peat was divided into three strong clusters, which were correlated with the different grades of suppressiveness achieved by the composts in the bioassay. Metabolite composition is governed by the extant microbial communities in the substrate and it is well-known that pathogen inhibition may be mediated by the secretion of antibiotics or antimicrobial compounds.[64,65] For instance, over two-thirds of all natural antibiotics are derived from *Streptomyces* spp.[65] Some species within this genus can produce antifungal compounds such as tuberdicidin, phosphalactomycin, and candicidin.[67,68] Also, the antibiotics produced by the biological control agent *Bacillus cereus* UW85 (zwittermicin A and kanosamine) appear to control species of *Phytophthora*.[69,70,71] Members of the family Pseudomonadaceae protect plants from fungal infection through the production of a putative chlorinated lipopetide,[72] while phosphate availability has been shown to regulate the biosynthesis of two antibiotics in *Serratia*.[73] It is important to underline that some bacterial strains, which are not biological control agents by themselves, can act synergically as part of microbial consortia.[74] These promising results obtained from the metabolomic analysis could promote, in a near future, the use of the compost metabolome as a predictive parameter of suppressiveness. However, further studies are required to reassert these preliminary results and increase the scant available data. Furthermore, metabolic profiling (the

identification of every peak) will help to clarify the involvement of the metabolites in the ability of composts to suppress *P. nicotianae*.

5. Conclusion

Our study enriches the understanding of the compost microbiome and metabolome and sheds light on the contribution of microorganisms to disease control. The presence of certain microbes with antifungal properties, such as the bacteria *Streptomyces* and *Bacillus* and the fungi *Fusarium* and *Zopfiella*, was not enough for all the composts to achieve a high level of suppression. Composts which were excessively stabilized, measured as high alkyl/O-alkyl ratios, may have lost their capability to support microbial activity and, consequently, their disease suppression potential. In this respect, higher levels of microbial activity were achieved in less stabilized composts, as measured by dehydrogenase activity. Although we have found parameters that seem to be good predictors of the disease suppressiveness of composts, we conclude that the integration of these parameters with others will substantially contribute to our understanding of the occurrence and persistence of compost-derived disease suppression.

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PUBLICATION 5

Changes induced by *Trichoderma harzianum* in suppressive compost controlling Fusarim wilt

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Changes induced by *Trichoderma harzianum* in suppressive compost controlling *Fusarium* wilt

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ABSTRACT

The addition of species of *Trichoderma* to compost is a widespread technique used to control different plant diseases. The biological control activity of these species is mainly attributable to a combination of several mechanisms of action, which may affect the microbiota involved in the suppressiveness of compost. This study was therefore performed to determine the effect of inoculation of *Trichoderma harzianum* (*T. harzianum*) on compost, focusing on bacterial community structure (16S rRNA) and chitinase gene diversity. In addition, the ability of vineyard pruning waste compost, amended (GCTh) or not (GC) with *T. harzianum*, to suppress *Fusarium* wilt was evaluated. The addition of *T. harzianum* resulted in a high relative abundance of certain chitinolytic bacteria as well as in remarkable protection against *Fusarium oxysporum* comparable to that induced by compost GC. Moreover, variations in the abiotic characteristics of the media, such as pH, C, N and iron levels, were observed. Despite the lower diversity of chitinolytic bacteria found in GCTh, the high relative abundance of *Streptomyces* spp. may be involved in the suppressiveness of this growing media. The higher degree of compost suppressiveness achieved after the addition of *T. harzianum* may be due not only to its biocontrol ability, but also to changes promoted in both abiotic and biotic characteristics of the growing media.

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1. Introduction

Composting has been widely accepted as one of the most feasible solutions for the treatment and valorization of organic wastes. It has contributed not only to enhancing environmental preservation, but also to the recovery of valuable resources [1]. The use of composts in horticulture and agriculture as growing media can reduce the use of peat, which is a non-renewable resource and conductive to soil borne diseases [2]. Suppressive composts have been commonly used to control plant diseases caused by soil-borne pathogens, such as *Pythium* spp. [3], *Fusarium* spp. [4] or *Phytophthora* spp. [1]. The disease suppression capacity of the compost is attributed to the activities of antagonist microorganisms and related to the stage of composting process [5]. Microbial community surviving the thermophilic phase of composting is not sufficient to support disease, thereby an active and specific microflora adapted to the available substrates following compost maturation or stabilization is essential [5]. Recolonization of composts after the heat-peak of the composting process before substantial colonization with mesophilic microorganisms, is the time of inoculation [6] and establishment of biological control agents (BCAs) at high densities to obtain induced suppressive compost [7]. Species of the genus *Trichoderma* have been described as potential BCAs due to their high antagonistic ability against several plant fungal pathogens, including *Fusarium* spp., *Phytophthora* spp., *Sclerotinia* spp., *Rhizoctonia* spp. and *Pythium* spp. [8–10]. Their biological control activity is mainly attributable to a combination of several mechanisms of action, which may include secretion of hydrolytic enzymes, such as chitinases and glucanases, which have been reported to be a key factor in the lysis of cell walls of phytopathogens [11] and anti-microbial compounds [12]. The rapid growth of these species allows them to directly compete for space and nutrients with phytopathogens [9], and indirectly by stimulating plant growth as well as inducing systemic resistance mechanisms in the plant [13]. *Trichoderma* populations can be established relatively easily in different types of soil [14]. Some authors have found that the inoculation of *Trichoderma* only modify slightly the microbial diversity of soils [14,15], while others have showed that the increase of soil microbial biomass after the addition of *Trichoderma*, contributes to a reduction of the biocontrol efficacy of this BCA [16]. However, the effect of inoculated *Trichoderma* on compost have not been deeply studied, and mainly, after the heat peak, when composts show a biological vacuum [4]. *Trichoderma* may induce chemical changes that indirectly exert a control on the dynamics of bacterial community structure.

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Chitin is the second most abundant natural biopolymer in nature and is widely distributed across diverse environments, comprising structures such as the cell wall of filamentous fungi [17]. Chitin degrading enzymes, chitinases, are also found in a wide variety of organisms, including fungi, plants, insects, crustaceans, and bacteria. Chitinases are glycosyl hydrolases that catalyzes the degradation of chitin, a linear β-1, 4-linked polymer of N-acetylglucosamine. Based on amino acid similarity, chitinases are classified into family 18 or 19 of glycosyl hydrolases [18]. The majority of known bacterial chitinases are grouped into family 18 group A [19]. The family 19 chitinases are also equally distributed within *Streptomycetes*. The screening of different chitinolytic bacteria community DNA samples may lead to the possible identification of diverse chitinase genes that may have enhanced chitinolytic properties and therefore have potential controlling plant fungal pathogens.

The goal of this study was to analyze the influence of the inoculation of *Trichoderma harzianum* on the bacterial community structure and chitinase gene diversity, through 16S rRNA and chitinase gene libraries, of compost against *Fusarium oxysporum* f. sp. *melonis* (FOM). Other characteristics of the growing media were also studied.

2. Materials and methods

2.1. Fungal strains and growth conditions

Composts were amended with the BCA *T. harzianum* T-78 (CECT 20714, Spanish Type Culture Collection). The isolate was incubated on potato dextrose agar (PDA, (Sharlau, Spain) 39 g L^{-1}, autoclaved at 121 °C for 20 min, and amended with 100 mg L^{-1} sterilized streptomycin (Sigma, USA)) at 28 °C for 5 days under no light conditions to obtain active growing microorganisms. Three discs (5 mm) of PDA were suspended in a flask containing 250 ml of potato dextrose broth (PDB, (Sharlau, Spain) 24 g L^{-1}, autoclaved at 121 °C for 20 min, and amended with 100 mg L^{-1} sterilized streptomycin) and incubated at 28 °C on a rotatory shaker (150 rpm) for 7 days under no light conditions. Conidia were recovered by centrifugation (5000 rpm, 20 min), rinsed twice with sterile distilled water and filtered through 101 quartz wool. The fungus was immobilized in bentonite following the protocol described by Bernal-Vicente [7]. The pathogen FOM was isolated from infected melon plants from a greenhouse nursery. Conidia were recovered as described above for *T. harzianum*.

2.2. Organic amendment

Two green composts containing 100% vineyard pruning wastes were used in this study: green compost (GC) and green compost inoculated at the beginning of the maturation process with *T. harzianum* 10^6 CFU g^{-1} (GCTh). Composting piles were made in open-air piles of 1 m^3 as described by Bernal-Vicente [7]. Once the composting process was finished (after 120 days), three samples of each compost pile were taken by mixing nine sub-samples from random sites on each pile. Samples were stored at −20 °C for DNA extraction and at 4 °C for chemical and microbiological analysis.

2.3. Physico–chemical and chemical parameters

Compost samples were analyzed for pH and electrical conductivity (EC) in a 1:5 (w/v) water-soluble extract in a conductivimeter and pH-meter (Crison mod. 2001, Barcelona, Spain). Total organic carbon (TOC) was determined by the method of Yeomans and Bremmer [20] and total organic nitrogen by the Kjeldahl method as modified by Bremner and Mulvaney [21]. Total P and K were determined in nitric-perchloric digestion extract (1:1), P by colorimetry and K by flame photometry using a Jenway PFP7 flame photometer (Essex, Englang) [22]. Other nutrients and heavy metals were determined by atomic absorption spectrometry (Vista Radial, Varian, Les Ulis, France). The analyses were performed in triplicate.

2.4. In vitro test

Serial destillated water dilutions of each compost extract (1:10 w/v) and peat were plated in nutrient agar (8 g L^{-1} nutrient broth, (Scharlau, Spain); 15 g L^{-1} technical agar (Scharlau, Spain) amended with nystatin (50 mg L^{-1}) autoclaved at 121 °C for 20 min, or sterilized streptomycin (100 mg L^{-1})). A plug (5 mm) of a 7-day-old mycelium of FOM was placed in the center of the petri dishes and incubated for 5 days at 28 °C. The radial growth of FOM was measured after five days of FOM growth in nutrient agar plates amended with the peat, GC and GCTh extracts. The ability of composts to suppress the pathogen was calculated as the reduction of FOM growth compared to the control (water amended plates). The analyses were performed in triplicate.

2.5. In vivo experiment

The experiment was carried out under greenhouse nursery conditions. Three treatments were assayed: GC compost (GC-treatment), GCTh compost (GCTh-treatment) and peat (peat-treatment) as growing media. Six polystyrene containers (10 wells per container) were used for each treatment, and each container was considered as one unit. One muskmelon (*Cucumis melo* L. cv. Giotto) seed was sown on each well and covered with vermiculite. Seeds were germinated in a growth chamber at 28 ± 1 °C and 90–95% relative humidity. After seeds germinated, the different containers were randomly distributed in a polyethylene-covered greenhouse under natural daylight conditions. Once the first true leaf appeared (15 days after germination), three of the six containers for each treatment were inoculated on the substrate with 2 mL of a FOM conidial suspension to achieve a final concentration of 10^4 conidia g^{-1} substrate. Control plants were inoculated with 2 mL of distilled water. Seedlings were irrigated according to need and harvested 30 days after inoculation (D.A.I).

The percentage of plants infected by FOM was measured by accounting FOM on melon stem. FOM was isolated from infected melon plants by cutting a piece from a melon stem (1 cm) and disinfecting it with sodium hypochlorite (1%) for 5 min. Each sterilized surface plant piece was put on PDA dishes and identified after 5 days at 28 °C in darkness. Disease severity of *Fusarium* wilt was rated on a scale of 1–4 (1. Healthy; 2. Yellowing; 3. Stem wilting; 4. Dead) based on Baayen and Van der Plas [23].

2.6. DNA extraction and PCR

Total DNA was extracted from 250 mg compost samples using the Fast DNA kit for soil (Q-Biogene, USA), following the modifications described by López-Mondéjar [24]. Samples were previously ground with liquid nitrogen and kept at −20 °C for DNA extraction. For the study of the bacterial community, bacterial universal primers 338f and 907r [25] were used for partial amplification of 16S RNA genes, obtaining a 570 bp final product. The PCR mixture (25 μl) contained a final concentration of 1× PCR buffer, 0.1 mg mL^{-1} bovine serum albumin (BSA, 5 mg mL^{-1}), 0.2 mM dNTPs mix, 0.2 μM of each primer, 1 U of DNA polimerase (1 U μL^{-1}, Biotools, Spain), 20 mM tetramethylammonium chloride (TMA) and 1 μL DNA. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, and

elongation at 72 °C for 1 min. A final elongation was performed at 72 °C for 10 min in a Takara PCR Thermal Cycler (Takara, Japan). Products were checked by electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide staining (10 mg mL^{-1}).

For the study of family 18 chitinolytic bacteria, degenerated PCR primers GA1F/GA1R [26] targeted to a gene fragment from family 18 group A chitinases were used, resulting in a 440 bp final product. The PCR mixture (25 μL) contained a final concentration of 1× PCR buffer, 0.1 mg mL^{-1} BSA (5 mg mL^{-1}), 0.2 mM dNTPs mix, 0.2 μM of each primer, 1 U of DNA polimerase (1 U μL^{-1}, Biotools, Spain), 0.1 μM TMA and 100 ng μL^{-1} of extracted DNA. The thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 10 s and elongation at 72 °C for 1 min. A final elongation was performed at 72 °C for 10 min in a Takara PCR Thermal Cycler (Takara, Japan) [26]. Products were checked as previously described.

2.7. T. harzianum T-78 quantification

T. harzianum T-78 quantification was estimated in composts before setting up the experiments by quantitative real-time PCR (qPCR) in a total volume of 8 μL using LightCycler™ (Roche Applied Science, Germany), following the protocol described by López-Mondéjar [24]. The quantification was performed in triplicate.

2.8. Cloning and sequencing

PCR products (three for each set of primers) of each compost sample were mixed and purified using a QUIAquick PCR Purification Kit (Qiagen, Germany) following the manufacturer's instructions. The purified products were cloned with the pCR®II TA cloning kit for sequencing (Invitrogen, USA) according to the manufacturer's instructions. The appropriate presence of inserts was determined by PCR with the primer sets 338f/907r and GA1F/GA1R, for the 16S rRNA region and chitinase gene region respectively, and by electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide staining (10 mg mL^{-1}). Clone inserts were grouped according to their *restriction fragment length polymorphism* (RFLP) using four-base pair recognizing restriction enzymes (HhaI and HaeIII), and representative clones containing different RFLP patterns were sequenced using a 3730xI DNA Analyzer (Applied Biosystems, USA). The obtained sequences were checked by Chromas Lite 2.01 to eliminate primers and vector regions.

2.9. Phylogenetic analysis of 16S rRNA and chitinase gene sequences

Sequence identities of 16S rRNA were determined with the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) [27]. The Bellerophon Chimera Check was used to determine potential chimeric sequences [28]. The search for similar sequences was carried out with BLAST (Basis Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST) in GenBank (NCBI database, USA), and those found to be the most similar (at least 97% sequence similarity) were used for the subsequent phylogenetic analysis. Sequences were aligned using ClustalW [29], and phylogenetic trees were constructed using MEGA4 [30] by the neighbor-joining method [31] based on the bootstrap analysis with a total of 1000 replicates [32].

For the analysis of the chitinase gene, sequence identities were determined with BLAST as well as with similar sequences previously deposited in the NCBI database (sequences displaying 78–99% identity with previously identified chitinase genes). The phylogenetic analysis was performed as described for 16S rRNA sequences.

The Shannon index ($H' = \Sigma p_i \times (\ln\ p_i)$) and Simpson index ($1 - D = 1-[\Sigma n_i \times (n_i - 1)/(N \times (N - 1))]$) were calculated to estimate the diversity of both samples. The relative abundances of bacterial phyla were defined by calculating the number of sequenced clones from each phylum with respect to the total sequences.

The 16S rRNA gene sequences have been deposited under accession number JQ736135–JQ736244 in GenBank (NCBI database, USA). The chitinase gene sequences have been deposited under accession numbers JQ906200–JQ906265 in GenBank (NCBI database, USA).

2.10. Statistical analysis

Data were subjected to one-way ANOVA analysis. Statistical analyses were performed using SPSS 19.0 software (SPSS Inc.). When the F-statistic was significant, Tukey's post hoc test ($P < 0.05$) was used to separate means.

3. Results

3.1. Compost characterization

The main physico-chemical, chemical and microbiological characteristics of composts and peat are shown in Table 1. Both pH and EC showed significant differences depending on the treatment (Table 2). Peat showed the lowest values of pH and EC, while among composts, the highest pH and CE was observed in compost without *T. harzianum* (GC). Composts showed higher values of nutrient contents than peat (Table 1). Compost with *T. harzianum* (GCTh) showed significant higher values of total N, K and Fe than compost without *T. harzianum* (GC), while opposite results were observed for total C and P (Table 1 and 2). Compost CGTh showed the presence of *T. harzianum* (10.42 log copies ITS g^{-1}), while compost GC and peat did not show any copies of *T. harzianum*.

3.2. In vitro test

The percentage of inhibition of mycelial growth of *F. oxysporum* f. sp. *melonis* (FOM) found in selective media for fungi and bacteria

Table 1
Physico-chemical, chemical, and microbiological characteristics of composts and peat.

Parameter	GC Th[a]	GC[b]	Peat
pH	7.82 ± 0.12b	8.50 ± 0.19a	5.93 ± 0.03c
EC (1:5; 25 °C)[c] (dS/m)	0.89 ± 0.07b	1.06 ± 0.09a	0.54 ± 0.02c
Total organic C (%)	38.28 ± 0.3b	41.37 ± 1.20a	14.77 ± 0.06c
C/N ratio	23.82 ± 0.10c	27.32 ± 0.05b	43.45 ± 0.17a
Total N (g/kg)	16.10 ± 0.20a	15.10 ± 0.40b	9.60 ± 0.70c
Total K (g/kg)	12.30 ± 0.40a	8.40 ± 0.80b	0.60 ± 0.01c
Total P (g/kg)	1.50 ± 0.05b	2.0 ± 0.20a	0.30 ± 0.01c
Ca (g/kg)	52.20 ± 6.50a	36.80 ± 1.30b	0.74 ± 0.30c
Mg (g/kg)	18.10 ± 1.80a	8.40 ± 1.40b	0.70 ± 0.02c
Na (g/kg)	1.30 ± 0.10a	1.20 ± 0.10a	0.40 ± 0.01b
Cu (mg/kg)	13.03 ± 0.59b	19.93 ± 1.83a	10.32 ± 1.38b
Fe (mg/kg)	3485.51 ± 156a	2571.42 ± 171b	413.67 ± 6.05c
Mn (mg/kg)	121.69 ± 6.85a	74.04 ± 6.77b	22.74 ± 0.97c
Pb (mg/kg)	3.43 ± 0.33b	15.56 ± 1.30a	1.16 ± 0.004c
Zn (mg/kg)	34.47 ± 2.68b	54.39 ± 4.71a	5.70 ± 0.32c
T. harzianum (log copies ITS[d] g^{-1})	10.42 ± 0.29	ND[e]	ND

For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($P < 0.05$).
[a] GCTh: green compost amended with *T. harzianum*.
[b] GC: green compost.
[c] EC: electrical conductivity.
[d] ITS: internal transcribed spacer copies.
[e] N.D.: non detected.

Table 2
Results from one-way ANOVA (treatment).

	F	*P*
pH	330.178	<0.001
EC (1:5; 25 °C)[a] (dS/m)	49.468	<0.001
Total organic C (%)	1249.501	<0.001
Total N (g/kg)	1696.087	<0.001
Total K (g/kg)	414.283	<0.001
Fe (mg/kg)	415.428	<0.001
Inhibition of FOM[b] in fungi[c] selected media	67.836	<0.001
Inhibition of FOM in bacteria[d] selected media	85.303	<0.001
Infected stems by FOM	244.808	<0.001
Disease severity index	68.310	<0.001

[a] EC: electrical conductivity.
[b] FOM: *Fusarium oxysporum* f. sp. *melonis*.
[c] Fungi selected media: nutrient agar+nystatin.
[d] Bacteria selected media: nutrient agar+streptomycin.

was significantly influenced by treatments (Fig. 1, Table 2). In fungal culture media, growth inhibition of FOM reached values of 51% and 53% for GCTh and CG respectively, compared to the growth inhibition of FOM for peat (20%). In bacterial culture media, growth inhibition of FOM reached values of 59% and 54% in GCTh and GC respectively, compared to the growth inhibition of FOM for peat (29%). Regardless of the media used, no significant differences were observed in the inhibition of the growth of FOM with the addition of *T. harzianum* to compost (GCTh) compared to GC (Fig. 1).

3.3. In vivo experiment

The percentage of stems infected by FOM was significantly influenced by treatment (Table 2). Plants grown in GCTh-treatment showed the lowest percentage of stems infected by FOM, reaching values of 10%, while the stem infection rate of plants grown in GC-treatment reached 25% and in peat-treatment 75% (Fig. 2A). Seedlings grown in both composts (GC-treatment and GCTh-treatment) presented significantly lower disease severity compared to those grown in peat-treatment. Disease severity values were 1.5 and 2.1 for GCTh-treatment and GC-treatment respectively, while plants grown in peat-treatment showed a value of 3.0 (Fig. 2B).

3.4. 16S rRNA libraries

A preliminary study performed in triplicate of the bacterial community structures of composts through denature gradient gel electrophoresis (DGGE) showed changes in composts when *T. harzianum* was added (data not shown). On the basis of the DGGE results, 16S rRNA gene clone libraries from both composts (GCTh and GC) were constructed. A total of 192 clones were randomly selected. Since identical RFLP profiles were obtained, a total of 125 clones were sequenced.

One hundred and nineteen OTUs (sequence similarity 100%) over the region of the 16S rRNA gene were identified in both composts and classified into seven phyla and a group of unclassified bacteria (Fig. 3). Proteobacteria and Actinobacteria largely dominated the bacterial communities of both composts. The GCTh clone library was classified into six phyla and a group of unclassified bacteria. The largest phyla were Proteobacteria (48.7%) [α-Proteobacteria (33.0%), δ-Proteobacteria (8.7%) and γ-Proteobacteria (7.0%)], Actinobacteria (19.1%) and Bacteriodetes (11.3%). The GC clone library was only classified into three phyla and a group of unclassified bacteria. Among these phyla were Proteobacteria (41.2%) [α-Proteobacteria (30.4%), β-Proteobacteria (2.9%), δ-Proteobacteria (4.3%) and γ-Proteobacteria (2.9%)], Actinobacteria (30.4%) and Firmicutes (1.4%).

3.5. Chitinase gene libraries

Chitinase gene libraries were constructed to study the bacterial Family 18 subgroup A chitinase gene diversity of both composts (Table 3). A total of 129 clones were selected randomly for RFLP. Since few identical RFLP profiles were obtained 92 clones were sequenced. A total of 57 OTUs (sequence similarity ⩾99%) over the region of the chitinase gene were identified in both composts. Sequences displayed 78–99% identity with previously identified chitinase genes and were distributed into two different phyla, Actinobacteria (50.7%) and Proteobacteria (6.7%), and a group of unclassified bacteria. The majority of chitinolytic Actinobacteria belonged to *Streptomyces* spp. (53.8% and 32.3% in GCTh and GC respectively) along with species of *Micromonospora* (11.9% in GCTh) and *Amycolaptosis* (10.2% in GCTh).

3.6. Diversity index

For 16S rRNA gene sequence and chitinase gene library analysis, diversity indices were calculated according to phylum affiliation (Table 4). The diversity indices showed greater diversity in GCTh than in GC. Analysis of the chitinase gene sequence library showed lower diversity in GCTh than in GC.

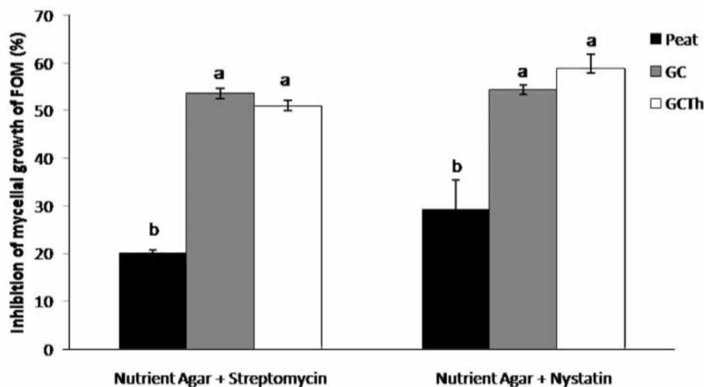

Fig. 1. *In vitro* inhibition of mycelial growth of *Fusarium oxysporum* (*in vitro* test) in peat, compost (GC) and compost amended with *T. harzianum* (GCTh). For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($P < 0.05$). Error bars represent standard error.

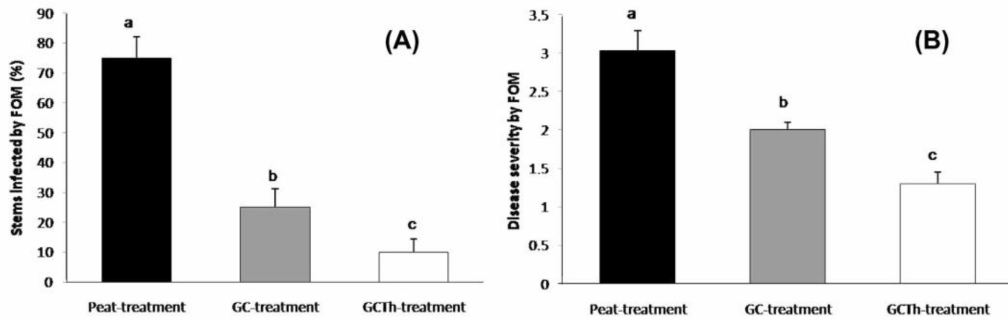

Fig. 2. Percentage of infected stem by FOM (A) and disease severity of *Fusarium* wilt (B) in the different assayed treatments: Peat, compost (GC) and compost amended with *T. harzianum* (GCTh) (*in vivo* experiment). For each parameter, data followed by the same letter are not significantly different according to Tukey's post hoc test ($P < 0.05$). Error bars represent standard error.

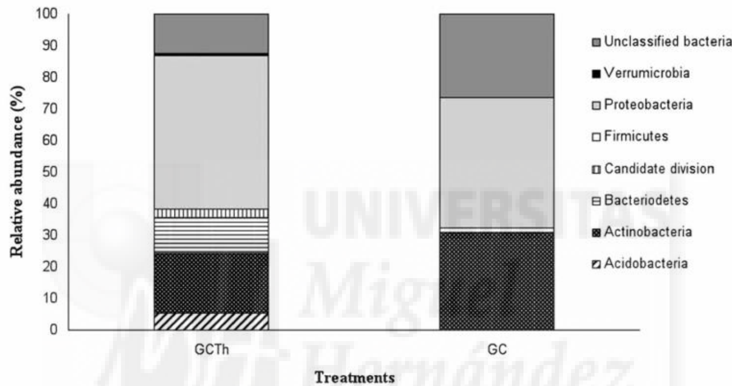

Fig. 3. Relative abundance of bacterial phyla based on 16S rRNA gene clone libraries of compost amended with *T. harzianum* (GCTh) and compost (GC).

4. Discussion

The amendment of a specific biological control agent (e.g. *T. harzianum*) to compost can lead to a substrate with a broader –range suppressive effect [33] due to the mechanisms of action used by this BCA [34], through changes in the biotic and abiotic substrate characteristics promoted by the BCA [35], or through a combination of such mechanisms.

The biocontrol activity of composts analyzed through *in vitro* tests was different from that found under *in vivo* conditions, although in both cases, composts showed higher biocontrol activity than peat against FOM. This fact is in concordance with other studies in which composts provided more successful disease suppression compared to peat [24,36,37]. No significant differences in the inhibition of mycelial growth of FOM with non-amended and amended compost with *T. harzianum* (GC, GCTh) were found. However, *in vivo*, the percentage of stems infected by FOM and disease severity was significantly lower in the treatment amended with *T. harzianum* (GCTh-treatment) than in the non-amended treatment (GC-treatment). Several authors have pointed out the inability of *in vitro* assays to produce accurate solutions [4,38], either due to the lack of interaction between plants, rhizosphere environment [38], the interaction *Trichoderma*-plant [39] or the fact that it is difficult for many species of *Trichoderma* to express fungal secondary metabolite genes under standard laboratory conditions [40]. These findings highlight *in vivo* experiments are required to obtain accurate conclusions regarding the suppression capacity of *Trichoderma*-amended composts.

From the point of view of fertility both compost showed some fertilizing value to be used as a growing media [4,8], even GCTh showed higher values of N and P than GC. In this study, the addition of *T. harzianum* to compost (GCTh) led to a significant decrease in pH and EC values compared to results in GC. *Trichoderma* spp. produces organic acids, such as gluconic, citric or fumaric acids, that decrease soil pH [34,35], promoting *T. harzianum* growth [41]. pH likely plays a role, directly or indirectly, in the suppression of plant diseases through its impact on microbial activity [42]. Specifically, the pH of growing media is a determinant of *Fusarium* wilt severity. This fact is associated with the availability of macro- and micro-nutrients, which are important for the growth, sporulation and virulence of *F. oxysporum* [43]. To give an example, high pH values in certain composts achieve antifungal activity against *Fusarium* wilt in tomato and carnation [44,45]. The analyzed composts attained high pH values, considered a positive characteristic of organic amendments in terms of reducing diseases caused by *Fusarium* spp. [43–45].

These high pH values reduce the availability of nutrients such as iron in organic growth media [46], inducing siderophore production and competition for this mineral. This is a mechanism used by *Trichoderma* spp. which results in explicit nutrient competition among microorganisms, specifically against *F. oxysporum* [47,48]. Several authors have shown the importance of the availability of specific nutrients for the biocontrol of *Fusarium* wilt induced by fluorescent *Pseudomonas spp*., *T. harzianum* and *T. asperellum* as a result of iron competition [44,47,49,50].

Table 3
Chitinase gene sequences analysis of composts.

OTU (sample[a])	% Similarity	Closest sequence match (accession number)	Phylogenetic group
4 (GCTh)	78	*Cellvibrio japonicus* Ueda 107 (CP000934.1)	Proteobacteria
18 (GCTh)	98	*Janthinobacterium lividum* (U07025.1)	Proteobacteria
89 (GC)	86	*Lysobacter enzymogenes* (DQ888611.1)	Proteobacteria
56 (GC)	85	*Lysobacter enzymogenes* (AY667480.1)	Proteobacteria
46 (GC)	81	*Stenotrophomonas maltophilia* (AF014950.1)	Proteobacteria
6 (GCTh)	87	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
11 (GCTh)	89	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
34 (GC)	87	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
61 (GC)	90	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
73 (GC)	89	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
81 (GC)	89	*Actinoplanes* sp. SE50/110 (CP003170.1)	Actinobacteria
30 (GC)	87	*Actinosynnema mirum* DSM 43827 (CP001630.1)	Actinobacteria
60 (GC)	87	*Actinosynnema mirum* DSM 43827 (CP001630.1)	Actinobacteria
40 (GC)	83	*Amycolaptosis mediterranei* S699 (CP002896.1)	Actinobacteria
44 (GC)	86	*Amycolaptosis mediterranei* S699 (CP002896.1)	Actinobacteria
79 (GC)	83	*Amycolaptosis mediterranei* S699 (CP002896.1)	Actinobacteria
80 (GC)	84	*Amycolaptosis mediterranei* S699 (CP002896.1)	Actinobacteria
84 (GC)	84	*Amycolaptosis mediterranei* S699 (CP002896.1)	Actinobacteria
53 (GC)	87	*Micromonospora* sp. L5 (CP002399.1)	Actinobacteria
58 (GC)	83	*Micromonospora* sp. L5 (CP002399.1)	Actinobacteria
51 (GC)	83	*Micromonospora aurantiaca* ATCC 27029 (CP002162.1)	Actinobacteria
85 (GC)	85	*Micromonospora aurantiaca* ATCC 27029 (CP002162.1)	Actinobacteria
12 (GCTh)	85	*Streptomyces* sp. Sirex AA-E (CP002993.1)	Actinobacteria
41 (GC)	83	*Streptomyces* sp. Sirex AA-E (CP002993.1)	Actinobacteria
48 (GC)	84	*Streptomyces* sp. Sirex AA-E (CP002993.1)	Actinobacteria
87 (GC)	84	*Streptomyces* sp. Sirex AA-E (CP002993.1)	Actinobacteria
45 (GC)	96	*Streptomyces coelicolor* A3(2) (AL939108.1)	Actinobacteria
32 (GC)	81	*Streptomyces flavogriseus* ATCC 33331 (CP002475.1)	Actinobacteria
5 (GCTh/GC)	89	*Streptomyces griseobrunneus* (AY641546.1)	Actinobacteria
25 (GCTh/GC)	90	*Streptomyces griseobrunneus* (AY641546.1)	Actinobacteria
26 (GCTh)	80	*Streptomyces hygroscopicus* subsp. *Jinggangensis* 5008 (CP003275.1)	Actinobacteria
42 (GC)			
71 (GC)	79	*Streptomyces hygroscopicus* subsp. *Jinggangensis* 5008 (CP003275.1)	Actinobacteria
88 (GC)			
39 (GC)	88	*Streptomyces roseoflavus* (AY392156.1)	Actinobacteria
66 (GC)	84	*Streptomyces roseoflavus* (AY392156.1)	Actinobacteria
82 (GC)	82	*Streptomyces roseoflavus* (AY392156.1)	Actinobacteria
9 (GCTh)	83	*Streptomyces scabiei* 87.22 (FN554889.1)	Actinobacteria
13 (GCTh)	90	*Streptomyces scabiei* 87.22 (FN554889.1)	Actinobacteria
54 (GC)	83	*Streptomyces scabiei* 87.22 (FN554889.1)	Actinobacteria
8 (GCTh)	99	*Streptomyces thermoviolaceus* (D14536.1)	Actinobacteria
83 (GC)	85	Streptosporangium roseum DSM 43021 (CP001814.1)	Actinobacteria
20 (GCTh)	83	Thermobispora bispora DSM 43833 (CP001874.1)	Actinobacteria
22 (GCTh)	83	Thermobispora bispora DSM 43833 (CP001874.1)	Actinobacteria
67 (GC)	83	Thermobispora bispora DSM 43833 (CP001874.1)	Actinobacteria
78 (GC)	83	Thermobispora bispora DSM 43833 (CP001874.1)	Actinobacteria
7 (GCTh)	86	Uncultured bacterium gene, cloneMH20 (AB361987.1)	Unclassified
10 (GCTh)	82	Uncultured bacterium gene, cloneSA19 (AB362103.1)	Unclassified
14 (GCTh)	83	Uncultured bacterium gene, cloneKK26 (AB361886.1)	Unclassified
68 (GC)	95	Uncultured bacterium gene, cloneYH06 (AB362061.1)	Unclassified
70 (GC)	95	Uncultured bacterium gene, cloneST21 (AB362035.1)	Unclassified
75 (GC)	78	Uncultured bacterium clone TDchi1 (GQ202084.1)	Unclassified
90 (GC)	90	Uncultured bacterium gene, cloneMH03 (AB361971.1)	Unclassified
91 (GC)	84	Uncultured bacterium gene, cloneHA16 (AB361745.1)	Unclassified

[a] GCTh: green compost inoculated with *T. harzianum*; GC:green compost.

Table 4
Bacterial diversity indices based on 16S rRNA and chitinase gene libraries of composts.

	Shannon–Wiener index (H′)		Simpsons index (1-D)	
	GCTh[a]	GC[b]	GCTh	GC
16S rRNA gene sequences	1.95	1.42	0.83	0.77
Chitinase gene sequences	1.36	1.53	0.29	0.93

[a] GCTh: green compost inoculated with *T. harzianum*.
[b] GC: green compost.

In particular, we know that pH may strongly influence soil bacterial communities [51,52]. High pH soils typically have higher relative abundances of Actinobacteria and Bacteriodetes with lower abundances of Acidobacteria compared with more acidic soils [53]. In our study, this pattern holds in the case of Actinobacteria and Acidobacteria but not in the case of Bacteriodetes. Rousk et al. [51] pointed out that the incremental differences in soil bacterial community composition with pH were insignificant above pH 6.8. Factors other than pH may also be driving the bacterial community patterns showed in Fig. 3. Changes in quantities of organic carbon added to soil can have considerable influences on microbial communities. In this sense, compost GCTh shows a lower C content as well as higher relative abundance of Bacteriodetes and Proteobacteria groups, which have been putatively identified as being copiotrophic taxa (those taxa that characteristically grow and multiply in high C environments). Just as in the case of Bacteriodetes, the presence of *T. harzianum* in GCTh promoted the appearance or

increase of certain bacterial communities such as γ-proteobacteria and Verrumicrobia, all of them related to chitin-amended soils [54,55] (Fig. 3). The potential death of part of the *T. harzianum* mycelia present in GCTh could imply an increase in chitin and oligomers available in the medium [56] promoting the abundance of these groups despite the lower C rate. The higher N content showed in GCTh could imply a higher relative abundance of Proteobacteria spite of being identified as being copiotrophic taxa. A similar pattern has been observed in other studies of microbial communities involving different levels of N amendments on soil [57]. Conversely, oligotrophic microbes such as Acidobacteria, may be important components in bacterial communities where C is limited as a result of low C flux or depletion of C as a result of competition [58]. Thus, the presence of *T. harzianum* may create an oligotrophic environment in compost as a function of competitive interactions. Moreover, the low levels of easily biodegradable substances present in cellulose-enriched GCTh and GC composts may enhance the competitiveness between microorganisms [56,59], maintaining both composts in a competitive state [10]. These shifts in the composition of compost microbial communities inoculated with *T. harzianum* are likely associated with shifts in the diversity of these communities. The diversity indices that were influenced either by rare taxa, such as the Shannon index H', or by the most abundant taxa, such as the Simpson index (1/D) [60], showed greater diversity in GCTh than in GC. As has been demonstrated previously [53,57], compost pH may be responsible for the 16S bacterial community diversity. Other authors have also observed higher microbial diversity after the addition of *T. harzianum* to soil [61].

Disease suppression by composts is mainly attributed to the biotic factor [6,62]. Analysis of 16S rRNA clone libraries showed that phyla Proteobacteria and Actinobacteria dominated the bacterial community in the composts. Several authors reported that the presence of Actinobacteria is typical of suppressive composts as they may work as BCAs [63–65]. Thereby, to understand the capacity of compost to suppress soil-borne plant diseases, it is important to study chitinase genes [66]. Analysis of the chitinase gene composition was performed using a set of primers developed based on a dataset biased in favor of the *Streptomyces* group. Some species of *Streptomyces* are well-known for their ability to degrade the cell walls of soil-borne plant fungi through the production of chitinases and antibiotics [66–68]. We therefore expected the chitinase gene libraries of our composts to be dominated by Actinobacteria with species related to *Streptomyces*. In the current study, the addition of *T. harzianum* to the GCTh compost caused an increase in the relative abundance of species of *Streptomyces* spp., which is in accordance with the increased suppression of FOM under *in vivo* conditions. The cell wall-degrading enzymes of *T. harzianum* and *Streptomyces* spp. are produced during logarithmic growth, and especially when the nutrient supply is limited [68]. The characteristics of the growing media (vineyard pruning wastes) present in this study may benefit the growth of these antifungal microbes more than it benefits FOM.

The loss of chitin gene diversity in GCTh might be due to the simultaneous presence of *T. harzianum* and *Streptomyces*, since both efficiently compete with other microorganisms for space and nutrients [69,70]. The competitiveness between antagonistic genera and microflora of matured composts could be enhanced under low levels of easily biodegradable substances.

5. Conclusions

Vineyard pruning waste composts (GC and GCTh) are suitable to be used as suppressive growing media against FOM. The incorporation of *T. harzianum* not only increased the biocontrol capacity of this compost (GCTh) compared to GC, but also induced changes in the biotic and abiotic characteristics of the compost. Changes in the physico-chemical characteristics of the growing media could induce changes in bacterial community composition and increase the relative abundance of species of *Streptomyces* spp. and therefore, the suppressiveness of GCTh compost. These results should lay the groundwork to optimize the use of biological control agents and appropriate source materials so as to achieve high levels of suppressiveness in composts. In addition, the use of plant experiments is required to obtain realistic conclusions concerning the suppression capacity of composts amended with *Trichoderma* species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pestbp.2013.06.001.

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IV

Chapter IV. General Discussion

IV. GENERAL DISCUSSION

The production of pepper in Murcia is practically all localized in "El Campo de Cartagena". One of the main problems in this growing area has been the losses associated with the presence of *P. capsici* during recent decades (Tello & Lacasa, 1997; Guerrero *et al*., 2004). However, in the last few years *P. nicotianae* has been found in the same greenhouses where *P. capsici* was considered the causal agent of Phytophthora root rot (Guerrero, 2012). The control of *P. nicotianae* is important, not only because it is a threat to a broad range of hosts but also because there are no effective treatments (Erwin & Ribeiro, 1996; Kamoun *et al.*, 2014). Based on these observations we decided to study the presence of *P. nicotianae* and *P. capsici* in one of the most important pepper-growing areas in southeast Spain, "El Campo de Cartagena". PCR-based methods, which have proved to be a sensitive technology, offer several advantages over the traditional ones (Schena *et al.*, 2004). Although several primers have been designed to detect *P. nicotianae* using conventional PCR (PCR), in most cases it remained difficult to establish their specificity (Li *et al.*, 2011; Huang *et al.*, 2010; Meng *et al.*, 2010). Moreover, qPCR technologies have emerged as a major tool to detect and study phytopathogenic fungi and oomycetes (Sanzani *et al.*, 2010). These techniques are more sensitive, more accurate, and more specific than the conventional techniques (Lievens *et al.*, 2006). Moreover, qPCR enables the elimination of the post-amplification processing steps needed in PCR, reducing the time and cost of analyses as well as the use of harmful substances utilized to stain DNA in electrophoretic gels (Schena *et al.*, 2013). In Publication I, a TaqMan system of probe and primers in the ITS region of

the oomycete *P. nicotianae* was developed. The probe included an MGB fluorophore in order to increase its melting temperature, which was lower than required due to its short length. The ITS region is one of those most used to detect and quantify fungi, since it is a multicopy gene with high variability (White *et al.*, 1990). This region has been used previously by other authors to develop specific probes and primers to detect a wide range of phytopathogens (Silvar *et al.*, 2005; López-Mondéjar *et al.*, 2010; Lievens *et al.*, 2006). With this system it was possible to detect all the isolates of *P. nicotianae* tested, without amplifying the rest of the fungi and oomycetes. It is worth mentioning that *P. nicotianae* has become the main casual agent of Phytophthora root rot in "El Campo de Cartagena" since it was the species extracted from the pepper tissues, while *P. capsici* was not. The latter was determined using a TaqMan system which was published by Silvar *et al.* (2005) and designed in the ITS region. Nevertheless, *P. capsici* is still present in "El Campo de Cartagena". It was found in two out of the 15 greenhouses tested, coexisting with *P. nicotianae* in both greenhouses. For breeders, it is very important to take all this information into account, since the phenomenon of out-crossing may occur between these two species, as reported by English *et al.* (1999) under *in vitro* conditions.

Both morphological and molecular analyses determined that *P. nicotianae* was the main causal agent of Phytophthora root rot in the study area. One of the advantages of PCR-based techniques over morphological ones, as well as their rapidity, is the potential to detect a single target molecule in a complex mixture (Schena *et al.*, 2013). The TaqMan system developed for *P. nicotianae* allows one to detect the presence of the pathogen

at low levels, even before the onset of the symptoms (Lievens *et al.*, 2007). This characteristic qPCR is particularly useful in nurseries, considering the important role of the propagation of infected material in the spread of soil-borne pathogens (Spies *et al.*, 2011). The transplantation of infected plants not exhibiting clear symptoms favors pathogen dispersal, making the elimination of the pathogen from the field environment extremely difficult (López-Mondéjar *et al.*, 2012). Indeed, once a disease has emerged in a crop, losses can be severe, especially in greenhouses in which many plant species are simultaneously and repeatedly cultivated with limited crop rotation (Blaya *et al.*, 2014b). In this sense, an early and accurate detection of plant pathogens can be considered a cornerstone for optimization of management strategies (Sanzani *et al.*, 2013).

The use of qPCR allowed quantification of the concentration of pathogen in terms of ITS copies μL^{-1}, through the development of a standard curve. The quantification is based on the assumption that standard and environmental DNAs are PCR-amplified with the same efficiency. However, the presence of inhibitory substances (typically co-extracted with environmental DNA) such as humic acids, fulvic acids, and polysaccharides may reduce this efficiency (Tsai & Olson, 1991; Huang *et al.*, 2009). It is worth mentioning that the selection of an appropriate extraction kit should depend on the characteristics of the kind of matrix involved as well as on the intended downstream application (Sagova-Mareckova *et al.*, 2008; Dineen *et al.*, 2010). Several DNA extraction methods have been used in the present Doctoral Thesis and all of them yield pure DNA. Firstly, the FastDNA® SPIN kit for soil (Q-Biogene, Carlsbad, CA, USA) was used to extract DNA from

soil samples in Publications I, II, III, and V. In the case of plant samples, the CTAB protocol was the method chosen to extract DNA in Publications I and II. Finally, a non-commercial method, based on phenol and chloroform extraction followed by isopropanol precipitation (Hartl & Seiboth, 2005), was used to extract DNA from the mycelia of fungi in pure cultures, in Publications I and II. The CTAB protocol is useful for isolation of DNA from tissues containing high amounts of polysaccharides, since CTAB is able to bind them - facilitating their removal from the solution (Clarke, 2009). These procedures have been extensively used in other studies (Clarke, 2009; Huang *et al.*, 2009; Dineen *et al.*, 2011).

Digital PCR offers a unique approach to qPCR for measuring nucleic acids (Vogelstein & Kinzler, 1999; Dube *et al.*, 2008). This technique allows absolute quantification of target genes without any standards and, thus, it is considered to be less susceptible to PCR inhibitors present in DNA extracts (Hoshino & Inagaki, 2012). In this sense, in Publication II, we tested the suitability of dPCR to detect and quantify *P. nicotianae* in different environmental samples (soils, stems, and roots) in comparison to qPCR, the current method of choice in this field (Publication I). Although dPCR has recently been used for clinical diagnosis purposes (Kim *et al.*, 2014; Wang *et al.*, 2014; Whale *et al.*, 2013), there do not seem to be any studies in the field of plant pathology, although there are a few regarding environmental matrices (Hoshino & Inagaki, 2012). The dPCR along with the TaqMan system developed in Publication I allowed the detection of low levels of *P. nicotianae* ITS copies, regardless of the type of sample. The dPCR was able to quantify accurately as few as 195 copies μL^{-1} in soils and 143 copies μL^{-1}

in stems. Moreover, dPCR estimated more copies μL^{-1} than qPCR in all root samples and most stem samples. Since these types of samples were less susceptible to inhibition in dPCR than in qPCR, lower dilutions were required, reducing the possibility of obtaining concentrations below the detection limit in samples with low-level copy numbers. In this respect, an internal positive control was used to verify that the DNA extracts were not influenced by inhibitors and provided inhibition-free quantification results (Hoshino & Inagaki, 2012). Linear regression analysis showed a high correlation between the results obtained from the two techniques (R^2 0.874-0.999), which is in concordance with previous studies comparing these technologies (Kim *et al.*, 2014). The results reported in Publication II highlighted the use of dPCR as a suitable and promising alternative to qPCR, being able to quantify accurately the presence of the pathogen without the need for standard curves, and verified the potential of the primers and probe designed in Publication I. It is also worth mentioning the rapidity of both techniques, which are able to provide results in less than 24 hours, including nucleic acids extraction, compared to the 5-6 days needed when using culture techniques such as plate dilution in selective media.

In the present Doctoral Thesis, molecular techniques have been used not only to detect and quantify *P. nicotianae* but also to study the genetics of its population in “El Campo de Cartagena” (Publication I). Knowledge of the genetics of populations of plant pathogens may provide information about the evolutionary potential of pathogens to overcome management strategies (Southwood *et al.*, 2012). In this case, the use of mitochondrial markers was our choice to characterize the population, since they have been proposed for

the study of clonally reproducing populations (Mammella *et al.*, 2011; Martin *et al.*, 2012). Two regions were studied but only one, *trnY/rns*, showed differences, which were due to single-nucleotide polymorphisms. The four haplotypes detected could suggest asexual reproduction, leading to a lack of diversity among the isolates (Mammella *et al.*, 2011). The morphological study of the isolates obtained from infected stems revealed that all of them belonged to the same mating type, A2. Given that *P. nicotianae* is a heterotallic species, sexual combination among isolates is impossible or difficult, in the absence or low presence of one of the mating types. As reported previously by other authors (Silvar *et al.*, 2005; Mammella *et al.*, 2011), neither the phylogenetic groups nor the mitochondrial haplotypes were correlated with any of the phenotypic traits.

The use of composts in horticulture as growing media can contribute to waste recycling and to reducing the use of other, more harmful fertilizers (Pérez-Murcia *et al.*, 2005; Suárez-Estrella *et al.*, 2013). The results from Publications III, IV, and V showed that all the agricultural and agro-industrial composts tested in the present Doctoral Thesis were able to be used as partial substitutes for peat in potting media, as postulated by other authors (Kavroulakis *et al.*, 2005; Pane *et al.*, 2011). Specifically, in Publication III, plants grown in compost–based substrates (compost:peat 1:1) presented good agronomic values, similar to or even higher than in peat. This feature could be due to the higher macro- and micronutrient contents found in the agricultural and agro-industrial composts. Currently, peat is the organic substrate utilized most for the preparation of potting mixtures. However, its use in horticulture will probably be discouraged because of its limited

sustainability and the negative impact on global climatic changes associated with its extraction (Carlile, 2009). Moreover, peat is hardly ever suppressive against soil-borne pathogens such as *Fusarium oxysporum* and *Phytophthora* spp. None of the composts from Publication IV was conducive to Phytophthora root rot, nor were composts from Publications III and V conducive to Fusarium wilt, and only one compost from Publication III was conducive to Phytophthora root rot. These results are in concordance with previous studies which showed the ability of composts to suppress Fusarium wilt (Borrero *et al.*, 2004; Ros *et al.*, 2005; Bernal-Vicente *et al.*, 2008) or Phytophthora root rot (Termorshuizen *et al.*, 2006; Ntougias *et al.*, 2008). However, the level of suppression achieved was variable among composts and was related to the pathogen to be controlled. It is well-known that the ability of composts to suppress phytopathogenic agents varies, without consistency against various pathogens. In a study involving the use of 36 composts against three pathosystems, the authors found that 49 % of the bioassays showed significant suppression and 14 % significant disease enhancement (Sheuerell *et al.*, 2005). In a comparable study with 18 composts and seven pathosystems, Termorshuizen *et al,* (2006) showed that in 54 % of the cases the composts achieved significant disease suppression while only 3 % of the cases showed significant disease stimulation. These authors highlighted that no single compost showed significant disease suppression against all pathogens; neither was there a pathogen that was affected similarly by all composts. In this sense, we observed in Publication III that compost suppressive of Fusarium wilt (T-C3) did not show a suppressive effect against Phytophthora root rot and, by contrast, compost which was suppressive against *P. capsici* (T-C2) did not show any control of

F. oxysporum. In this respect, some authors pointed out that one of the main drawbacks of the use of composts is the lack of predictability (Hadar & Papadopoulou, 2012), which hinders their practical use (van Elsas & Postma, 2007). Therefore the studies presented in Publications III and IV were performed, to try to select compost characteristics which may be used to predict suppressiveness. Developing criteria to predict whether a particular compost will suppress a pathogen, in terms of a single crop, is of high relevance for growers and compost producers (Termorshuizen *et al.*, 2006). However, this task has been difficult to achieve due to the many interactions of contributing factors (Bonanomi *et al.*, 2010).

Phytophthora species are often claimed to be highly sensitive to microbial nutrient competition (Hoitink & Boehm, 1999; Avilés *et al.*, 2011). In the case of pathogens whose control has been related to the general suppression model, some promising predictive parameters (already correlated with disease suppression) could be those which measure general microbial activity such as fluoroscein diacetate (FDA) hydrolysis, basal respiration, and dehydrogenase activity. The dehydrogenase activity of composts from Publications III and IV was measured: this activity was positively correlated with the control effect of the composts against Phytophthora root rot. However, it was not correlated with Fusarium wilt control (Publication III). In this Doctoral Thesis, it was observed that the suppressive nature of *Fusarium oxysporum* may be the result of complex interactions between abiotic characteristics of the growth media and the microbial population, as proposed by other authors (Borrero *et al.*, 2004; Castaño *et al.*, 2011). The high pH values in the composts of Publication V were considered a positive

characteristic in terms of reducing diseases caused by *Fusarium* spp., since pH modifies the availability of macro- and micronutrients - which are important for the growth, sporulation, and virulence of *F. oxysporum* (Jones *et al.*, 1991). High pH values in certain composts achieved antifungal activity against Fusarium wilt in tomato, carnation, and melon (Borrero *et al.*, 2004, 2009). We also observed a positive correlation between Fusarium wilt suppression and the NAGase, chitinase, and protease activities. These results could indicate a potentially high level of colonization by chitinolytic-enzyme-producing microorganisms (López-Mondéjar *et al.*, 2012). The suppression of Fusarium wilt has been related also to the presence of specific antagonists such as actinomycetes (Ntougias *et al.*, 2008). Thus, the Gram-positive:Gram-negative ($Gram^+$:$Gram^-$) ratio indicated the preferential development of $Gram^+$ bacteria in Fusarium-wilt-suppressive compost (C3), which may be interpreted as a shift towards an enriched actinomycete community. On the other hand, the monounsaturated:saturated ratio, which may indicate the C availability in soils, as reported by some authors (Bastida *et al.*, 2008), was higher in the compost C2 that was suppressive of Phytophthora root rot. Higher C availability could promote higher microbial activity, as was observed in this publication (Bastida *et al.*, 2008). The PLFA technique showed significant differences in the microbial community structure of the different composts (Klamer & Baath, 1998; Ebersberger *et al.*, 2004). The microbial community structure and activity are important factors that have been related to compost suppressiveness (Hoitink & Fahy, 1986). Molecular techniques have rapidly been adopted for studies aimed at understanding the structure and function of microbial communities. In Publications IV and V, clone libraries and metagenomics, respectively, were

used to elucidate the different communities present in the composts. Both techniques have been used previously to characterize microbes in composts (Partanen *et al.*, 2010; Dougherty *et al.*, 2012; DeGannes *et al.*, 2013a,b; Yeh *et al.*, 2013) and have revealed the identity of uncultured and previously-unknown composting microorganisms (Garbeva *et al.*, 2004; Handelsman, 2004; Franke-Whittle *et al.*, 2009). In this Doctoral Thesis the ability of these techniques to detect and quantify shifts in the microbial community structure of composts has been proven. Both methods reported that Proteobacteria and Actinobacteria largely dominated the bacterial communities, taxa that have been commonly associated with compost. The fungal community of the composts was only analyzed in Publication IV, and three phyla were detected, the Ascomycota being the most dominant. *Fusarium*, *Aspergillus*, and *Penicillium* were present as common saprophytic fungi on food wastes (Anastasi *et al.*, 2005; Neher *et al.*, 2013).

Fungal populations have been reported as the main contributors to the biological suppressivity of compost (Hardy & Sivasithampram, 1995). In Publication IV, the *P. nicotianae* incidence was negatively correlated with the presence the Ascomycota phylum. The relative abundance of this phylum was higher in suppressive composts COM-A and COM-B. Within this phylum, the two most-frequent orders recorded were Sordariales and Hypocreales, represented with species such as *Zopfiella* and *Fusarium*. The Hypocreales order is especially rich in fungal parasites and antagonists (Jeffries & Young, 1994). Probably, the higher proportion of vineyard pruning wastes incorporated into composts COM-A and COM-B led to a higher development of fungi associated with hardwood compost, as reported

by Neher *et al.* (2013). Both composts also showed a higher relative abundance of *Streptomyces*. Some species within this genus are well-known for their ability to degrade the cell walls of soil-borne plant pathogens through the production of chitinases and antibiotics (Hoster *et al.*, 2005; Kawase *et al.*, 2006; Susi *et al.*, 2011). Publications IV and V show that the activity of this genus may be affected by the materials used to produce the compost, since cell-wall-degrading enzymes are especially produced when the nutrient supply is limited (Susi *et al.*, 2011). In Publication V, the organic fraction of the composts was evaluated by SP-MAS ^{13}C NMR. The relative abundances of carbohydrate structures (60-110 ppm) and aliphatic structures (0-45ppm) were positively correlated with the Phytophthora root rot incidence. Moreover, the alkyl/O-alkyl ratio, which is considered as a sensitive index of the stabilization and humification of organic matter (Pane *et al.*, 2011), was higher in conducive substrates. This shows that these substrates were more stabilized - a feature that is related negatively to disease suppression, since such materials are not able to support microbial activity and, thus, suppression is lost (Hadar & Mandelbaum, 1986; Widmer *et al.*, 1998). The organic matter present in compost influences not only the metagenome of compost, but also its metabolome (Castaño *et al.*, 2011). For this reason we studied the metabolome of the composts included in Publication IV. The resulting principal component analysis clustered substrates in three groups, related to the levels of suppression achieved in the bioassays. It seems that the secondary metabolites produced by the microbial community present in composts COM-A and COM-B could lead to the suppression of Phytophthora root rot. Reuben *et al.* (2008) reported that some antagonistic microorganisms are able to produce and secrete a broad array of

antimicrobial compounds; these include *Streptomyces* and *Bacillus*, which were present in these composts (Publication IV).

Moreover, among the mechanisms involved in the suppression of pathogens, the activation of disease-resistance genes in plants has become one of the most-promising strategies in the control of plant pathogens using composts (Yogev *et al.*, 2010). In Publication II, the levels of one hormone related to systemic acquired resistance (SAR), salicylic acid (SA), and one related to induced systemic resistance (ISR), jasmonic acid (JA), were evaluated. We also measured the plant hormone abscisic acid (ABA), which is considered to act as a negative regulator of disease resistance (Flors *et al.*, 2008). In spite of the activation of the defense pathway in muskmelon and pepper seedlings, a susceptible plant-pathogen interaction was observed and this mechanism is not the one involved in the compost supressiveness reported in this Publication (Publication III).

One route that converges on the production of effective composts is the development of suppressive composts by an additional fortification with specific microbial agents (Hadar & Papadopoulou, 2012). In Publication V, we evaluated the effects of the addition of *T. harzianum* to compost (GCTh) and found that it improved the suppressive effect obtained, in comparison with non-inoculated composts (GC). Composts fortified with *Trichoderma* spp. have been used to control a wide array of plant pathogens (Trillas *et al.*, 2006; López-Mondéjar *et al.*, 2010; Sant *et al.*, 2010). *Trichoderma* spp. have been widely used in agriculture (Papavizas, 1985; Chet, 1987) - specifically *T. harzianum*, whose antagonistic effect has been demonstrated against several soil-borne plant pathogens (López-Mondéjar *et al.*, 2010). However,

the suppressive effect of *T. harzianum* was only observed under *in vivo* conditions, whereas no differences were observed between the two composts *in vitro*. These results show the inability of *in vitro* assays to produce accurate conclusions (Ros *et al.*, 2005; Avilés *et al.*, 2011).

On the other hand, some authors have reported that the inoculation of *T. harzianum* into the soil only modifies slightly its microbial diversity while others have observed an increase in soil microbial biomass (Bae *et al.*, 2002; Cordier *et al.*, 2007; Savazzini *et al.*, 2009). However, the effect of the addition of *T. harzianum* to compost after the temperature peak of the composting process has not been previously studied. In Publication V, we evaluated the effect of *T. harzianum*, not only on the biotic characteristics of composts but also on the abiotic ones. The bacterial community structure and the chitinase gene diversity were analyzed through 16S rRNA and chitinase gene libraries. This technique was able to reveal differences between the two composts, such as an increase in the relative abundance of species of the genus *Streptomyces* and the appearance of other bacterial communities (γ-proteobacteria and Verrumicrobia) related to chitin-amended soils (Sato *et al.*, 2010) in GCTh compost, compared to GC. Not only the presence of *T. harzianum*, but also the presence of these communities may be related to the control of Fusarium wilt. Moreover, *T. harzianum* promoted other changes in the compost - such as lower pH, EC, and organic carbon content and higher levels of N and K.

V

Chapter V. Conclusions / Conclusiones

V. CONCLUSIONS

1. The development of molecular tools for faster and more precise identification of *P. nicotianae* is vital for tracking unintended spread of this species and as such, is essential for integrated disease management strategies to control Phytophthora root rot. The specific detection and accurate quantification of this oomycete was achieved using the qPCR and dPCR techniques along with the primers Nic-F1 and Nic-R4 and the MGB TaqMan probe Nic-Pro, designed in the ITS region of *P. nicotianae*. The efficacy of these techniques was proved in pure culture and in different environmental samples such as soil, compost, peat, and plant tissues. The dPCR displayed similar results compared to qPCR although in the case of plant tissues, dPCR seemed to be more sensitive as well as less susceptible to inhibition. These features made dPCR an attractive alternative for measurement of low copy DNA in the field of plant pathology.

2. *P. nicotianae* replaced *P. capsici* in pepper greenhouses of "El Campo de Cartagena" as the main casual agent of Phytophthora root rot. However, both species still coexist in few greenhouses. It seems that the population of *P. nicotianae* in this area is the result of a succession of a clonal lineage, since only one mating type was found. The different haplotypes detected by analyzing individually the mitochondrial region *trnY/rns* were not correlated with any phenotypic trait.

3. The use of agricultural and agro-industrial composts could replace the use of peat in nurseries as well as reducing organic waste disposal in landfills. Plants grown in compost-based media showed good agronomic values compared to peat and in some cases, higher capacity to reduce Fusarium wilt in muskmelon and Phytophthora root rot in pepper, although this suppressiveness was pathogen-dependent.

4. The different raw agro-industrial wastes and diverse combination of them used during the composting process, led to composts with different organic matter composition and degrees of stabilization, as revealed by ^{13}C-NMR, which exerted a control in the extant microbiota in composts. Suppression of Phytophthora root rot was lost in compost excessively stabilized. Therefore, the proper selection of raw materials is an important strategy in order to control this disease.

5. Specific enzymatic activities such as NAGase, chitinase, and protease could be used as potential indicators of compost suppressiveness against Fusarium wilt, as well as the general enzymatic activity, measured as the dehydrogenase activity, could predict the potential of compost to suppress Phytophthora root rot. These parameters may be useful to evaluate the level and specificity of the suppression effect, representing a step toward an accurate prediction of compost suppressiveness.

6. Insights into the microbial community structure of composts were obtained by metagenomic analysis. The presence of certain antagonistic microbes such as *Streptomyces* and *Bacillus* among bacteria, and the fungi *Fusarium* and *Zopfiella* among fungi, was not enough to achieve a high level of suppressiveness in all composts. The organic matter composition of some composts did not contribute to the maintenance of the microorganisms involved in the suppressive phenomena, as a result of the high degree of stabilization, or the encrustation of cellulose by lignin.

7. The study of metabolome may be a promising predictor of compost suppressiveness of Phytophthora root rot. Some of the secondary metabolites reported in the suppressive composts may be responsible of the successful suppressiveness achieved. Deeping into the metabolite profiles can further contribute to the identification of the sources of variability in compost to disease control. Moreover, the integration of this parameter with others, such as metagenomics, will be useful to reveal the species and the functions of the microbial community that are associated with disease suppression.

8. The addition of *T. harzianum* during composting process improved the efficacy of compost to reduce Fusarium wilt. This effect could be related to the direct action of *T. harzianum,* or to the changes induced by this BCA in the compost, such as the increase in the relative abundance of *Streptomyces* spp., or the variations in its abiotic characteristics (pH, C, N and Fe levels). The presence of *T.*

harzianum promoted an increase in the 16S rRNA library diversity and caused a decrease in the chitinase gene library diversity.

9. Plant experiments are required to obtained realistic conclusions concerning the suppression capacity of composts. *In vitro* analyses did not show differences in the mycelia growth inhibition of *F. oxysporum* among composts, while compost with *T. harzianum* showed the highest control of Fusarium wilt under *in vivo* conditions.

CONCLUSIONES

1. El desarrollo de herramientas moleculares para identificar *P. nicotiane* de forma más rápida y precisa, es de gran relevancia para evitar la expansión de esta especie, y es esencial para llevar a cabo estrategias de control integrado de la tristeza del pimiento. La detección específica y la cuantificación precisa de este oomiceto, se realizó mediante el uso de las técnicas qPCR y dPCR, junto con los cebadores Nic-F1 y Nic-R4 y la sonda TaqMan Nic-Pro, diseñados en la región del ITS. La efectividad de estas técnicas se demostró tanto en cultivo puro como en muestras ambientales, tales como suelo, compost, turba y material vegetal. La dPCR mostró resultados similares a la qPCR, siendo incluso más sensible y menos susceptible a la inhibición en muestras de planta. Estas características convierten a la dPCR en una alternativa interesante para la cuantificación de un bajo número de copias de ADN en el campo de la fitopatología.

2. *P. nicotianae* sustituyó a *P. capsici* en los invernaderos de pimiento de "El Campo de Cartagena" como el principal agente causante de la tristeza del pimiento. Sin embargo, actualmente ambas especies todavía coexisten en un par de invernaderos. Parece que la población de *P. nicotianae* en esta zona ha evolucionado como resultado de la sucesión de una línea clonal, puesto que todos los aislados tienen el mismo tipo de compatibilidad de cruce. Los diferentes haplotipos detectados mediante el análisis individual de la región mitocondrial *trnY/rns*, no estaban correlacionados con ninguna característica fenotípica.

3. El uso de compost de origen agrícola y agro-industrial puede reemplazar a la turba en semilleros así como reducir el depósito de residuos orgánicos en vertederos. Las plantas crecidas en medios de cultivo basados en compost presentaron buenas características agronómicas en comparación con las crecidas en turba. Además en algunos casos estas plantas mostraron una mayor capacidad para reducir la fusariosis vascular del melón y la tristeza del pimiento, aunque dicha supresividad estaba relacionada con el patógeno a controlar.

4. La tipología de los residuos agro-industriales y las distintas combinaciones de los mismos usados durante el compostaje, dieron lugar a compost con diferente composición química y distinto grado de estabilización, según fue revelado por ^{13}C-NMR, características que podrían conllevar cierto control en la microbiota de los compost. El control de la tristeza del pimiento disminuyó en el caso de los compost excesivamente estabilizados. Por esto, la correcta selección de los materiales de partida es importante para controlar esta enfermedad.

5. Actividades enzimáticas específicas tales como la NAGasa, chitinasa y la proteasa pueden ser usadas potencialmente como indicadores de la capacidad de los compost para controlar la fusariosis vascular del melón, así como la actividad deshidrogenasa, es útil para predecir el potencial supresivo de los compost contra la tristeza del pimiento.

Estos parámetros son útiles para evaluar el nivel y especificidad del efecto supresivo de los compost, siendo un paso más hacia la predicción de la supresividad de los sustratos orgánicos.

6. Mediante un análisis metagenómico se obtuvo una nueva perspectiva sobre la comunidad microbiana presente en un grupo de compost con distintos niveles de supresividad. La presencia de ciertos microoganismos antagonistas como las bacterias *Streptomyces* y *Bacillus*, y los hongos *Fusarium* y *Zopfiella*, no fue suficiente para que todos los compost pudieran controlar *P. nicotianae*. La composición de la materia orgánica en alguno de los compost no contribuyó a mantener activa la comunidad involucrada en el fenómeno de supresión, como resultado de un alto grado de estabilización de los materiales o el encrustamiento de la celulosa por la lignina.

7. El estudio del metaboloma es un factor prometedor para predecir la supresividad de los compost contra la tristeza del pimiento. Algunos de los metabolitos observados en ciertos compost podrían ser responsables del potencial supresivo alcanzado. Una profundización en la caracterización de los metabolitos detectados podrá contribuir a la identificación de las fuentes de variabilidad de los compost en el control de enfermedades. Además, la integración de esta técnica junto con otras como la metagenómica, permitirá conocer qué especies y

qué funciones de la comunidad microbiana están asociadas con la supresividad.

8. La adición de *T. harzianum* durante el proceso de compostaje mejoró la eficacia del compost para control la fusariosis vascular del melón. Este efecto pudo estar relacionado con la acción directa de *T. harzianum*, o bien por los cambios inducidos por este ACB en el compost, como el incremento de la abundancia relativa de *Streptomyces* sp., o las variaciones en sus características abióticas (pH, niveles de C, N y Fe). La presencia de *T. harzianum* aumentó la diversidad de la librería de clones del 16S ARNr y disminuyó la de la librería de clones del gen quitinolítico.

9. Los ensayos con plantas son necesarios para obtener conclusiones realistas en cuanto a la capacidad supresiva de los compost. El análisis llevado a cabo bajo condiciones *in vitro* no mostró diferencias significativas en la inhibición del crecimiento de *F. oxysporum*, mientras que bajo condiciones *in vitro*, el compost inoculado con *T. harzianum* mostró un mayor control de la fusariosis vascular.

VI

Chapter VI. References

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