Induction of resistance in pepper against *Verticillium dahliae* by the application of PO212

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Doctoral Thesis 2019

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PhD in Cellular and Molecular Biology

UNIVERSIDADE DA CORUÑA

Departamento de Bioloxía

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Que la presente tesis doctoral titulada "Induction of resistance in pepper against *Verticillium dahliae* by the application of PO212" presentada por la graduada **Marta Lois Alvedro** ha sido realizada en el Departamento de Biología de la Universidad de A Coruña bajo su dirección, y dentro del Programa de Doctorado de Biología Celular y Molecular, y cumple las condiciones exigidas para aspirar al título de Doctor en Biología,

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Agradecimientos

En primer lugar me gustaría agradecer a mis padres, por apoyarme día tras día en esta etapa, ya sea preocupándose sobre la comida que llevaría al día siguiente a la facultad, como preguntándose si era necesario llevarme o recogerme todos los días al laboratorio (incluidos ciertos festivos y fines de semana). Sin vosotros hubiera sido mucho más difícil (y sin Tranvias de A Coruña también).

A mis hermanos, Saleta y Alex, por consentir todos mis caprichos de hermana pequeña (os lo compensaré cuando sea mayor y trabaje) y a Patri, por interesarse siempre sobre mi trabajo y lo que es más importante, entenderlo.

A mis tutores, José y Javi, por su paciencia y esfuerzo diarios. A José, por su empeño y curiosidad constantes, y por hacerme ver que los cafés desde las 9 de la mañana hasta las 10 también son trabajo. A Javi, por sus grandes conocimientos en informática que me han salvado de más de un apuro, y por ayudarme siempre que lo he necesitado tanto dentro como fuera del laboratorio. Sin vosotros no hubiera conseguido aumentar mi capacidad para aguantar chistes malos.

A Geli, porque siempre has tenido buenas palabras hacia mí, y por enseñarme a disfrutar de toda experiencia que se ponga por delante. Gracias por la confianza depositada en mí.

A Fede, por introducirme en el mundo de la ópera y las películas clásicas de terror (sin éxito), y siempre respetando mis gustos musicales y deportivos.

A Cristina Silvar, por el crosstalk de cotilleos y confesiones que han hecho mucho más llevaderas todas las tardes en el laboratrio.

A Néstor, por ofrecerme su ayuda siempre que la he necesitado, por su ímpetu y capacidad de transmitir, y por hacer que todo parezca más sencillo.

A la profesora Teresa Lino-Neto, por acogerme dentro del grupo BioISI (Biosystems and Integrative Sciences Institute) en el Plant Functional Biology Centre (CBFP) de la Universidade do Minho de Braga. Gracias por tu generosidad y entusiamo en aquellas inolvidables salidas de campo, y por todos aquellos platos típicos portugueses que obteníamos como recompensa.

A todos los profesores y compañeros del CBFP, Manuela, Rui, Shweta, Francisca, Eunice, Ana Teresa, Mário y Ana Maria, y en especial a Daniela, Helena, Sara y Rómulo, por hacerme sentir una más. A Daniela, por dejarme entrar en su vida y ofrecerme su amistad. A Helena, por esos cafés antes de empezar a trabajar en los que conseguíamos arreglar el mundo. A Sara, por ser nuestra mamá. A Rómulo, por ser nuestro papá y mi fiel compañero del dueto de maldades.

A mis dos apoyos fundamentales en toda esta etapa, Laura y Alba, juntas formamos el trío del piso 4. A Laura, gracias por hacer siempre de cualquier problema un pequeño bache fácil de sobrepasar, por todas esas mañanas de compras en tiendas de ropa de alta gama, las tardes en Sardiñeira en las que hacíamos mucho más que deporte, y los cafés de confesiones interminables. Ahora podremos competir por el tan ansiado puesto en Harvard. A Alba, por estar siempre ahí para ayudarme, sin esperar nada a cambio y con una sonrisa, por responder de forma efectiva a todas mis preguntas (laboratoriales, informáticas, o de cultura general), y por elogiarme con ser la primera persona a la que casi mato de la risa (emesis incluida). Aunque eso no lo sabes, gracias a mí eres una adoradora de Satán. Gracias a las dos por ser mis amigas, y por ser parte de mi vida tanto dentro como fuera del laboratorio.

A Manu y Jorge, mis fieles compañeros de Estrella Galicia y mecánicos personales. Sin vosotros las mañanas en el laboratorio no serían lo mismo (y las noches tampoco). A Manu, gracias por hacerme ver más allá, tus elegantes piropos y tu capacidad de aguante. A Jorge, gracias por tu ayuda y saber escuchar en todo momento, y por mantenerme siempre informada de las novedades musicales. Sois tan grandes por dentro como por fuera.

A Diego, por sus gestos de cariño diarios, y su capacidad para olvidarse de todo lo negativo (y de lo positivo también).

A Tania, por su siempre buena disposición para ayudar, desde el comienzo de mi máster hasta ahora, y por aquellos viajes hasta Sardiñeira.

A Nuria, por ser una compañera más de cafés y, por supuesto, de cursos del CUFIE.

A todos los demás compañeros del laboratorio con los que he coincidido en esta etapa, Ana Monteagudo, Carlos Caramelo, Teresa, Esther, Alexander y Carlos García. De todos vosotros he aprendido.

A Nani y Miriam, de la Unidad de Biología Molecular de los SAI (Servizos de Apoio á Investigación), por el trabajo realizado y su siempre buena disposición, a la Unidad de Técnicas Instrumentales de Análisis por su trabajo que ha contribuido de forma importante a la etapa final de la tesis, y a Victor Flors y Victoria Pastor, de la Universitat Jaume I, por su también notable trabajo realizado.

A Jesús Collar, del Laboratorio Agrario e Fitopatolóxico de Galicia (Lafiga) de Mabegondo, por su ayuda y el gran trabajo realizado con la identificación fúngica.

A Irene, por hacer más interesantes nuestras comidas en el piso 4, y por mantenerme siempre informada de su agenda social, y de sus hábitos culinarios.

A Cristina Pardo, por su sinceridad y muestras de cariño, y por enseñarme todo lo que sé sobre moda (hasta en el día de su tesis).

A Rafa, por todas esas horas en el microscopio sin esperar nada a cambio (ni siquiera una hamburguesa), por descubrirme las tecamebas y demás micromundos, y subirme la autoestima.

A Lorena, por haber revolucionado el laboratorio con su alegría, simpatía y locura, y por todas aquellas tardes de trabajo que tuvieron su recompensa.

A María Carreras, por ser mi primera alumna (y la más aventajada), y por dejarme ser su profe en nuestro año fuerte. Siempre nos quedará la casa rural.

A Andrea, Trini y Fran por haber estado ahí de una forma u otra (y con muchos kilómetros de por medio).

A mis compañeras de gimnasio, Noelia y Leti, por ser mi vía de escape, ya sea yendo al Marineda como a cenar a la Dolce, y por estar siempre actualizadas en cuanto a fenoles y ligninas. Y a Miguel también, porqué no.

A mi segunda familia, Alberto, Ánxela, Alex, Lara, Iara, Aida, Sofía y Gonzalo.

A Carlos, por apoyarme y escucharme cada vez que lo necesitaba, y sobre todo por su paciencia infinita. Gracias a ti creo en mi.

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Preface

Food production needs to be increased to sustain an increasing human population that has already reached 7.6 billion of people and it could reach 9.8 billion in 2050. This implies to improve plant disease management through strategies different from pesticide application. In the last years, the use of biological control agents has been implemented in order to reduce the use of pesticides and its environmental impact.

In this thesis we studied the effect of the biocontrol agent PO212 as an inducer of resistance in pepper plants against the vascular fungus *Verticillium dahliae*. In addition, we studied a possible complement to PO212 in biological control: arbuscular mycorrhizal fungi in soils of several Galician pepper greenhouses, which can be responsible of suppressiveness to *V. dahliae* and the oomycete *Phytophthora capsici*.

This research was supported by grants RTA2013-00060-C05-01, RTA2013-00060-C05-02 from Spanish Ministry of Economy and Competitiveness-INIA co-financed with FEDER funds from the European Union and RTA2017-00019-C03-03 fom Spanish Ministry of Science, Innovation and Universities-INIA.

Some work from this thesis has been carried out through pre-doctoral research stays in the Plant Functional Biology Centre (CBFP) of the University of Minho (Braga, Portugal), supported by IACOBUS and INDITEX-UDC scholarships.

Some work from this thesis has already been submitted to a scientific journal as well as showed in scientific meetings:

- **Lois, M., Veloso, J., García, T., Carrillo-Barral, N., Larena, I., Díaz, J.** (2019). *Penicillium rubens* strain 212 induces resistance to *Verticillium dahliae* in pepper (submitted, to be resubmitted attending the referees comments).

Following, there is a list of the communications to scientific meetings:

- **Veloso J., García T., Lois M., Larena I., Díaz J.** (2015). Two fungi inducing resistance to *Verticillium dahliae* in pepper: Fo47 and PO212. PR-Proteins and Induced Resistance Against Pathogens and Insects. Aachen (Germany). Poster.

- **Lois, M., Veloso, J., García, T., Larena, I., Díaz, J.** (2016). PO212 induces resistance in pepper against *Verticillium dahliae*. XVIII Congreso de la Sociedad Española de Fitopatología. Palencia (Spain). Oral communication.

- **Lois, M., Almagro, L., Bernal, M.A., Veloso, J., García, T., Larena I., Díaz, J.** (2017). Eliciting plant defences in *Capsicum annuum* cell cultures with PO212. Congreso Nacional de Biotecnología (BIOTEC 2017). Murcia (Spain). Poster.

- **Lois, M., Díaz, J., Lino-Neto, T.** (2017). Arbuscular mycorrhizal fungi from greenhouse pepper crops as potential biocontrol agents of *Verticillium dahliae*. Proteção das Plantas 2017. 2º Simpósio SCAP de Proteção das Plantas. 8º Congresso da Sociedade Portuguesa de Fitopatologia. 11º Encontro Nacional de Proteção Integrada. Santarém (Portugal). Oral communication.

- **Lois, M., Veloso, J., García, T., Larena, I., Díaz, J.** (2017). PO212 induces resistance to *Verticillium dahliae* in pepper. Proteção das Plantas 2017. 2º Simpósio SCAP de Proteção das Plantas. 8º Congresso da Sociedade Portuguesa de Fitopatologia. 11º Encontro Nacional de Proteção Integrada. Santarém (Portugal). Poster.

- **Lois, M., Larena, I., Fernández, N., Veloso, J., García, T., Pastor, V., Flors, V., Díaz, J.** (2018). PO212 induced resistance in pepper: new insights on phenolic defense responses. XIX Congreso de la Sociedad Española de Fitopatologia. Toledo (Spain). Poster.
- **Fernández, N., Lois, M., Veloso, J., García, T., Carrillo, N., Larena, I., Díaz, J.** (2019). *Penicillium rubens* strain PO212 induces resistance in pepper: phenolic metabolism and transcriptomics. XXIII Meeting of the Spanish Society of Plant Physiology/XVI Spanish Portuguese Congress of Plant Physiology. Pamplona (Spain). Poster.

Abstract

The biological control agent (BCA) *Penicillium rubens* (formerly *Penicillium oxalicum*) strain 212 (PO212) was employed as inducer of resistance in pepper (*Capsicum annuum* L.) against the vascular pathogen *Verticillium dahliae* Kleb. PO212 protected pepper plants against *V. dahliae*. The observed induced resistance in pepper involves cell wall reinforcement with an increase in lignin as well as other defenses as PR proteins. Signaling of the defense response triggered in pepper or tomato by PO212 is mediated by hydrogen peroxide, salicylic acid, ethylene and jasmonic acid. Induced resistance is one of the mechanisms by which PO212 protects pepper against *V. dahliae*. The combination of PO212 with the fungal BCAs known as arbuscular mycorrhizal fungi (AMF) can improve biological control effectiveness. Soil suppressiveness to *V. dahliae* and the oomycete *Phytophthora capsici* in pepper greenhouses appears to be related with AMF community.

Resumen

El agente de control biológico (ACB) *Penicillium rubens* (antes *Penicillium oxalicum*) cepa 212 (PO212) se empleó como inductor de resistencia en pimiento (*Capsicum annuum* L.) contra el patógeno vascular *Verticillium dahliae* Kleb. Se demostró que PO212 confiere protección en las plantas de pimiento contra *V. dahliae*. La resistencia inducida observada en pimiento implicó el reforzamiento de la pared celular con un aumento en lignina, y el incremento de varias defensas de tipo bioquímico como las proteínas PR. La señalización de la respuesta de defensa desencadenada por PO212 en pimiento o tomate parece estar mediada por peróxido de hidrógeno, ácido salicílico, etileno y ácido jasmónico. La resistencia inducida es uno de los mecanismos por los que PO212 ejerce su protección en pimiento contra *V. dahliae*. La combinación de PO212 con los ACBs fúngicos conocidos como hongos micorrícicos arbusculares (HMA) puede mejorar la efectividad del control biológico. La supresividad del suelo a *V. dahliae* y al oomiceto *Phytophthora capsici* en invernaderos de pimiento parece estar relacionada con la comunidad de HMA.

Resumo

O axente de control biolóxico (ACB) *Penicillium rubens* (antes *Penicillium oxalicum*) illado 212 (PO212) foi empregado como inductor de resistencia en pemento (*Capsicum annuum* L.) contra o patóxeno vascular *Verticillium dahliae* Kleb. Demostrouse que PO212 protexe as plantas de pemento contra *V. dahliae*. A resistencia inducida observada en pemento implicou o reforzamento da parede celular cun aumento en lignina e o incremento de varias defensas de tipo bioquímico como as proteínas PR. A sinalización da resposta de defensa desencadeada por PO212 en pemento ou tomate parece estar mediada por peróxido de hidróxeno, ácido salicílico, etileno e ácido jasmónico. A resistencia inducida é un dos mecanismos polos cales PO212 exerce a sua protección en pemento contra *V. dahliae*. A combinación de PO212 cos ACBs fúnxicos conocidos como fungos micorrícicos arbusculares (FMA) pode mellorar a efectividade do control biolóxico. A supresividade do solo a *V. dahliae* e ao oomiceto *Phytophthora capsici* en invernadeiros de pemento parece estar relacionada coa comunidade de FMA.

General introduction

Introduction

Plants are the pillar of all life on Earth and an indispensable resource for human well-being and, in general, for the vast majority of terrestrial inhabitants. As photosynthetic organisms, plants provide the energy and carbon to sustain most of organisms, influence the climate and regulate the water cycle through transpiration. In addition, food and many drugs come directly or indirectly from plants, as well as other materials such as fibers and wood. Plants constantly deal with different types of stress, which sometimes lead to important losses in their production and affect their survival. Nowadays, pathogens are by far the most important factor affecting plant health, causing severe diseases in many plant species. Plant diseases are caused by fungi, oomycetes (fungal-like organisms), bacteria, viruses, phytoplasmas, parasitic plants and nematodes, and they are the reason for great annual losses in crop production worldwide (Agrios, 2005). If human population continues to increase at the current rate, it will reach 9.8 billion in 2050 (ONU, 2017), and it is estimated that by then there will be no food available to feed such a population (Ray *et al.*, 2013). Therefore, food production needs to be increased, and this means to improve plant disease management. One of the most promising strategies to achieve this goal is to take advantage of the plant resistance.

Plant defense mechanisms – induced resistance

Plants have been able to develop a broad range of strategies to defend themselves against different biotic stresses. These strategies are part of the plant innate immune system, which is based on preformed (non-specific, passive) and induced (specific, active) defense responses (Jones & Dangl, 2006; Zeilinger *et al.*, 2016; Bacete *et al.*, 2018). Preformed defense responses include structural barriers (cuticles, trichomes and preformed cell walls), and compounds with antimicrobial activity (phytoanticipins)

(Agrios, 2005; Zeilinger *et al.*, 2016; Bacete *et al.*, 2018). However, plant cell wall (mainly composed by cellulose, hemicelluloses and pectins) is part of both, constitutive and inducible defense mechanism; when pathogens attempt to colonize plant tissues, they have to overcome the plant cell wall, which changes its physical properties and chemical composition (e.g. lignin, xylans) as a response to the pathogen (Miedes *et al.*, 2014; Bacete *et al.*, 2018). Many pathogens are able to penetrate and break down the plant cell wall by secreting cell wall-degrading enzymes (CWDEs) (e.g. cellulases, pectinases, hemicellulases) and using mechanical force (e.g. appressoria) (Miedes *et al.*, 2014; Zeilinger *et al.*, 2016; Bacete *et al.*, 2018). When the pathogen penetrates into the plant, induced defense responses are activated to prevent the pathogen establishment. Induced defense responses are initiated through recognition of microbial compounds called pathogen-, microbe- or herbivore-associated molecular patterns (PAMPs, MAMPs or HAMPs) by specialized membrane-associated receptor-like kinases (RLKs) or receptor-like proteins (RLPs) the so-called pattern-recognition receptors (PRRs) (Jones & Dangl, 2006; Dempsey & Klessig, 2017; Klessig *et al.*, 2018; Saijo *et al.*, 2018). Examples of PAMPs/MAMPs are peptidoglycan, flagellin, chitin, ergosterol or β-glucans, which are present in cell walls of bacteria, fungi, oomycetes and plants (Jones & Dangl, 2006; Dempsey & Klessig, 2017; Bacete *et al.*, 2018; Klessig *et al.*, 2018). There are also endogenous plant-derived signals released as a consequence of cell wall degradation, the so-called damage-associated molecular patterns (DAMPs) (e.g. pectins, oligogalacturonides, β-1,3-glucans) (Dempsey & Klessig, 2017; Bacete *et al.*, 2018). This first layer of immunity is called MAMP-triggered immunity (MTI), or PAMP-triggered immunity (PTI), whose objective is to limit the growth of the intruder (Jones & Dangl, 2006; Zeilinger *et al.*, 2016; Dempsey & Klessig, 2017; Klessig *et al.*, 2018) (Fig. 1). After PTI, a series of events takes place, including the activation of

Mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) through the BRI1-Associated Receptor Kinase (BAK1), stomatal closure through the activation of the abscisic acid (ABA) signaling pathway (associated with root colonization by the pathogen), oxidative burst (production of reactive oxygen species -ROS-), apoplast alkalinization, cell wall strengthening near the site of pathogen penetration (e.g., callose, enriched appositions -papillae-), salicylic acid and ethylene production, expression of Pathogenesis-Related (PR) proteins and defense-related enzymes (e.g. Phenylalanine ammonia-lyase -PAL- and Lypoxygenase -LOX-), production of antimicrobial compounds (e.g. phytoalexins), or calcium (Ca^{2+}) influx from extracellular spaces and changes in free cytosolic (Ca^{2+}) concentrations (Fig. 1) (Jones & Dangl, 2006; Lehmann *et al.*, 2015; Dempsey & Klessig, 2017; Bacete *et al.*, 2018; Ali *et al.*, 2018; Klessig *et al.*, 2018; Saijo *et al.*, 2018).

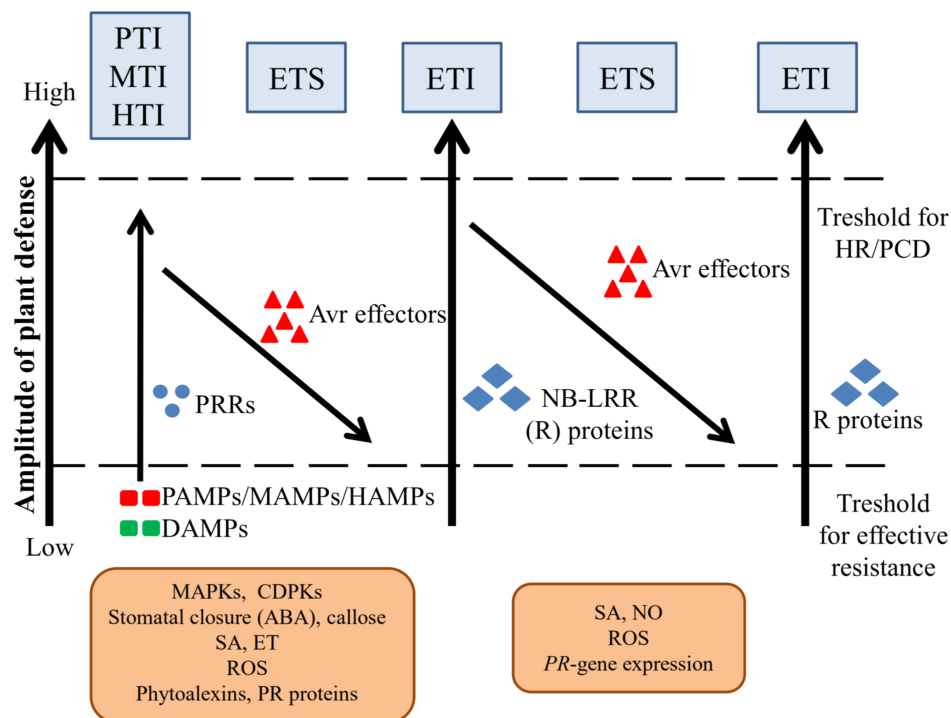

Figure 1. The zig-zag model of plant immune system. The amplitude of plant defense increases with PAMP, MAMP or HAMP-triggered immunity (PTI, MTI, HTI) and effector triggered immunity (ETI) and decreases with effector-triggered susceptibility (ETS). Pathogen, microbe

or herbivore-associated molecular patterns (PAMPs/MAMPs/HAMPs, red squares) or DAMPs (green squares) are recognized by plant pattern-recognition receptors (PRRs, blue circles) and activate PTI/MTI/HTI. During PTI/MTI/HTI, the Mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) pathways are activated, an oxidative burst occurs, and antimicrobial compounds are produced. Some pathogens deliver effectors (Avr, red triangles) that interfere with PTI/MTI/HTI and cause ETS. Some plant genotypes recognize one of the effectors by R proteins (blue diamonds), starting the ETI. ETI involves a hypersensitive response (HR) with programmed cell death (PCD) at the site of infection, in addition to salicylic acid (SA) and nitric oxide (NO) production. Selective pressure benefits pathogen isolates that have lost that effector, and gained new effectors (red triangles), that suppress ETI. Selective pressure acts upon plant genotypes than recognize these new effectors restoring ETI. Adapted from Jones & Dangl (2006).

Pathogens can suppress PTI/MTI by delivering effectors (virulence molecules, proteases, toxins) into the host cells. This state of PTI-suppression is known as effector-triggered susceptibility (ETS) (Jones & Dangl, 2006; Zeilinger *et al.*, 2016; Dempsey & Klessig, 2017) (Fig. 1). In this situation, some plants are able to recognize directly or indirectly pathogen effectors through host-specific proteins called Resistance (R) proteins (most of them are intracellular), and effectors are then called avirulence (Avr) factors/proteins. Genes encoding for R proteins usually contain a nucleotide-binding site-leucine-rich repeat (NBS-LRR) domain, but others contain a C-terminal leucine-rich repeats (LRRs). According to the "guard hypothesis", R proteins interact with the target (the guardee protein) of their corresponding pathogen effector and activate resistance, so the recognition of effectors by R-proteins can be achieved by direct interaction between the R and Avr proteins, or indirectly through the guardee protein. Thus, the second layer of immunity is initiated, and is called effector-triggered immunity (ETI; also called *R* gene-mediated resistance) (Jones & Dangl, 2006;

Zeilinger *et al.*, 2016; Dempsey & Klessig, 2017; Klessig *et al.*, 2018) (Fig. 1). ETI is faster and stronger than PTI, and often yields a hypersensitive response (HR) with programmed cell death (PCD) at the site of infection, in addition to SA (and/or its glucoside SAG) and nitric oxide (NO) production and the expression of plant defense genes (e.g. *PR*-genes). Like PTI, ETI is also related with the activation of MAPKs, the synthesis of phytoalexins and the increase in intracellular Ca^{2+} concentrations (Jones & Dangl, 2006; Lehmann *et al.*, 2015; Dempsey & Klessig, 2017; Ali *et al.*, 2018; Klessig *et al.*, 2018). The production of ROS (e.g. hydrogen peroxide -H_2O_2-) is required for HR, and HR limits the pathogen access to water and nutrients (Lehmann *et al.*, 2015; Ali *et al.*, 2018). ETI also is usually associated with necrotic lesion formation, which may help to restrict pathogen movement from the infection site (Dempsey & Klessig, 2017; Klessig *et al.*, 2018). Days or weeks after defense activation in the infected (local) tissue, PTI and ETI can also induce immune responses in the uninfected (systemic) tissues of the plant (e.g. production of PR proteins and SA), leading to induced resistance (Dempsey & Klessig, 2017; Klessig *et al.*, 2018).

Currently, induced resistance is one of the most economically and eco-friendly strategies against plant diseases (Ab Rahman *et al.*, 2018). Induced resistance is a plant defense response triggered by biotic, chemical or physical inducers that prevents or delays disease (Oliveira *et al.*, 2016; Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018). This defense mechanism is activated when plants contact with inducers. The term inducer (also kwon as elicitor) refers to any molecule or compound capable of activating defenses in the plant. Inducers can be substances of pathogenic/microbial origin, compounds released by the plant against pathogen attack or synthetic compounds (Oliveira *et al.*, 2016).

In agriculture there are several inducers that have been successfully used to control plant disease. A key characteristic of these inducers is that they are systemic or cause a systemic response. These inducers trigger the activation of the defense response not only in the plant part that has been treated but systemically on the whole plant. There are two types of systemic induced resistance that an inducer can trigger: systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Ab Rahman *et al.*, 2018). Currently, mycorrhiza-induced resistance (MIR) is considered other type of induced resistance, triggered by mycorrhizal fungi (Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018). In this work we will consider MIR as a variation of ISR since ISR and MIR are both triggered by beneficial microbes and similar defenses are activated in both (Zeilinger *et al.*, 2016).

Systemic Acquired Resistance (SAR)

SAR was first described by Ross as the phenomenon in which uninfected systemic plant parts develop resistance in response to a localized infection (Ross, 1961). It confers a broad-spectrum and long-term systemic resistance (Fu & Dong, 2013; Ab Rahman *et al.*, 2018; Klessig *et al.*, 2018). This mechanism produces mobile signals released from the site of infection causing localized necrosis and also they are translocated (apparently through the vasculature) to distal parts of the plant to protect against subsequent pathogen attack. In other words, SAR allows plant to acquire a systemic defense "memory" against upcoming infection, and this "memory" can last for weeks to months (Mauch-Mani *et al.*, 2017; Klessig *et al.*, 2018). SAR can be induced by both biotic (virulent and avirulent pathogens) and abiotic stresses, and is usually effective against biotrophic (which require living host tissue) and hemibiotrophic pathogens. This type of induced resistance is associated with the accumulation of SA in the uninfected tissues and the expression of PR proteins (Fig. 2) (Fu & Dong, 2013; Dempsey & Klesssig,

2017; Ab Rahman *et al.*, 2018; Klessig *et al.*, 2018). During SAR there are also cell wall modifications (e.g. lignin deposits) and production of phytoalexins, which are generated by healthy cells adjacent to necrotic cells (Oliveira *et al.*, 2016; Ab Rahman *et al.*, 2018). SA links to the transcription factor Non-expressor of Pathogenesis-related protein 1 (NPR1; also named Non-Inducible Immunity 1 [NIM1] or SA Insensitive 1 [SAI1]), which is the major regulator of SA and SAR and activate a large set of *PR* genes (Fu & Dong, 2013; Dempsey & Klessig, 2017; Klessig *et al.*, 2018). When SA-mediated defense is activated in infected plants, changes in the cellular redox status reduce cytosolic NPR1 (oligomers) to monomers that are transported to the nucleus and consequently activate defense-related genes such as *PR-1*. SA also induces the initiation of a MAPK cascade, histone modifications, and the DNA repair machinery (Fu & Dong, 2013; Dempsey & Klessig, 2017; Klessig *et al.*, 2018). Even though NPR1 is the central orchestrator of SAR activation, other paralogs of NPR1, NPR3 and NPR4, have been found to contribute to SAR activation. NPR3 and NPR4 are able to bind SA with different affinities and they contribute to NPR1 stability. It was thought that NPR3 and NPR4 were the interacting point between NPR1 and SA allowing NPR1 to respond to the different SA concentrations but recently, Ding *et al.* (2018) reported that NPR1 is also able to bind SA independently of NPR3/NPR4. Other SA-binding proteins (SABPs) have been identified, and show different affinities for SA, therefore certain immune responses are activated via SA-dependent pathway(s) independently form NPR1 (Dempsey & Klessig, 2017). Lately, Yuan *et al.*, (2017a) reported that SA binds to CATALASE2 to inhibit auxin (IAA) and jasmonic acid (JA) biosynthesis against biotrophs. Although SA is present in the phloem sap, it is not itself the systemic SAR signal (Vernooij *et al.*, 1994; Klessig *et al.*, 2018). Several candidates have been listed to putatively act as the long-distance SAR signal, namely methylated SA (methyl

salicylic acid -MeSA), the chaperone DIR1 (Defective in Induced Resistance), the nine carbon (C9) dicarboxylic acid azelaic acid (AzA), AzA insensitive (AZI1), glycerol 3-phosphate (G3P)-dependent factor, the diterpenoid dehydroabietinal (DA), the lysine derivative pipecolic acid (Pip), IAA, NO and ROS- free radicals and galactolipids (Gao *et al.*, 2015; Dempsey & Klessig, 2017; Klessig *et al.*, 2018).

On the other hand, SAR is characterized by the accumulation of PR proteins in distant uninfected tissues, which can protect the plant from a new pathogen attack (Oliveira *et al.*, 2016; Ab Rahman *et al.*, 2018). PR proteins may be acidic (generally located in the intercellular spaces) or basic (generally located in the vacuole), but they have similar functions (Van Loon & Van Strien, 1999). PR-proteins are categorized into 17 families according to their properties and functions, including PR-1, β-1,3-glucanases (PR-2), chitinases (PR-3, PR-4, PR-8, PR-11), thaumatin-like proteins (PR-5) and peroxidases (PR-9) (van Loon & van Strien, 1999). PR-1 function was recently reported by Gamir *et al.* (2017); PR-1 is capable to sequestrate sterols from pathogens due to its sterol-binding activity, therefore inhibiting their growth. β-1,3-glucanases and chitinases are two important hydrolytic enzymes that are expressed coordinately in order to degrade β-1,3-glucan- and chitin-fungal cell walls respectively (Van Loon &Van Strien, 1999; Oliveira *et al.*, 2016). Peroxidases are involved in many physiological processes; they participate in phytoalexin synthesis, mediate oxidative burst (ROS) and, in addition to laccases, catalize monolignol polymerization to form lignin and reinforce plant cell wall after contact with the pathogen (Lehmann *et al.*, 2015; Sorokan *et al.*, 2018). Apoplastic peroxidases are responsible of ROS production in the apoplast and lignin deposition (Sorokan *et al.*, 2018).

Induced systemic resistance (ISR)

ISR was first described in 1991 (Alström, 1991; Van Peer *et al.*, 1991; Wei *et al.*, 1991). This mechanism protects plants without causing necrosis in the infected tissues or eliciting plant defenses in spatially distant plant parts, but provides the plant a state of "alertness", called priming, before the potential pathogen attack (Mauch-Mani *et al.*, 2017). Priming allows the faster and stronger activation of defenses only if the pathogen is present. ISR is usually induced by root contact with plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) (including arbuscular mycorrhizal fungi -AMF-), but also by antibiotics, surfactants or chemicals (Pozo *et al.*, 2015; Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018). ISR is usually effective against necrotrophs (which kill host tissues), but also against herbivores as insects (Fig. 2) (Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018). During ISR several defenses are expressed after pathogen recognition, such as PR proteins (e.g. peroxidases), polyphenol oxidases (PPO), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD), proline, phenols, heat-shock proteins (HSPs) and LOXs (Lehmann *et al.*, 2015; Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018; Sorokan *et al.*, 2018). Similarly to SAR, it was shown that ISR also requires cytoplasmically localized NPR1 (Mauch-Mani *et al.*, 2017; Klessig *et al.*, 2018). ISR is jasmonic acid (JA)- and ethylene (ET)-dependent but other plant hormones as ABA may also play a role (Pozo *et al.*, 2015; Mauch-Mani *et al.*, 2017). Several genes are expressed during ISR, such as *MYC2*, R2R3-MYB-like transcription factor (*MYB72*) and Ethylene response factor 3 (*ERF3*), which are involved in JA (*MYC2*, *MYB72*) and ET (*ERF3*) signaling pathways respectively (Pozo *et al.*, 2015; Velivelli *et al.*, 2015; Martínez-Medina *et al.*, 2017).

An important part of ISR is priming. Priming has been firstly characterized in ISR but it has been later shown in SAR (Fig. 2). Priming is defined as an "alertness" status in

which plants respond in a faster and stronger way to the stress. This physiological status is characterized by low fitness costs, a memory of the stimuli, a more robust defense, and a better performance in the presence of the pathogen (Mauch-Mani *et al.*, 2017). Priming is linked to the resistance conferred by pathogens, PGPR and PGPF, and also by AMF, in addition to other natural (SA, JA, AzA, hexanoic acid -Hx-) and synthetic inducers (benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester -BTH-, its derivate Acibenzolar-S-methyl -ASM-, and 2.6-dichloroisonicotinic acid -INA-) (Oliveira *et al.*, 2016; Zine *et al.*, 2016; Mauch-Mani *et al.*, 2017; Ab Rahman *et al.*, 2018). Priming can be divided into three different phases. In the first phase (priming state), plants prepare its metabolism to respond more quickly to a given challenge (Mauch-Mani *et al.*, 2017). In the second phase (post-challenge primed state), there is a faster response induced against the pathogen (Mauch-Mani *et al.*, 2017). In the last phase (transgenerational primed state), the offspring of primed parental has a priming memory that allows them to respond more rapidly against the pathogen (Mauch-Mani *et al.*, 2017).

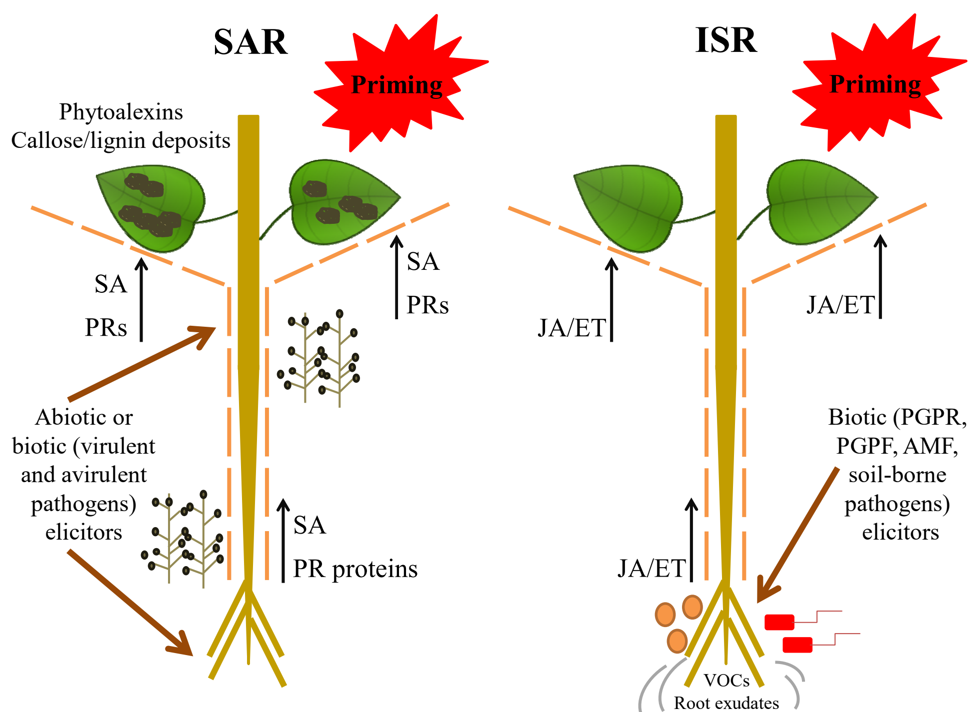

Figure 2. The two main types of induce resistance in plants. Systemic acquired resistance (SAR) is provoked by necrotizing pathogens which cause localized infection, but also by avirulent pathogens. Induced systemic resistance (ISR) is related to root colonization by plant growth-promoting rhizobacteria and fungi (PGPR and PGPF) and other non-pathogenic organisms. VOCs: volatile organic compounds. Adapted from Ab Rahman *et al.* (2018).

Verticillium dahliae Kleb.: the fungus and the disease

Verticillium is one of the earliest described genera of filamentous fungi and was established in 1817 by Nees von Esenbeck for *Verticillium tenerum*, now *Verticillium luteo-album* (Subramanian, 1971), and isolated from a stem of hollyhock (*Alcea rosea* L.) in Germany (Inderbitzin & Subbarao, 2014). The genus *Verticillium* belongs to the family *Plectosphaerellaceae* in the subclass Hypocreomycetidae of the class Sordariomycetes, which is in the phylum Ascomycota and order Hypocreales (Inderbitzin *et al.*, 2011; Inderbitzin & Subbarao, 2014).

Within this genus, 10 *Verticillium* species are distinguished (Inderbitzin *et al.*, 2011): *V. dahliae*, *V. albo-atrum*, *V. longisporum*, *V. tricorpus*, *V. theobromae*, *V. nubilum*, *V. nigrescens*, *V. alfalfa*, *V. nonalfalfae*, *V. zaregamsianum*, *V. isaacii* and *V. klebahnii*. The genus *Verticillium* was redefined with *V. dahliae* as the type species, so *Verticillium* species that belong to the same clade as *V. dahliae* are referred to as *Verticillium sensu stricto* (s.s.), which excludes *V. theobromae* (Inderbitzin & Subbarao, 2014).

The reproduction of *Verticillium* spp. appears to be strictly asexual; there was not found any sexual state, being all species potentially heterothallic (Inderbitzin *et al.*, 2011; Inderbitzin & Subbarao, 2014). *Verticillium* spp. produce survival structures called microsclerotia (black melanised clumps that are formed by budding of mycelial cells) which generally germinate and form hyphae. *Verticillium* hyphae grow toward roots and

penetrate root epidermis through the hyphopodium (Zhao *et al.*, 2016). Hyphae grow between epidermal cells and colonize xylem vessels due to fungal secretion of effector proteins (Klimes *et al.*, 2015; Zhou *et al.*, 2017). Once the fungus colonizes plant roots, hyphae grow upstream and conidia and survival structures (microsclerotia) are produced in the vascular system, thus impeding water and nutrient transport across the plant (Klimes *et al.*, 2015).

Among *Verticillium* spp., *V. dahliae*, *V. albo-atrum* and *V. longisporum* cause the most important agronomic diseases, and are the three causal agents of the so-called "Verticillium wilt" (Luo *et al.*, 2014). The remaining *Verticillium* species have narrower host ranges and restricted global distributions that cause diseases less important economically (Inderbitzin *et al.*, 2011; Inderbitzin & Subbarao, 2014). For example, *V. nubilum* only causes disease on tomato and potato (Inderbitzin *et al.*, 2011; Inderbitzin & Subbarao, 2014).

Verticillium dahliae Kleb. is an asexual, vascular-colonizing and soil-borne Deuteromycete fungus (Inderbitzin *et al.*, 2011). This phytopathogen has a broad host range, being the pathogen with more hosts among *Verticillium* spp. Approximately, 300 to 400 plant species are susceptible to this pathogen (Table 1), causing billions of dollars in annual crop losses. Herbaceous annuals and woody perennial hosts including crops, flowers, and vegetables are infected by *V. dahliae* under several environmental conditions (greenhouses, open field) and at any growth stage (Goldberg, 2003; Fradin & Thomma, 2006; Inderbitzin *et al.,* 2011; Inderbitzin & Subbarao, 2014; Keykhasaber *et al.*, 2018).

Table 1. Some horticultural crops attacked by *Verticillium dahliae*.

Family	Scientific name	Common name
SOLANACEAE	*Capsicum annuum*	Pepper
SOLANACEAE	*Solanum lycopersicum*	Tomato
SOLANACEAE	*Solanum melongena*	Eggplant
SOLANACEAE	*Solanum tuberosum*	Potato
BRASSICACEAE	*Brassica oleracea*	Cabbage
BRASSICACEAE	*Brassica oleracea* var. *italic*	Broccoli
BRASSICACEAE	*Brassica oleracea* var. *botrytis*	Cauliflower
ROSACEAE	*Prunus persica*	Peach
ROSACEAE	*Fragaria* x *ananassa*	Strawberry
ASTERACEAE	*Lactuca sativa*	Lettuce
ASTERACEAE	*Cynara scolymus* var. *scolymus*	Artichoke
VITACEAE	*Vitis vinifera*	Grapevine
OLEACEAE	*Olea europaea*	Olive tree
AMARANTHACEAE	*Spinacia oleracea*	Spinach
CUCURBITACEAE	*Cucurbita pepo*	Pumpkin
CUCURBITACEAE	*Citrullus lanatus*	Watermelon

V. dahliae is able to infect and colonize plants without triggering symptoms. In this way, *Verticillium* inoculum remains dormant and can initiate epidemics of Verticillium wilt disease (Keykhasaber *et al.*, 2018). Thus, *V. dahliae* is considered a hemibiotroph with a biotrophic phase within root xylem without a visible disease phenotype and a necrotrophic phase in the aerial parts of the plant (Sun *et al.*, 2014). *V. dahliae* was also considered as necrotroph due to its appressoria, which are used to infect plants (Zeilinger *et al.*, 2016). There are controversies about *V. dahliae* life style, and authors have not reached a consensus yet. When symptoms caused by *V. dahliae* occur (commonly known as "wilt disease symptoms"), these consist of leaf loss of turgor, abscission and epinasty, foliar chlorosis and necrosis, stunting, vascular discoloration and, in extreme situations, plant death (Fig. 3) (Goldberg, 2003; Fradin & Thomma,

2006; Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). The pathogen colonizes water-conducting tissues within a plant resulting in wilting and tissue necrosis at the end of the disease cycle (Fradin & Thomma, 2006; Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). Because symptoms can vary among hosts, there are no unique symptoms that belong to all plants infected by this fungus, and true wilt does not always occur as a consequence of *Verticillium* infection (Fradin & Thomma, 2006). *V. dahliae* is a systemic pathogen, so symptoms can affect all parts of the plant. Generally, symptoms in the aerial part start in the lower leaves and spread acropetally and can affect the entire plant or be confined to one side (sectoring) (Fradin & Thomma, 2006; Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018).

Figure 3. Symptoms caused by *Verticillium dahliae* in pepper (*Capsicum annuum* L.) observed in the laboratory (A) and in the field (B). García (2018).

V. dahliae causes a monocyclic disease, which means that only one cycle of disease and inoculum production takes place during a growing season (Fradin & Thomma, 2006). The disease is called vascular wilt, and occurs as a result of the presence and activity of the pathogen in the xylem tissues of the plant (Yadeta & Thomma, 2013; Klimes *et al.*,

2015; Keykhasaber *et al.*, 2018). *V. dahliae* infection consists of two phases (Fig. 4). In the first phase (Phase I; asexual development), *V. dahliae* microsclerotia (spreading structures) germinate as a response to root exudates and the fungus enters the plant root through epidermis or wounds to reach immature xylem elements (Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). After crossing the endodermis, the second phase (Phase II; disease cycle) begins, in which hyphal proliferation and conidia production (budding) occur; conidia germinate and colonize the vascular elements and the upstream vessels through the sap stream and they are trapped in pit cavities or at vessel end walls (trapping sites) (Yadeta & Thoma, 2013; Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). When foliar senescence takes place, *V. dahliae* enters a saprophytic stage and colonizes the surrounding nonvascular tissues in shoots and roots, so that new microsclerotia are produced in the dying stems and leaves and will constitute a new source of inoculum in the soil (Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). While the host plant is alive, *V. dahliae* usually colonizes only host xylem vessels, and the hyphae compete with plant tissues for nutrients. When the host dies, the fungus colonizes senescent tissues (Klimes *et al.*, 2015; Keykhasaber *et al.*, 2018). Microsclerotia are the main inoculum in the field, where they can survive for 10-15 years even in the absence of a host (Wilhelm, 1955). Generally, the density of microsclerotia in the soil is host-dependent and is proportional to the incidence of the disease (Carroll *et al.*, 2017). Thus, *V. dahliae* is able to remain in a dormant phase in the soil as microsclerotia (Keykhasaber *et al.*, 2018).

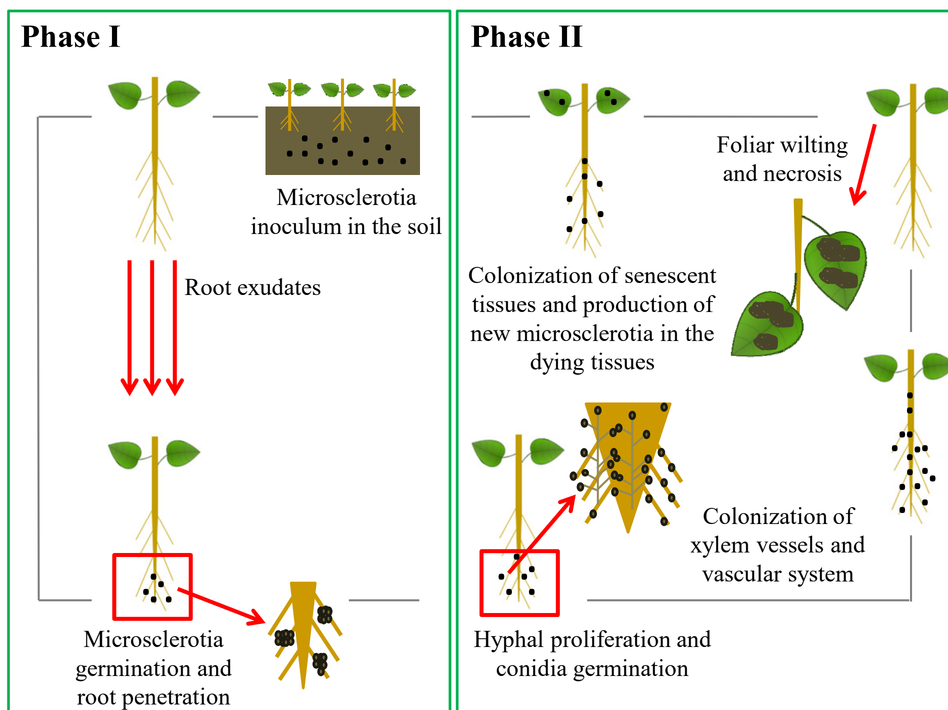

Figure 4. *Verticillium dahliae* disease cycle. Adapted from Klimes *et al.* (2015).

Different strategies have been used to reduce the disease caused by *V. dahliae*, and some of them are more effective than others. Currently it is claimed the necessity to find an effective control strategy of this pathogen without harming the environment and human health, a strategy economically viable, and easy to apply for farmers.

Current control strategies for Verticillium wilt

Plant disease control should allow a high crop production with a low impact on the environment and human health. Integrated Pest Management (IPM) strategies attempt to reduce the use of chemical products in modern agriculture and horticulture through biological control methods and the control of plant defense mechanisms. Moreover, IPM strategies look for achieve an adequate level of disease control and reduce the use of phytosanitary products taking into account the environmental impact and the legal and social implications of such control methods.

Control of the soil-borne disease caused by *V. dahliae* is difficult for different reasons. The disease cannot be treated once crops are affected due to the inaccessibility of *V. dahliae* once it reaches the vascular plant tissue, and the long persistence of its microsclerotia in the soil (Fradin & Thomma, 2006; Yadeta & Thomma, 2013; Daayf, 2015; Carroll *et al.*, 2017). Moreover, this fungus has a wide host range, so there is no single effective treatment for all affected crops by this pathogen. In addition, *V. dahliae* can be introduced in the soil via infested seeds and/or spread locally from field to field by harvesting crews or equipment (Daayf, 2015; Carroll *et al.*, 2017). Therefore, it is necessary to raise awareness among farmers about the importance of cleaning equipment and tractors before entering a new field to prevent the spread of soil-borne pathogens such as *V. dahliae*.

Plant diseases can be controlled through chemical, mechanical and biological ways, but the use of pesticides is the most widespread practice. Thus, soil fumigation with fungicides is used to reduce the inoculum of *V. dahliae* in the soil, but its application is restricted due to the environmental and ecological risks, as well as the appearance of resistances to the fungicides used (Daayf, 2015; Carroll *et al.*, 2017). Methyl bromide was the main soil fumigant to treat Verticillium wilt, but its use was banned under the Montreal Protocol on Substances that Deplete the Ozone Layer in 2005 due to environmental concerns (Carroll *et al.*, 2017).

Currently, control of Verticillium wilt is focused on different IPM strategies, including crop rotation (Carroll *et al.*, 2017). This practice is generally of limited use for Verticillium wilt management due to the broad host range and the long-term persistence of *V. dahliae* microsclerotia. Biofumigation (use of biologically active plant substances), soil solarization (mulching the soil and covering it with tarp to trap solar energy), soil steam sterilization, flooding, anaerobic soil disinfestation (anaerobic decomposition of

organic soil amendments), use of fertilizers consisting of growing plants that are ploughed back into the soil (green manures), organic amendments or crops grown for the protection and enrichment of the soil (cover crops) are also common IPM strategies to control soil-borne pathogens (Daayf, 2015). For example, flooding (Sanogo *et al.*, 2008) and anaerobic soil disinfestation (Butler *et al.*, 2009) proved to be effective in suppressing *V. dahliae* in pepper. However, all these environmentally friendly approaches have their specific drawbacks and limitations, both economic and practical (expensive equipment, high temperature and water requirements, site-specific variability) (Daayf, 2015; Carroll *et al.*, 2017).

Grafting on resistant rootstocks is commonly used to protect plants against soil-borne pathogens. However, this technique is not always effective in controlling Verticillium wilt (Morra & Bilotto, 2006; Johnson *et al.*, 2014). It has been shown that grafting can trigger the emergence of new races of the pathogen and changes its population (Colla *et al.*, 2012). Genetically resistant varieties and development of transgenic varieties also play an important role in solving the Verticillium wilt problem (Vallad *et al.*, 2006; Daayf, 2015). In resistant tomato varieties, resistance against Verticillium wilt caused by race 1 is mediated by the *Ve* locus, which comprises *Ve1* and *Ve2* genes; however, no resistant varieties against race 2 have been found (Vallad *et al.*, 2006). In *C. annuum* there is an important number of genes conferring resistance against various pathogens (Wang & Bosland, 2006); nevertheless, to our knowledge, no resistance genes to *V. dahliae* have been found in *C. annuum*. However, Barchenger *et al.* (2017) recently found an allele-specific cleaved amplified polymorphic sequence (CAPS) in a *Ve1* homolog of *C. annuum*; this probably will help in the future to improve Verticillium wilt resistance in pepper.

The exogenous application of natural compounds that activate plant defenses and, therefore, induce resistance such as DL-3-aminobutyric acid (3-ABA), MeJA (Li *et al.*, 1996) or ASM (Zine *et al.*, 2016) has been used successfully to control Verticillium wilt. On the other hand, biocontrol makes use of BCAs (living organisms that directly or indirectly control other ones), which are mainly bacteria (i.e., actinomycetes, PGPR) and fungi (i.e., entomopathogenic fungi, root endophytic fungi, PGPF, AMF, ectomycorrhizas), to reduce the severity and incidence of several diseases in many fruits and vegetables of economic importance (Garmendia *et al.*, 2004; Elsharkawy *et al.*, 2012; Martínez-Beringola *et al.*, 2013; Maketon *et al.*, 2014; Chemeltorit *et al.*, 2017; Lozano-Tovar *et al.*, 2017; Zhang *et al.*, 2018). The BCAs were regulated at the European level by the European Regulation 1107/2009 and the European Directive 2009/128/EC where the use of pesticides was reduced to reach a sustainable level by combining it with biocontrol and other new control techniques (Colla *et al.*, 2012). Biocontrol can take place through different mechanisms: antibiosis, (hyper/myco)parasitism, biofilm formation, competition for space and nutrients, prevention of tissue colonization by the phytopathogen and induced resistance (Deketelaere *et al.*, 2017; Ghorbanpour *et al.*, 2018). All these mechanisms of biocontrol can act alone or in combination.

Biological control agents (BCAs)

Currently, there is a global trend to increase the use of BCAs in crop protection because of their easy accessibility and large-scale propagation, without negative repercussions on the environment or human health.

Several microorganisms have been reported as BCAs against *V. dahliae* (Deketelaere *et al.*, 2017), and they offer an eco-friendly alternative to chemical synthetized fungicides.

Among these biocontrol organisms there are several fungi (Larena *et al.*, 2003[a]; Veloso & Díaz, 2012; Sun *et al.*, 2014; Demir *et al.*, 2015; Carrero-Carrón *et al.*, 2016; Lozano-Tovar *et al.*, 2017; Zhang *et al.*, 2018), bacteria (Tjamos *et al.*, 2004; Yang *et al.*, 2014; Gómez-Lama Cabanás *et al.*, 2017; Yuan *et al.*, 2017b; Zhang *et al.*, 2018) and oomycetes (Al-Rawahi *et al.*, 1998; Rekanovic *et al.*, 2007). The mechanisms of action of these BCAs against *V. dahliae* include antibiosis (Tjamos *et al.*, 2004; Carrero-Carrón *et al.*, 2016; Lozano-Tovar *et al.*, 2017), competition for nutrients or space (Veloso *et al.*, 2015; Lozano-Tovar *et al.*, 2017), parasitism (Al-Rawahi *et al.*, 1998; Tjamos *et al.*, 2004; Ruano-Rosa *et al.*, 2016), and induced resistance (Garmendia *et al.*, 2004; Veloso & Díaz, 2012; Gómez-Lama Cabanás *et al.*, 2017; Yuan *et al.*, 2017b).

Biological control refers to any direct or indirect action by the BCA that lead to a reduction in the disease (Cook & Baker, 1983). In this way, BCAs can directly act on the pathogen, or indirectly on the host plant. Direct interactions with the pathogen include many of the mechanism of action mentioned above, namely antibiosis, competition for nutrients and space and parasitism. These direct processes are found in non-pathogenic species of plant pathogens or antagonistic living organisms occupying the same ecological niche as the pathogen (Dutta, 2015). Many BCAs belong to these non-pathogenic antagonistic organisms.

An ideal BCA should be able to survive in the environment and colonize different organic substrates with high cell viability during a long period of time, produce large amounts of inoculum in the fastest way possible, and germinate and grow faster than the pathogen (Vázquez *et al.*, 2013; Larena *et al.*, 2014). The BCAs should be ecologically competitive, act against a wide spectrum of diseases, persist in soil after its application with a similar population size to that of the native population, and do not imply a risk for the other non-target microorganisms present in the ecosystem, in addition to do not

negatively influence human and animal health or the environment (Vázquez *et al.*, 2013; Larena *et al.*, 2014). Moreover, the BCA has to be easily prepared, transported and applied, stable at room temperature, and economically profitable for farmers (Vázquez *et al.*, 2013; Larena *et al.*, 2014; Yang *et al.*, 2014). Thus, a dry product is more suitable to achieve all these features, and it has also less weight to ship and diminishes the risk of contamination (Larena *et al.*, 2003a).

BCAs can be mass produced for agriculture in an economically profitable and ecologically convenient way by liquid or solid fermentation (Janisiewicz & Jeffers, 1997; Rojan *et al.*, 2011). Solid fermentation is mainly used for fungal BCA production, and depends on several factors such as moisture content and water activity, pH, temperature, substrate, particle size, and aeration (Janisiewicz & Jeffers, 1997; Rojan *et al.*, 2011). The BCA formulation also needs to preserve a similar biocontrol activity as the fresh microorganism against the pathogen during storage and transportation, and work on a wide host range and different cultivars (Janisiewicz & Jeffers, 1997; Yang *et al.*, 2014). To maintain the stability of the BCA, it is also necessary to take into account the contamination by other microorganisms that could modify the pH or produce enzymes that damage the BCA. The additives used in the formulation process cannot have biological activity or affect the activity of the BCA, be harmless to the environment, and should mix efficiently with the BCA. The use of additives can improve plant-BCA interaction, BCA colonization, and the capacity of adaptation of the BCA (Guijarro *et al.*, 2008; Vázquez *et al.*, 2013).

The European Regulation 546/2011 for the evaluation and authorization of phytosanitary products containing microorganisms demands the development of methods for BCA detection and quantification in the field in the presence of the native population (Larena & Melgarejo, 2009), since introduced biocontrol organisms with a

high fitness may have broad consequences in the ecosystem when the displaced microbiota are involved in the cycling of nutrients (Gullino *et al.*, 1995). These methods should be selective at strain level, since many BCAs belong to species that commonly inhabit plants and soils (Larena & Melgarejo, 2009; Vázquez *et al.*, 2013; Larena *et al.*, 2014). BCA detection by qPCR and their combination with a semi-selective medium allows to quantify BCA growth and viability, and monitor the biopesticide once it has been applied (Larena & Melgarejo, 2009; Vázquez *et al.*, 2013; Larena *et al.*, 2014). Denaturing Gradient Gel Elecrophoresis (DGGE) is also an economically profitable technique to analyze changes in soil microbial communities in a sample (either soil or plant material) over time (Cleary *et al.*, 2012).

Generally, BCAs have more than one mechanism of action; the combination of various BCA strains with different ecological requirements and/or modes of action can improve biological control effectiveness in variable environmental conditions and reduces the risk of the development of resistance by the pathogen (Olivain *et al.*, 2004; Velivelli *et al.*, 2015; Chemeltorit *et al.*, 2017; Zhang *et al.*, 2018). Moreover, a single BCA probably will not be active in all soil environments in which it is applied or against all pathogens that attack the host plant and, in addition, may not have the same efficacy against all the pathotypes present in the population (Olivain *et al.*, 2004; Chemeltorit *et al.*, 2017; Zhang *et al.*, 2018). The BCA effectiveness also depends on pathogen virulence and disease severity, which are influenced by the climatic conditions; sometimes the most favorable environmental and/or climatic conditions for the development of the pathogen may not be the same as those required for the effective action of the BCA (Vázquez *et al.*, 2013; Larena *et al.*, 2014).

Plant and soil microbiota present where the pathogen is located can be a source of potential BCAs, since they are adapted to the same environmental conditions and can

compete for nutrients and/or occupy secondary lesions and displace the pathogen. BCAs are first selected and isolated according to their antibiotic production and root colonization abilities (Ruano-Rosa *et al.*, 2016; Gómez-Lama Cabanás*et al.*, 2017; Lozano-Tovar *et al.*, 2017). The study of BCA persistence in the roots helps to know the optimal number of BCA applications and also the proper time of application.

The introduction of antagonistic microorganisms into the rhizosphere or in any part of a given ecosystem can be carried out by applying high amounts of the BCA(s) by spraying or irrigation, or by applying low amounts of the BCA(s) by immersion of the roots, multiplying and persisting throughout all or part of the plant life cycle (Angelopoulou *et al.*, 2014; Ruano-Rosa *et al.*, 2016; Gómez-Lama Cabanás *et al.*, 2017). BCAs can also be applied directly to the seeds, watering on soil, coating wounds, and transplant soil plugs (Angelopoulou *et al.*, 2014). When the BCA is applied to the seeds, the plant can sustain the BCA's growth thanks to the organic compounds released in root exudates (Chemeltorit *et al.*, 2017). On the other hand, the application of BCAs to transplant soil plugs allows to develop stable populations in the seedling rhizosphere that would then persist in the field and help young plants to resist pathogen attack after transplantation in the field. Moreover, transplant soil plugs would be easier in practice and less expensive and time-consuming for crops that are initially grown in the greenhouse or nursery and then transplanted in the field for protection against soil-borne pathogens such as *V. dahliae* (Angelopoulou *et al.*, 2014).

The process of commercialization of biological products can be a disadvantage for biological control development, due to the high patent and registration costs of new products, especially in EU. The US Environmental Protection Agency (EPA) created the "Biopesticide Pollution and Prevention Division" (BPPD) to accelerate this process and reduce the costs involved. Most of the biological products currently registered are fungi-

and bacteria-based (Calvo *et al.*, 2017). Currently, the vast majority of products commercially available in Spain are bioinsecticides, and the registered biofungicides are mainly used to control diseases caused by soil- and air-borne pathogens, and a few post-harvest diseases (https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/fitos.asp; Table 2); only two of them are used to combat the diseases caused by *Verticillium* spp. (Table 2).

Table 2. Biofungicides currently registered in Spain.

Biofungicide	Organism(s)-based	Disease(s) controlled
Bioten®	*Trichoderma asperellum* ICC012 + *Trichoderma gamsii* ICC080	*Phytophthora* spp., *Verticillium* spp., *Sclerotinia* spp.
Polyversum	*Pythium oligandrum* M1	*Botrytis cinerea*, *Sclerotinia* spp., *Podosphaera fusca*
AQ-10	*Ampelomyces quisqualis*	Powdery mildew
Blossom protect	*Aurebasidium pullulans* DSM 14940 + *Aurebasidium pullulans* DSM 14941	*Botrytis cinerea*, *Penicillium* spp., *Pezicula* spp.
Botector	*Aurebasidium pullulans* DSM 14940 + *Aurebasidium pullulans* DSM 14941	*Botrytis cinerea*
Serenade AS	*Bacillus subtilis*	*Botrytis cinerea*
Serenade Max	*Bacillus subtilis* QST 713	Anthracnose, Powdery mildew, *Botrytis cinerea*, *Monilinia* spp., *Xanthomonas* spp., *Sclerotinia* spp., *Pseudocercospora ceratoniae*
Serenade Aso	*Bacillus subtilis* QST 713	*Alternaria* spp., *Botrytis cinerea*, *Sclerotinia* spp., Powdery mildew
Contans WG	*Coniothyrium minitans*	*Sclerotinia* spp.
Prestop	*Gliocladium catenulatum* J1446	*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani*, *Botrytis cinerea*, *Didymella bryoniae*

Cedomon Cerall	*Pseudomonas chlororaphis*	Seed-borne fungal diseases
Mycostop	*Streptomyces greseoviridis*	*Fusarium* spp.
T34 Biocontrol	*Trichoderma asperellum*	*Pythium aphanidermatum*
Tusal	*Trichoderma harzianum* T25 + *Trichoderma atroviride* T11	*Sclerotinia* spp., *Phytophthora* spp., *Fusarium* spp., *Pythium* spp., *Rizoctonia solani*
Trianum G Trianum P	*Trichoderma harzianum* Rifai T-22	*Pythium* spp.
Blindar Remedier	*Trichoderma asperellum* ICC012 + *Trichoderma gamsii* ICC080	*Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Pythium* spp., *Phytophthora* spp., *Armillaria mellea*, *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp.
Amylo-X WG	*Bacillus amyloliquefaciens* subesp. *plantarum* D747	*Botrytis cinerea*, Powdery mildew, *Sclerotinia* spp., *Monilinia* spp., *Stemphylium* spp.
Proradix	*Pseudomonas* sp. DMZ 13134	*Rhizoctonia solani*, *Helminthosporium solani*
Vintec	*Trichoderma atroviride* SC1	*Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Eutypa lata*

Fungi (both pathogenic and non-pathogenic) are the most active organisms in the rhizosphere. Because of their characteristics, fungi are potential BCAs; they antagonize most plant pathogens, insects and weeds; they are easily grown in culture media, produced in large quantities and released into the environment as spores or mycelia without causing any damage to other microorganisms; they are able to survive for long periods of time as resting structures and germinate when the conditions are appropriate;

their biologically active compounds are effective at low concentrations and not persistent, causing no harms to the environment, so they can be used directly or indirectly through their substances, or to design analogue substances equally effective (Vázquez *et al.*, 2013; Villarino *et al.*, 2016).

This thesis is mainly focused on the tripartite interaction *Capsicum annuum*-V*erticillium dahliae-Penicillium rubens* 212 (PO212), so in the following subsection we summarized different aspects of the fungal genus *Penicillium* and its use in plant protection.

The genus *Penicillium*

Penicillium is a diverse genus of opportunistic and filamentous fungi occurring worldwide and growing in various substrates, from soil to food (Visagie *et al.*, 2014). According to Pitt (1980), *Penicillium* genus belongs to the Fungi kingdom (true fungi), Ascomycota phyle, Euascomycetes class, Eurotiales order, *Trichocomaceae* family. However, now it is considered that this genus belongs phylogenetically to the *Aspergillaceae* family in the Trichocomaceae clade (Visagie *et al.*, 2014).

More than 1000 *Penicillium* species have been described based on morphological characters; however, many of them are not used today because descriptions were incomplete according to modern criteria, and some species are now considered to be synonyms of others. Currently, 354 *Penicillium* species are accepted (Visagie *et al.*, 2014).

Most *Penicillium* species are saprophytic. However, *P. digitatum*, *P. italicum* and *P. expansum* are well-known pathogens with necrotrophic lifestyles that cause important damages and yield losses in post-harvest fruits (Pimenta *et al.*, 2008; Calvo *et al.*, 2017). There are also common food spoilers in *Penicillium* genus, *P. biforme*, *P.*

fuscoglaucum, *P. carneum* and *P. paneum* (Ropars *et al.*, 2016). Other *Penicillium* spp. are used in industry for food production, such as *P. roqueforti* and *P. camembertii*, which have been used to make cheese (Leistner, 1990), and *P. nalgiovense*, which is used for fermenting sausages (Bernáldez *et al.*, 2013). *P. decumbens*, meanwhile, is used for biorefinery (Ropars *et al.*, 2016).

Other commonly occurring mould in indoor environments is *P. rubens*, the currently producer of the β-lactam antibiotic penicillin, which is known for its antimicrobial properties (Domínguez-Santos *et al.*, 2017). The original producer of penicillin was *P. notatum*, which was discovered by Fleming in 1929 (Houbraken *et al.*, 2011). *P. rubens* was considered as a synonym of *P. chrysogenum*; however, now they are closely related but separated species (Houbraken *et al.*, 2011).

Several *Penicillium* sp. strains have demonstrated to control different plant pathogens, mainly fungi (Table 3), but also oomycetes, bacteria, virus, nematodes and insects (Larena *et al.*, 2003ab; Thuerig *et al.*, 2006; Tamm *et al.*, 2011; Elsharkawy *et al.*, 2012; Martínez-Beringola *et al.*, 2013; Maketon *et al.*, 2014).

Table 3. Control of fungal pathogens by different *Penicillium* strains in various plant species.

***Penicillium* strain**	**Plant species**	**Pathogen (s)**	**Reference**
P. citrinum	Chickpea (*Cicer arietinum*)	*Botrytis cinerea*	Sreevidya *et al.*, 2015
P. oxalicum BZH-2002	Cucumber (*Cucumis* sativus)	*Cladosporium cucumerinum*	Peng *et al.*, 2004
P. chrysogenum	*Arabidopsis thaliana*	*Botrytis cinerea* *Alternaria brassicicola*	Thuerig *et al.*, 2006
P. chrysogenum	*Grapevine (Vitis vinífera)*	*Uncinula necator*	Tamm *et al.*, 2011
P. chrysogenum	Orchard Apple (*Malus domestica*)	*Venturia inaequalis*	Tamm *et al.*, 2011
P. sp. NICS01	Sesame (*Sesamum indicum*)	*Fusarium* spp.	Radhakrishnan *et al.*, 2013
P. sp.	Chestnut (*Castanea* sp.)	Chestnut blight (*Cryphonectria*	Akilli *et al.*, 2011

		parasitica)	
P. purpurogenum	Tomato	*Fusarium oxysporum* f. sp. *lycopersici*	Larena & Melgarejo, 1996
P. rubens 212 (PO212)	Melon (*Cucumis melo*) Watermelon (*Citrullus lanatus*)	*Fusarium oxysporum* f. sp. *melonis* *Fusarium oxysporum* f. sp. *niveum*	De Cal *et al.*, 2009
P. rubens 212 (PO212)	Tomato	*Fusarium oxysporum* f. sp. *lycopersici* *Verticillium dahliae* *Verticillium albo-atrum* *Botrytis cinerea*	Larena *et al.*, 2003ab; Sabuquillo *et al.*, 2005
P. rubens 212 (PO212)	Strawberry (*Fragaria* x *anannasa*)	*Powdery mildew (Podosphaera aphanis)*	De Cal *et al.*, 2008
P. frequentans	Peach (*Prunus persica*)	*Monilinia laxa*	Guijarro *et al.*, 2008
P. frequentans	Sugar beet (*Beta vulgaris*)	*Cercospora beticola*	El-Fawy *et al.*, 2018
P. sp.GP15-1	Cucumber	*Rhizoctonia solani* *Colletotrichum orbiculare*	Hossain *et al.*, 2014
P. purpurogenum	Peach	*Monilinia laxa* *Fusarium oxysporum* f. sp. *lycopersici*	Larena & Melgarejo, 1996
P. citrinum BTF08	Banana (*Musa* sp.)	*Fusarium oxysporum* f. sp. *cubense*	Ting *et al.*, 2012
P. simplicissimum CEF-818	Cotton (*Gossypium hirsutum*)	*Verticillium dahliae* Vd080	Yuan *et al.*, 2017b
P. goetzii	*Pinus ponderosa*	*Dothistroma septosporum*	Ridout & Newcombe, 2016
P. digitatum	Melon Watermelon	*Macrophomina phaseolina*	Boughalleb-M´Hamdi *et al.*, 2018
P. chrysogenum	*Pinus radiate*	*Fusarium circinatum*	Romón *et al.*, 2008
P. oxalicum	Potato (*Solanum tuberosum*)	*Verticillium dahliae* *Colletotrichum coccodes*	Farber *et al.*, 2018
P. chrysogenum *P. polonicum*	Potato	*Fusarium sambucinuma* *Fusarium solani*	Mejdoub-Trabelsi*et al.*, 2017
Penicillium sp.MNT8	Eggplant (*Solanum melongena*)	*Verticillium dahliae*	Narisawa *et al.*, 2002

To our knowledge, no *Penicillium* strain has been used to induce resistance in pepper against Verticillium wilt. *Penicillium* will be tested in this thesis as a potencial BCA to control Verticillium wilt in pepper cv. Padron. In the following section we briefly

remark the main characteristics of pepper cv. Padron and studies of induced resistance against *V. dahliae* in pepper.

Capsicum annuum L.

Pepper (*Capsicum annuum* L.) is one of the main annual crops worldwide, especially in Spain, were pepper yield and production increased in the last years, with a yield of 652622 hg/ha and a production of 1283546 tonnes in 2017 (FAOSTAT, 2019). The geographical origin of pepper is located in Peru and Bolivia, and later it was extended to the rest of America and domesticated in South and Central America (Perry *et al.*, 2007). It belongs to the *Solanaceae* family and the *Capsicum* genus. *Capsicum* spp. is categorized into 40 species (García *et al.*, 2016), being some of them cultivated species (*C. frutescens*, *C. baccatum*, *C. pubescens* and *C. chinense*), but *C. annuum* is the species of greatest economic importance.

This herbaceous plant can be consumed fresh as a vegetable accompaniment, or in dry powdered form as a condiment or coloring, and can be grown as an annual or as perennial crop in the field or in greenhouses (Srinivasan, 2016). Peppers are a good source of antioxidants, and contain carotenoids (provitamin A), ascorbic acid (vitamin C), tocopherols (vitamin E), flavonoids, phenolic compounds, derivatives of cinnamic acid and capsaicinoids, being all of them health-related metabolites (Topuz & Ozdemir, 2007; Aza-González *et al.*, 2011).

Fruit peppers differ in composition and level of their metabolites (Topuz & Ozdemir, 2007). For example, hot pepper varieties contain capsaicinoids. These alkaloids are responsible for the spicy taste (pungency) of this type of peppers, and the amount of capsaicinoids in a fruit depends on the genotype, the developmental stage and the growth conditions (Lee *et al.*, 2005; Aza-González *et al.*, 2011). Moreover,

capsaicinoids have antimicrobial, anticancer, antiarthritic, anti-inflammatory and anti-obesity/diabetic properties, reduce the pain caused by several discomforts (headaches, neck pain, mucositis), and are part of multiple products available on the market (hair-loss-prevention shampoos, self-protection aerosol sprays, pain relieving creams and oils) (Veloso *et al.*, 2014; Srinivasan, 2016). The most common capsaicinoid is capsaicin, followed by dihydrocapsaicin (Veloso *et al.*, 2014).

“Padrón” peppers (*Capsicum annuum* L. var. *annuum* cv. Padron) is a pepper ecotype originally cultivated in “Padrón” region (Galicia, N.W. Spain). They are well known by their content of capsaicinoids, which give them their characteristic hot flavor (although not all of its fruits are spicy at the commercial stage). This Galician ecotype of pepper was included into the Protected Designation of Origin and the Protected Geographical Indication for agricultural products provided by the EU Regulation No 1151/2012, and designated as “Herbón” PDO (Protected Designation of Origin). Some of the other Galician ecotypes of pepper, “Couto”, “Mougán”, “Oímbra” and “Arnoia” got the recognition of PGI (Protected Geographical Indication) (Casal, 2010; Taboada *et al.*, 2010).

The climate of Galicia differs from that of central and southern Spain, and the techniques of cultivation are different, so the content of various metabolites of *C. annuum* L. var. *annuum* cv. Padron may vary. The fruit is a berry and is commercialized when immature and eaten by frying it in oil (López-Hernández *et al.*, 1996). It is much richer than sweet peppers in fibers (cellulose, hemicellulose, lignin, cutin), monosaccharides (D-glucose, starch, D-fructose, pectin) (López-Hernández *et al.*, 1996), vitamin C, organic acids (malic acid, quinic acid, oxalic acid, citric acid, fumaric acid), pigments (chlorophyll a, chlorophyll b) and carotenoids (lutein, β-carotene).

There is a lot of literature showing that Verticillium wilt can be greatly reduced in pepper using induced resistance, especially if this mechanism is initiated in the roots, since *V. dahliae* primarily colonizes these tissues (Deketelaere *et al.*, 2017). Heavy metals such as copper (Chmielowska *et al.*, 2010) have been proved to induce resistance in pepper against *V. dahliae*. Organic materials such as whey and humic acid (Demir *et al.,* 2015), and organic amendments (García-Mina *et al.*, 1996; Goicoechea *et al.*, 2004; Pascual *et al.*, 2009) have been used to induce resistance in pepper against *V. dahliae*. Veloso *et al.* (2014) showed that capsaicin and N-vanillylnonanamide, two capsaicinoids, were effective in controlling Verticillium wilt through induced resistance. However, most studies in which induced resistance has been used to protect pepper against *V. dahliae* have been focused on using BCAs, especially bacteria (e.g. *Streptomyces*, *Bacillus*; Turhan, 1981; Sanogo *et al.*, 2017) and fungi (e.g. AMF, non-pathogenic *Fusarium* strains, *Trichoderma*; Garmendia *et al.*, 2004; Díaz *et al.*, 2005; Ślusarski & Pietr, 2009; Veloso & Díaz 2012; Demir *et al.*, 2015), or fungal-like organisms (*Pythium oligandrum*; Al-Rawahi & Hancock, 1998; Rekanovic *et al.*, 2007).

In this work, a biological approach is used to reduce the symptoms of Verticillium wilt in pepper plants. In Chapter 1, the aim was to test if *Penicillium rubens* (formerly *Penicillium oxalicum*) 212 (PO212) was able to protect pepper plants against Verticillium wilt. The protection ability of PO212 was studied through the quantification of symptoms as well as the biomass of the pathogen, the expression of several pepper defense related genes, the activity of certain enzymes, and the study of lignin deposition. In Chapter 2, pepper cell cultures were elicited with PO212 and several defense parameters were studied: gene expression, enzymatic activities, phenolic compounds, lignin, and H_2O_2 production after *Verticillium dahliae* inoculation. In Chapter 3, the main objective was to study the role of plant hormones and other signals

in the response induced by PO212 in pepper. The role of plant hormones in the response induced by PO212 was also tested using tomato mutants deficient in hormone accumulation or signaling and determining hormone levels in pepper roots by HPLC. In order to elucidate the role of other signals related with defense-signaling, H_2O_2 was also measured in pepper roots. In this chapter, PO212, previously transformed to express GFP fluorescent protein, was used to study the colonization of the pepper roots to further understand the interaction between the BCA and the plant. In Chapter 4, we looked for other potential BCA that could be combined in the future with PO212 in order to increase its effectiveness. Several pepper greenhouse soils were analyzed in terms of AMF diversity and abundance and suppressiveness potential to two of the main pathogens in pepper: *Verticillium dahliae* and *Phytophthora capsici*. Physicochemical properties were also analyzed in these soils. Disease monitoring and pathogen quantification were also analyzed in pepper plants grown in these soils. Detection of suppressive soils and the possible role of AMF in suppressiveness were discussed. Finally, a general discussion and a summary of the main conclusions of the thesis are presented.

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Objectives

The present thesis addresses the study of the soil-borne fungus *Penicillium rubens* Currie & Thom strain 212 (PO212) to determine its protective effect and the mechanism(s) of action in pepper plants. The objectives of this work were the following:

1- To test the ability of the biological control agent (BCA) PO212 (*Penicillium rubens* 212) to confer resistance against *Verticillium dahliae* in pepper plants (*Capsicum annuum* L.) (Chapter 1).

2- To characterize the PO212-induced resistance in pepper by quantifying the cell wall lignification, phenolic compounds, the pathogenesis-related protein 1 (PR-1) and 2 (β-1,3-glucanase), the peroxidase genes and activities, and the β-1,3-glucanase and chitinase activities (Chapter 1).

3- To determine the PO212-elicitation in pepper suspension cell cultures by quantifying biochemical defenses, lignin and the expression of genes involved in biochemical and structural defense and signaling (Chapter 2).

4- To elucidate the signals involved in the resistance induced by PO212 in Padron pepper roots against *Verticillium dahliae* by quantifying hydrogen peroxide levels, different plant hormones, expression of genes involved in hormone biosynthesis, as well as testing tomato mutants impaired in hormone accumulation or signaling (Chapter 3).

5- To study the direct effect of the BCA in pepper plants by conducting confocal microscopy assays (Chapter 3).

6- To detect putative suppressive soils to *Verticillium dahliae* and *Phytophthora capsici* in Galician pepper greenhouses and its relation with arbuscular mycorrhizal fungi (AMF) present in the mentioned soils (Chapter 4).

Chapter 1. *Penicillium rubens* strain 212 induces resistance to *Verticillium dahliae* in pepper

Part of this chapter is going to be resubmitted attending to the referees comments as: Lois, M., Veloso, J., García, T., Carrillo-Barral, N., Larena, I., Díaz, J. (2019). *Penicillium rubens* strain 212 induces resistance to *Verticillium dahliae* in pepper.

Introduction

Pepper (*Capsicum annuum* L.) is a crop with an outstanding economic value in Galicia (NW Spain). The soil-borne pathogen *Verticillium dahliae* causes a disease that affects pepper crops in Galicia, called Verticillium wilt (Veloso & Díaz, 2012), but is also a major problem for 300 to 400 plant hosts all over the world, causing heavy losses of high value crops (Carroll *et al.*, 2017; Inderbitzin *et al.*, 2018; Keykhasaber *et al.*, 2018). This fungus penetrates directly the plant roots and colonizes xylem vessels, obstructing them with its conidia and/or mycelium and leading to the development of symptoms as wilting, chlorosis, leaf epinasty and drying, dwarfism, as well as premature defoliation (Goicoechea *et al.*, 2000; Vallad & Subbarao, 2008; Yadeta & Thomma, 2013). *V. dahliae* is able to adapt to new hosts, which explains the continuous increase in the number of plants susceptible to this pathogen (Klimes *et al.*, 2015; Klosterman *et al.*, 2009). Besides conidia, *V. dahliae* also produces highly resistant structures, the microsclerotia, to survive in the soil in adverse conditions, and as a result the management of the disease is really challenging (Yadeta & Thomma, 2013).

One of the possible tools for Verticillium wilt management is the use of biological control agents (BCAs), alone or in the form of soil amendments containing such BCAs (Deketelaere *et al.*, 2017; Inderbitzin *et al.*, 2018). The commercial use of BCAs has become important because it provides long-term effective protection without a damaging impact to the environment or the human health (Demir *et al.*, 2015; Nollet & Rathore, 2015). The mode of action of these BCAs depends on two additive mechanisms: first, direct effects on the pathogen as mycoparasitism, secretion of antibiotics and competition for nutrients and space and, second, indirect effects by the activation of plant defenses or the stimulation of plant growth and vigor, therefore increasing the resistance of the plant to diseases (Deketelaere *et al.*, 2017). An extensive

review of the BCAs assayed against Verticillium wilt in different plant hosts can be found in Deketelaere *et al.* (2017). The fungus *Penicillium rubens* strain 212 (PO212, formerly *Penicillium oxalicum*) is an effective BCA in tomato against *Fusarium oxysporum* f. sp. *lycopersici* and *V. dahliae* (De Cal *et al.*, 1997; Larena *et al.*, 2003ab; Sabuquillo *et al.*, 2006; Villarino *et al.*, 2018). PO212 is a non-pathogenic, free-living and soil-borne fungus, and one of the mechanisms of action of this BCA is induced resistance (De Cal *et al.*, 1997; 2000).

The physiological mechanism of induced resistance involves genes encoding for phenylpropanoid pathway-enzymes or for PR proteins ("Pathogenesis-Related proteins") and results in a strong decrease in susceptibility to disease (Pozo *et al.*, 2002; van Loon *et al.*, 2006). In pepper the increase of several PR-proteins and PR-genes, such as β-1,3-glucanases (PR-2), chitinases (PR-3, PR-4, PR-8, PR-11) and peroxidases (PR-9), as well as the increase in phenolic compounds and phytoalexins, have been correlated with the resistance to Verticillium wilt (Chmielowska *et al.*, 2010; Veloso & Díaz, 2012). Genes encoding for many of these enzymes are also more expressed in *Penicillium*-treated plants to confer protection against other diseases (Murali *et al.*, 2013; Shimizu *et al.*, 2013). On the other hand, lignin deposition is a typical response to pathogen attack (Almagro *et al.*, 2009; Shigeto & Tsutsumi, 2016; Tobimatsu & Schutz, 2019), and plays a critical role in plant resistance to Verticillium wilt (Xu *et al.*, 2011).

In this work, PO212 was used in pepper plants to induce resistance against *V. dahliae*. We demonstrated that PO212 reduces Verticillium disease symptoms in pepper plants. Based on the measurement of several PR enzymes and genes, as well as soluble phenolic compounds and lignin, we prove that induced resistance is at least one of PO212 modes of action in pepper.

Materials and methods

Plant material

Seeds of *Capsicum annuum* var. Padron were disinfected in 0.1% (v/v) commercial bleach for 15 min, washed with tap water and incubated overnight in tap water at room temperature in darkness. Subsequently, seeds were sown in sterile vermiculite, and watered with nutrient solution (Hoagland & Arnon, 1950). Plants were grown in a growth chamber with a photoperiod of 16 h light at 25ºC and 8 h darkness at 18ºC during 24 days. Then, plants were treated with PO212.

Fungal material

The *Penicillium rubens* strain 212 (ATCC 201888) (PO212) belongs to the INIA collection. PO212 conidia were obtained and dried as indicated in Larena *et al.* (2003a), and stored at 4 ºC until their use.

The *Verticillium dahliae* isolate UDCVd53 belongs to the collection of FISAPLANT Group of the University of A Coruña, being periodically reisolated from infected plants in our laboratory to keep its virulence. This isolate was cultured on PDA (Potato Dextrose Agar) at 25 ºC in the dark.

PO212 treatment method

Conidia were gently dislodged in sterile nutrient solution (Hoagland & Arnon, 1950), and the conidial suspension was adjusted to 6×10^6 conidia·mL^{-1}. The conidial suspension was stirred for 3 hours at 150 rpm in a rotary shaker to ensure conidial hydratation. The conidial suspension was applied to the plants by root dipping during 24 hours, keeping the plants in the growth chamber with the temperature and photoperiod indicated above. The control plants were dipped in sterile nutrient solution. After 24

hours, plants of both treatments (PO212 and control) were transferred to fresh sterile nutrient solution an incubated for another 24 hours with the same temperature and photoperiod.

Inoculation with Verticillium dahliae

Inoculum preparation

The inoculum was obtained as described by Díaz *et al.* (2005). The concentration of the inoculum was adjusted to 10^6 conidia·mL^{-1}.

Challenge inoculation

After treatment with PO212-conidial suspension or nutrient solution alone as described above, the plants were inoculated with *V. dahliae* by dipping the roots for 45 minutes in the inoculum, following the protocol described in Veloso & Díaz (2012). Some plants were mock-inoculated with sterile distilled water. Plants were then transferred to a sterile mixture of soil/perlite (3:1, v/v). The number of wilted leaves was recorded weekly during 21 days post-inoculation (dpi). Stem length diameter, plant fresh weight, plant dry weight and the percentage of relative water content (RWC) were also recorded at 21 dpi. Four independent experiments were carried out with eight plants per treatment and per experiment.

Quantification of the biomass of the pathogen

Roots were sampled seven days after inoculation with *V. dahliae* from both PO212-treated and control plants. The roots were carefully washed with tap water. Each sample was a pool of roots from seven plants. The samples were frozen with liquid nitrogen and stored at -80 ºC. Total DNA extraction was performed according to Silvar *et al.* (2005). *V. dahliae* biomass was quantified according to Gayoso *et al.* (2007) with one modification: the primers used were: 5´-AGCATTCAGTTCAGAAGACGGA-3´ and

5′-CCGAAATACTCCAGTAGAAGG-3′. Plant DNA quantification was carried out with the primers for *CaBPR1* and the method described in Gayoso *et al.* (2007). *V. dahliae* colonization was calculated as the ratio of pathogen DNA to plant DNA. Five independent experiments were carried out.

Extraction and determination of enzyme activities

Roots were sampled 48 h after PO212-treatment (just before inoculation) and 24 h after inoculation with *V. dahliae*. The samples (six roots per sample) were frozen with liquid nitrogen and stored at -80 ºC. For enzyme extraction, samples were homogenized into a mortar at 4 ºC in 50 mM Tris HCl pH 7.5 buffer with 1 M KCl and adding 0.5 g of polyvinylpolypyrrolidone (PVPP) for each 10 g of fresh tissue. The extract obtained was centrifuged at 12857 xg and 4 ºC for 20 min. The supernatant was collected and desalted through a PD-10 Sephadex G-25 column (GE Healthcare). The eluate was stored at -80 ºC and was used for determining the enzyme activities and the protein content. Total proteins were determined as described in García *et al.* (2015).

Chitinase activity was quantified using glycol-chitin as substrate according to García *et al.* (2015). The enzyme activity was expressed as mU per mg of protein.

β-1,3-glucanase activity was quantified following the procedure described by García *et al.* (2015) for chitinase, but in this case laminarin was used as substrate. The reaction mixture consisted of 1% laminarin, 100 mM sodium acetate pH 5.0 and 85 µL of the sample. The mixture was incubated for 10 min at 37 ºC and then kept in ice for other 10 min. Then, 15 mM potassium ferricyanide in 0.5 M sodium carbonate was added and then boiled for 15 min. Finally, the absorbance was measured at 420 nm. A calibration curve was carried out with N-acetyl-glucosamine and the enzyme activity in the samples was expressed as mU per mg of protein.

Peroxidase activity was determined according to Ferrer *et al.* (1990). The enzyme activity in the samples was expressed as U per mg of protein (Díaz *et al.*, 2005). Three to four independent experiments were carried out depending on the enzyme (see figure legends for details).

Phenolic compounds and lignin

Roots were sampled 48 hours after PO212-treatment (just before inoculation) and 24 hours after inoculation with *V. dahliae*. The samples (six roots per sample) were frozen with liquid nitrogen and stored at -80 ºC. Soluble phenolics were extracted with 95% methanol (MeOH) as described by García *et al.* (2015). The phenolic content of each sample was determined using Folin-Ciocalteau reagent according to García *et al.* (2015). For total lignin analyses, fresh samples were weighed, fixed in boiling MeOH and stored in fresh MeOH at room temperature. Cell walls were extracted as previously described (Díaz & Merino, 1998). The final cell wall preparation was used for lignin determination by the acetyl bromide/acetic acid method according to García *et al.* (2015). Three to four independent experiments were carried out depending on the case (see figure legends for details).

Lignification assay

Roots from pepper were sampled 24 hours after inoculation with *V. dahliae*. Histochemical staining of secondary cell wall elements from pepper was realized according to Mitra & Loqué (2014).

Gene expression study

Roots were sampled 48 hours after PO212-treatment (just before inoculation) and 24 hours after inoculation with *V. dahliae*. The samples (six roots per sample) were frozen with liquid nitrogen and stored at -80 ºC. The protocol described in Veloso & Díaz

(2012) was followed to process the samples. We analyzed the expression of different genes involved in defense: a basic PR-1 protein (*CaBPR1*) (Gayoso *et al.*, 2007), a sesquiterpene cyclase (*CaSC1*) (Silvar *et al.*, 2008), a β-1,3-glucanase (*CaBGLU*) (Silvar *et al.*, 2008) and three peroxidases: *CaPO1*, *CaPO2* and *CanPOD* (García *et al.*, 2018; Wang *et al.*, 2013). The primers that were used are listed in Table 1.1.

Table 1.1. Primers used to quantify the target genes by real time qPCR.

Gene	Accession number	Reference			
			Name	Sequence	Amplicon
CaSC1	AF061285	Silvar *et al.* (2008)	*CaSCFW* *CaSCRV*	5'GCCTCCTGCTTCTGAATACC3' 5'TTAATATCCTTCCATCCCGACTC3'	**312 bp**
CaBPR1	AF053343	Gayoso *et al.* (2007)	*PR1FW* *PR1RV*	5'GTTGTGCTAGGGTTCGGTGT3' 5'CAAGCAATTATTTAAACGATCCA3'	**301 bp**
CaBGLU	AF227953	Silvar *et al.* (2008)	*GLUFW* *GLURV*	5'ACAGGCACATCTTCACTTACC3' 5'CGAGCAAAGGCGAATTTATCC3'	**226 bp**
CaPO1	AF442386	García *et al.* (2015)	*CAPO1FW* *CAPO1RV*	5'ACACTGGAAGCGTGAACAAT 3' 5'CAGCTTGCGCTAACATGAAC 3'	**333 bp**
CaPO2	DQ489711	García *et al.* (2018)	*CAPO2FW* *CAPO2RV*	5'TAGCACTAGAAGACGTCGGT 3' 5'TAATCATGGCAGCAGCGAAA 3'	**233 bp**
CanPOD	FJ596178	Lois *et al.*, 2019 (submitted)	*CanPODFW* *CanPODRV*	5'CACTGCTCCTTGATGACACA 3' 5'ACAACAGAGTCCCTAGCAGT 3'	**175 bp**
CaACT	AY572427	Silvar *et al.* (2008)	*CaACTFW* *CaACTRV*	5'ATCCCTCCACCTCTTCACTCTC3' 5'GCCTTAACCATTCCTGTTCCATTATC3'	**128 bp**

The actin gene was used as housekeeping gene (Veloso & Díaz, 2012). The RNA extraction and the reverse transcription were carried out following the protocol of the Bio-Rad Aurum-Total RNA Mini kit and iScript-cDNA Synthesis Kit, respectively. An

extensive description of qPCR reactions and data analysis was previously described in Veloso & Díaz (2012). At least two independent experiments were carried out.

Statistical analysis

All statistical analyses were performed using STATGRAPHICS 5.1 for Windows. The tests employed and the significance level are indicated in each figure. If possible, parametric tests were preferred, but if data did not fit normality of variance test even with transformations of the data, non-parametric tests were used instead. Statistically significant differences are indicated in the figures.

Results

PO212 reduces Verticillium wilt symptoms and colonization

Treatment with PO212 before inoculation with *V. dahliae* protected pepper plants against this pathogen, an effect observed by naked eye considering the extent of the dwarfism (Fig. 1.1A). Moreover, plants treated with PO212 and challenged with *V. dahliae* showed a significant reduction of wilt leaves percentage two and three weeks after inoculation (Fig. 1.1B). One week after inoculation, there was less *V. dahliae* biomass in the PO212-treated plants than in the control plants (Fig. 1.1C).

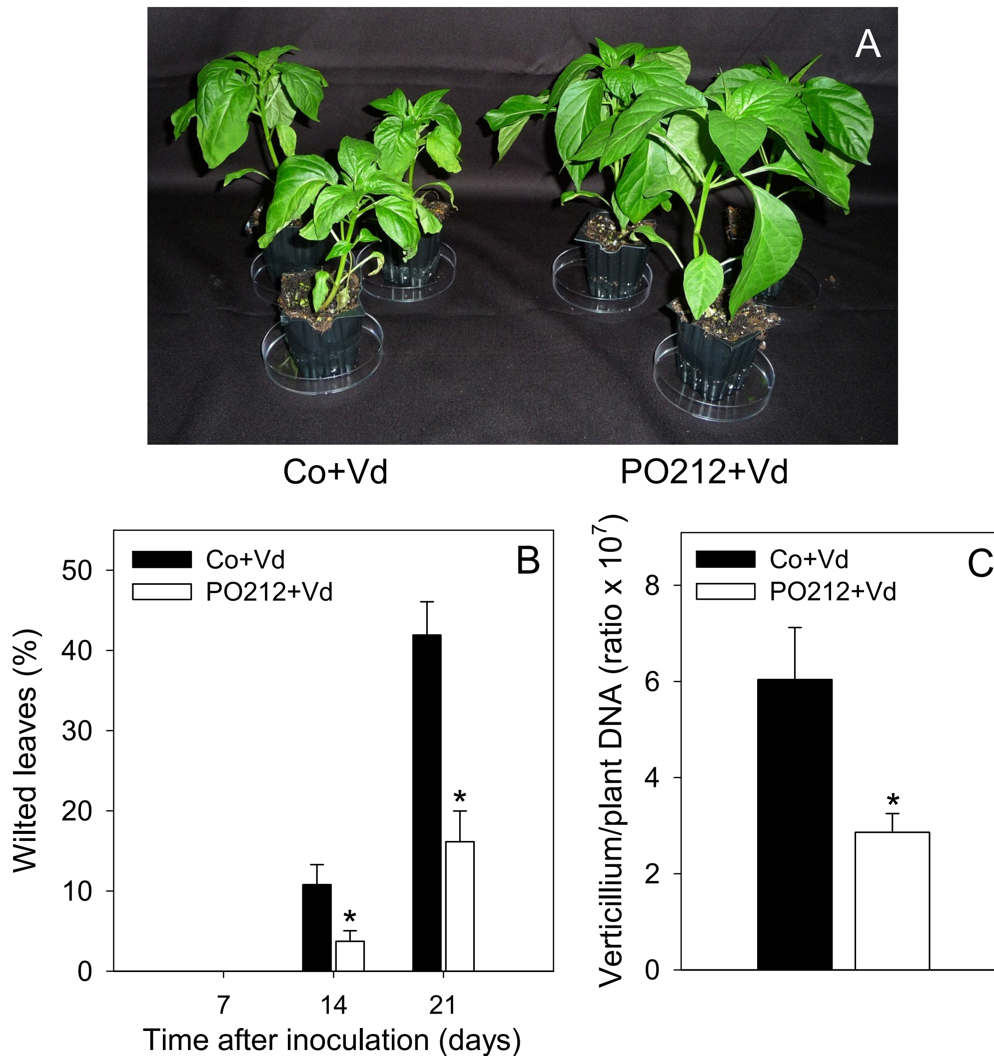

Figure 1.1. Verticillium wilt and colonization are alleviated by PO212 treatment. (A) View of some plants from a representative experiment, three weeks after inoculation with *V. dahliae*. (B) Percentage of wilted leaves recorded during three weeks after inoculation of pepper roots with *V. dahliae*. Data are means ± SE (standard error) of four independent experiments with eight plants per treatment and experiment (n=32). Asterisks indicate statistical differences respect to the control ($p < 0.05$) in a Mann-Whitney test. (C) *V. dahliae* colonization one week after inoculation expressed as the ratio between *V. dahliae* DNA and *C. annuum* DNA. Five independent experiments were performed (n=5). Data is the average ± SE. The asterisk means significant differences ($p < 0.05$) in a Mann-Whitney test. Co- Control, Vd- *V. dahliae*.

Stem length, plant fresh and dry weights and RWC were significantly higher in pepper plants treated with PO212 and inoculated with *V. dahliae* at the end of experiment (21dpi) in comparison with pepper plants only challenged with the pathogen (Fig. 1.2). Moreover, plant growth promotion effects were not observed between plants induced with PO212 and control plants (Fig. 1.2).

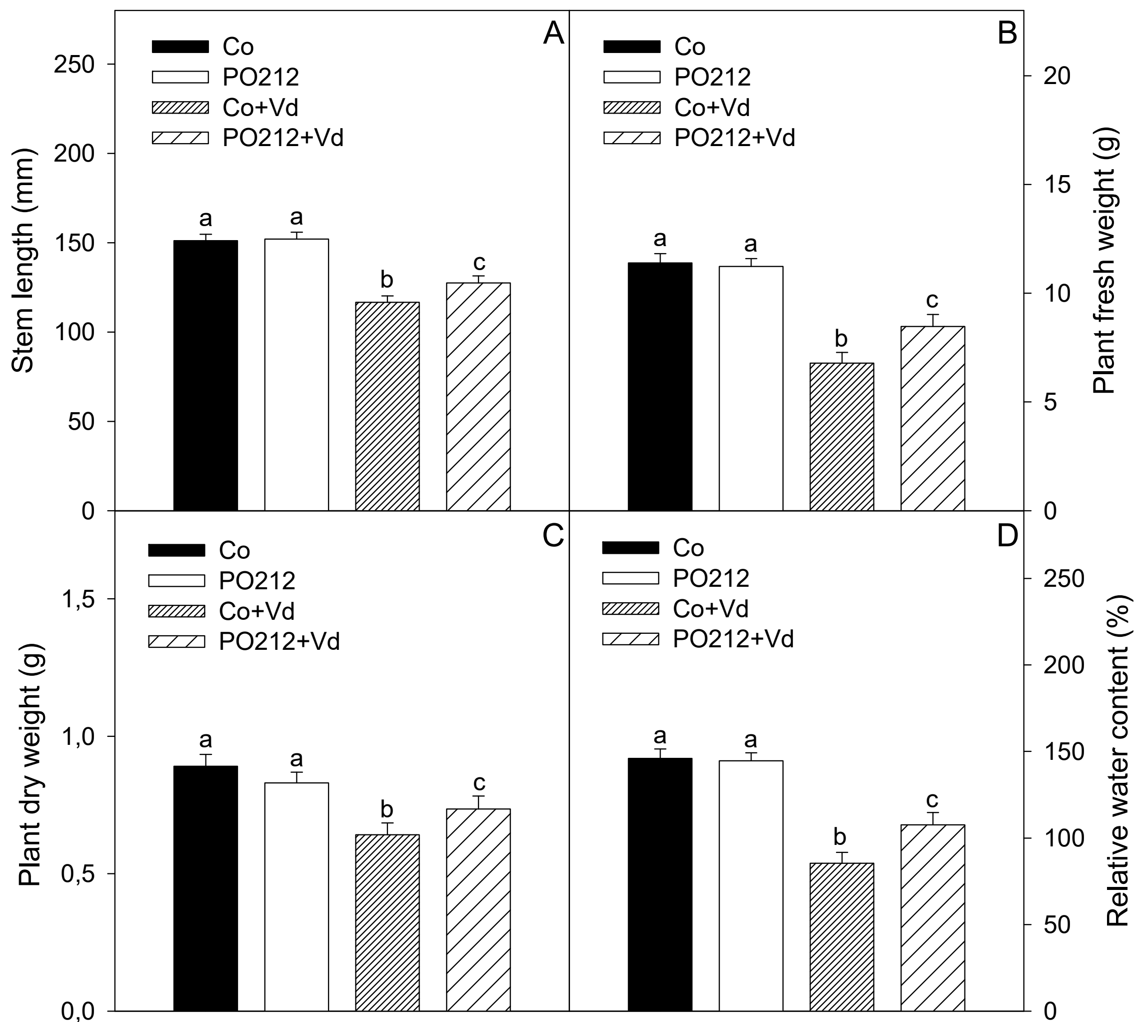

Figure 1.2. PO212 reduces the effect of Verticillium wilt in biomass and relative water content. (A) Stem length recorded three weeks after inoculation of pepper roots with *V. dahliae*. (B) Plant fresh weight recorded three weeks after inoculation of pepper roots with *V. dahliae*. (C) Plant dry weight recorded three weeks after inoculation of pepper roots with *V. dahliae*. (D)

Relative water content (RWC) recorded three weeks after inoculation of pepper roots with *V. dahliae*. Data are means ± SE (standard error) of four independent experiments with 8 plants per treatment and experiment (n=32). Different letters indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test followed by a Conover post-hoc test. Co- Control, Vd- *V. dahliae*.

PO212 induces biochemical defense at the enzyme and gene level

The activities of two PR-proteins, the enzymes β-1,3-glucanase and chitinase, were measured in the roots of pepper plants. Both β-1,3-glucanase and chitinase activities increased significantly respect to the control in the plants treated with PO212 before inoculation (Fig. 1.3A, C). After inoculation, the induction was also observed in both groups of plants treated with PO212 (mock-inoculated or inoculated with *V. dahliae*) when compared to the two groups of the control plants (Fig. 1.3B, D). *V. dahliae* inoculation itself increased the activity of both enzymes, as it is shown by significant differences between mock-inoculated plants and plants inoculated with *V. dahliae*, revealed by the two-way ANOVA test ($p < 0.05$).

Moreover, the expression of two PR-genes (*CaBPR1* and *CaBGLU*) and a sesquiterpene cyclase gene (*CaSC1*) involved in biosynthesis of the phytoalexin capsidiol, was determined in the roots. The three genes were induced significantly in PO212-treated plants both before and after inoculation with *V. dahliae* (Fig. 1.4).

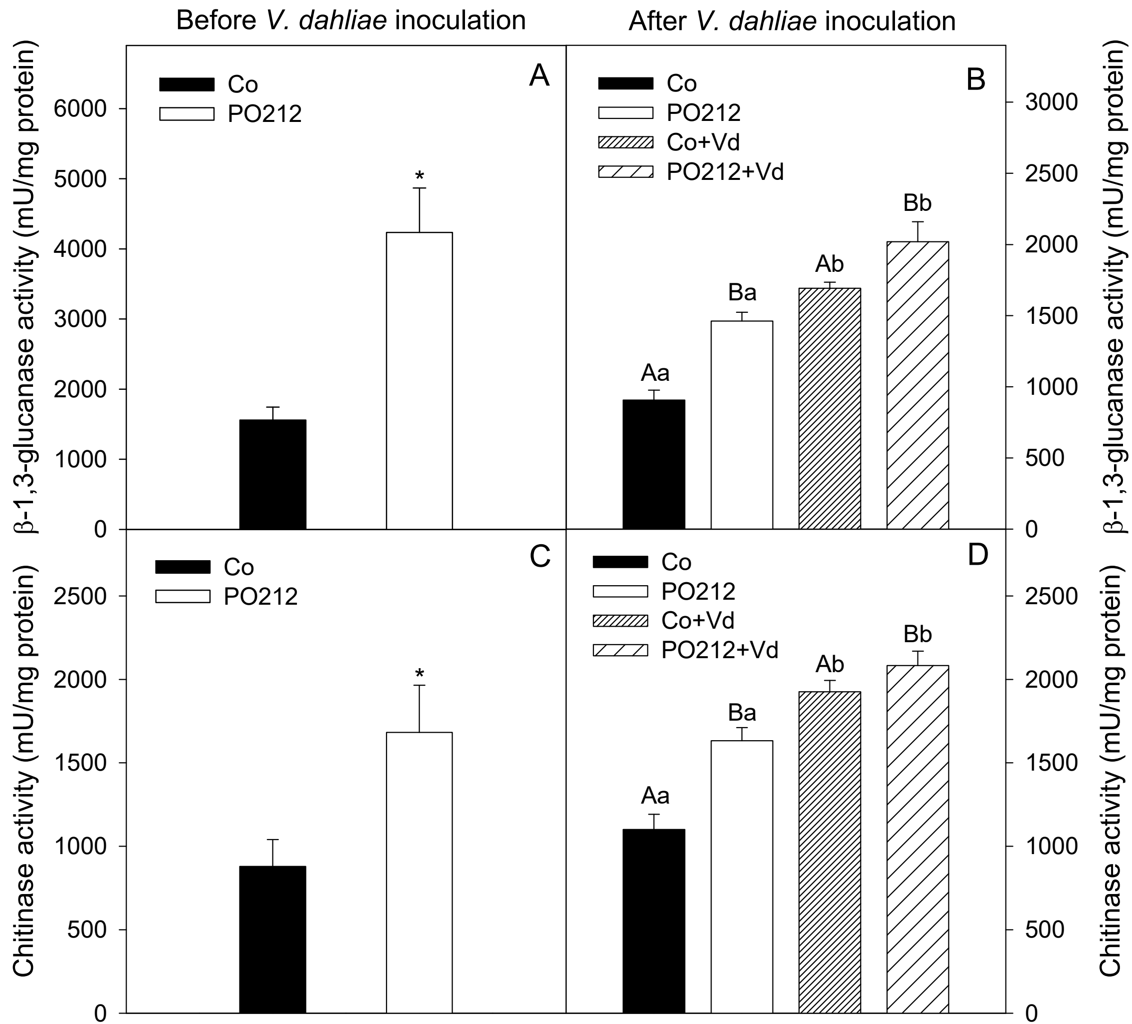

Figure 1.3. Induction of β-1,3-glucanase and chitinase activities by PO212 in the roots of pepper plants before and after inoculation with *V. dahliae*. (A) β-1,3-glucanase activity just before inoculation. (B) β-1,3-glucanase activity 24 hours after inoculation of pepper roots with *V. dahliae*. (C) Chitinase activity just before inoculation. (D) Chitinase activity 24 hours after inoculation of pepper roots with *V. dahliae*. Data are means ± SE of three (A, C) or four (B, D) independent experiments. In (A) and (C) asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. In (B) and (D) upper case letters indicate differences between treatments with or without PO212 and lower case letters indicate differences between treatments with or without *V. dahliae* in a two-way ANOVA test ($p < 0.05$). Co- Control, Vd- *V. dahliae*.

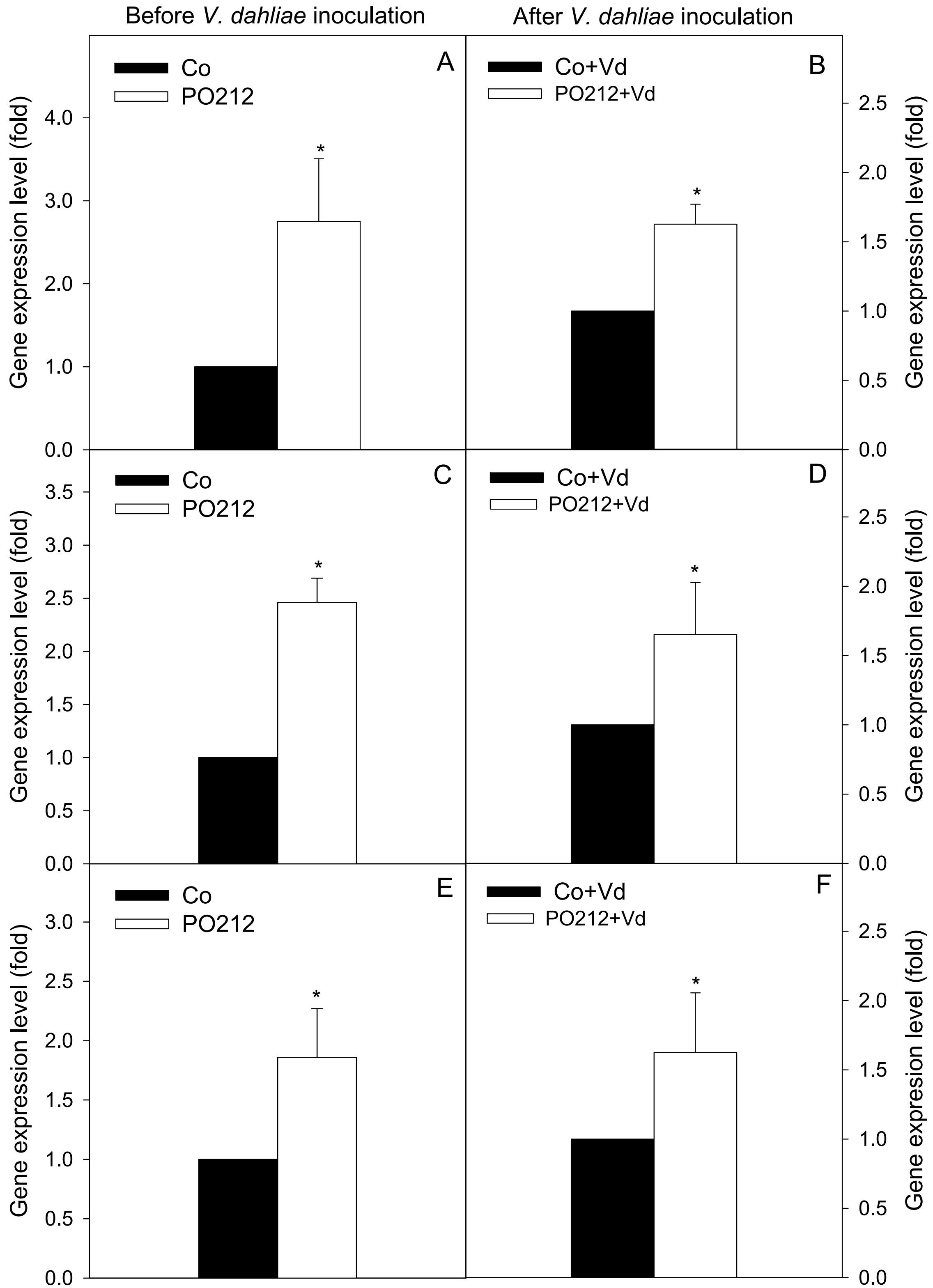

Figure 1.4. Effect of PO212 on relative expression in pepper roots of *CaBPR1*, *CaBGLU* and *CaSC1* genes before and after inoculation with *V. dahliae*. (A) *CaBPR1* expression before inoculation (n=3). (B) *CaBPR1* expression 24 hours after inoculation of pepper roots with *V.*

dahliae (n=5). (C) *CaBGLU* expression before inoculation (n=3). (D) *CaBGLU* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=5). (E) *CaSC1* expression before inoculation (n=3). (F) *CaSC1* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=5). Data are means ± SE. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

PO212 induces lignification

Before inoculation, the roots of PO212-treated plants showed more lignin (Fig. 1.5A). After inoculation, the roots of plants treated with PO212 and inoculated with *V. dahliae* showed more lignin that the other three groups: the groups of plants mock-inoculated (Control and PO212-treated) and the control plants inoculated with *V. dahliae* (Fig. 1.5B). However, the histochemical assay for lignin showed no appreciable differences in tissue staining between the control plants inoculated with *V. dahliae* and the PO212-treated plants subsequently challenged (Fig. 1.6). Soluble phenolics were less abundant in plants treated with PO212, regardless we consider plants before (Fig. 1.5C) or after inoculation (Fig. 1.5D).

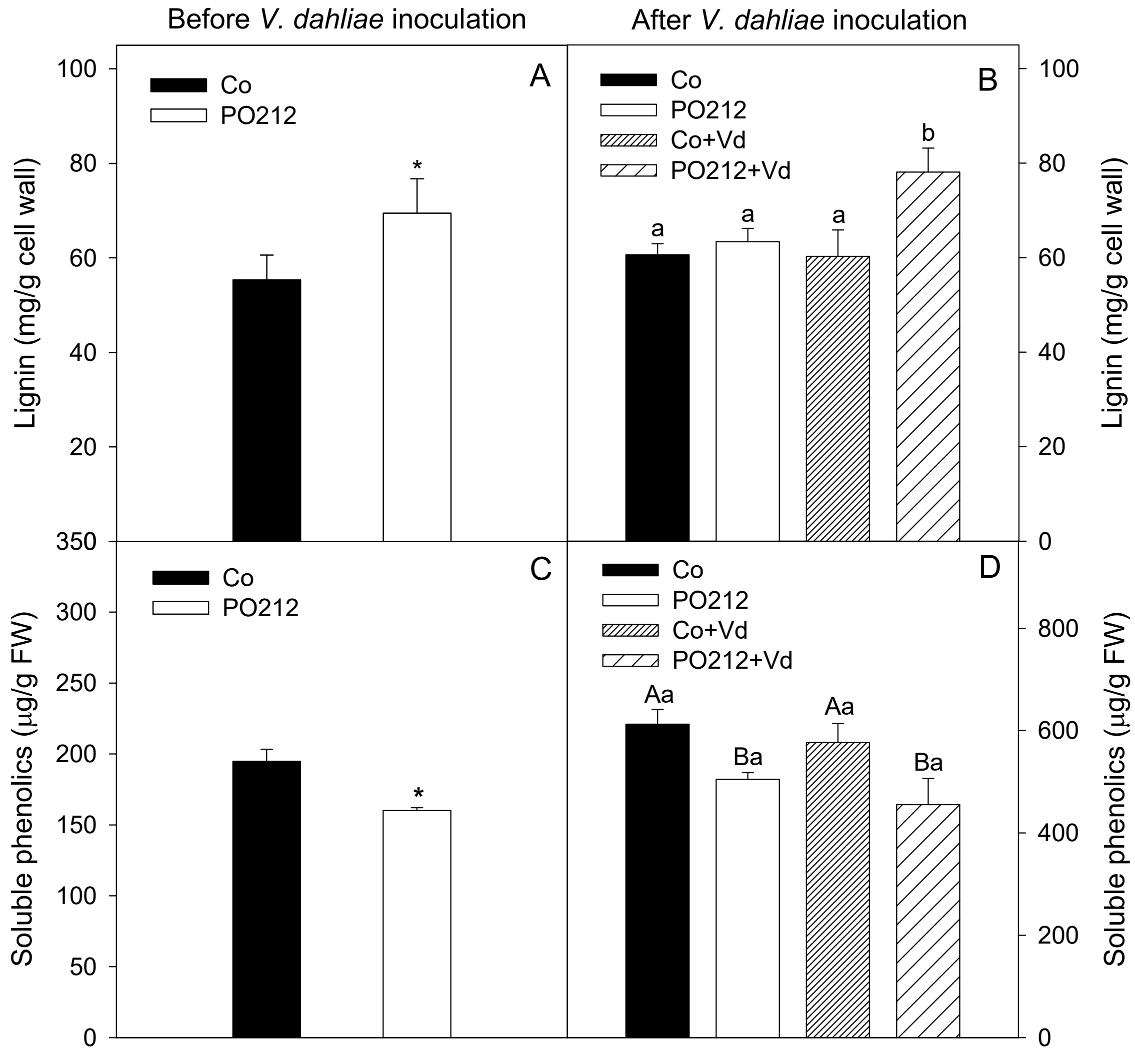

Figure 1.5. Effect of PO212 on lignin and soluble phenolics in the roots of pepper plants before and after inoculation with *V. dahliae*. (A) Lignin just before inoculation. (B) Lignin 24 hours after inoculation of pepper roots with *V. dahliae*. (C) Soluble phenolics just before inoculation. (D) Soluble phenolics 24 hours after inoculation of pepper roots with *V. dahliae*. Data are means ± SE of three (A, C, D) or four (B) independent experiments. In (A) and (C) asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. In (B) different letters indicate differences between treatments in one-way ANOVA and Duncan tests. In (D) upper case letters indicate differences between treatments with or without PO212 and lower case letters indicate differences between treatments with or without *V. dahliae* in a two-way ANOVA test ($p < 0.05$). Co- Control, Vd- *V. dahliae*.

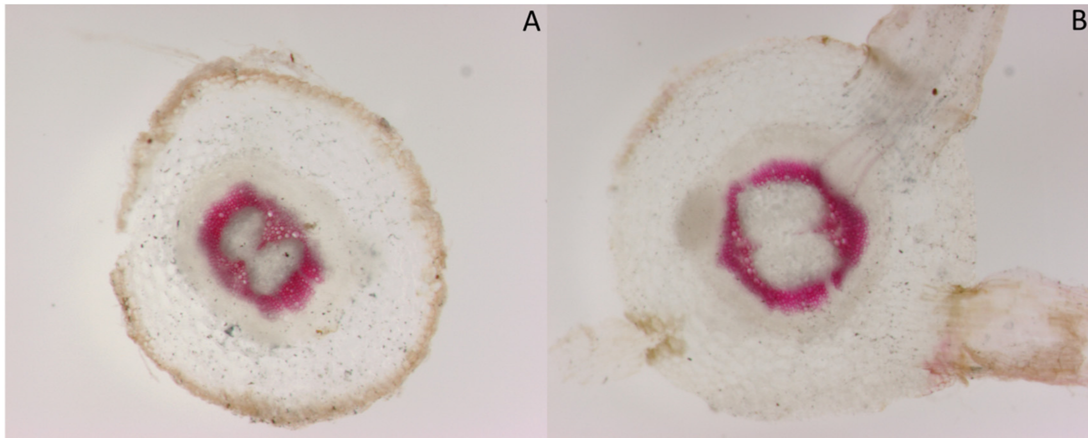

Figure 1.6. Effect of PO212 on lignin staining in the roots of pepper plants after inoculation with *V. dahliae*. (A) Lignin deposits in non-induced pepper roots 24 hours after inoculation with *V. dahliae*. (B) Lignin deposits in PO212-treated pepper roots 24 hours after inoculation with *V. dahliae*.

Because lignin is formed by polymerization of soluble phenolic monolignols, driven by peroxidases, we also measured peroxidase activity and the expression of three peroxidase genes: *CaPO1*, *CaPO2* and *CanPOD*. Peroxidase activity was higher in PO212-treated plants both before (Fig. 1.7A) and after *V. dahliae* inoculation (Fig. 1.7B). However, the expression of the three peroxidase genes was higher before pathogen challenge (Fig. 1.8A, C, E), but not after *V. dahliae* inoculation (Fig. 1.8B, D, F).

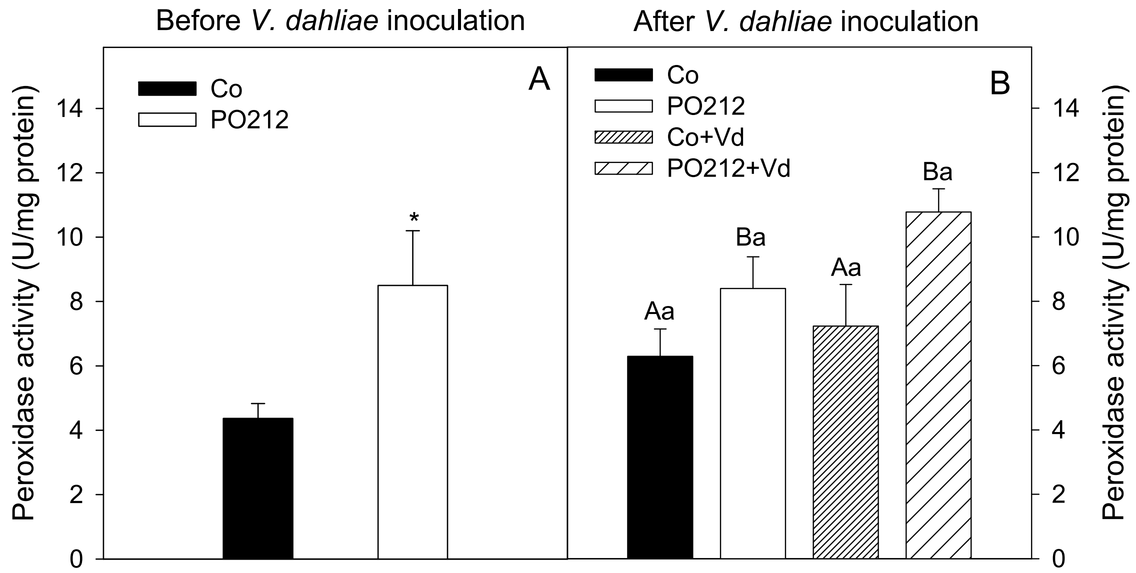

Figure 1.7. Induction of peroxidase activity by PO212 in the roots of pepper plants before and after inoculation with *V. dahliae*. (A) Peroxidase activity just before inoculation. (B) Peroxidase activity 24 hours after inoculation of pepper roots with *V. dahliae*. Data are means ± SE of three (A) or four (B) independent experiments. In (A) asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. In (B) upper case letters indicate differences between treatments with or without PO212 and lower case letters indicate differences between treatments with or without *V. dahliae* in a two-way ANOVA test ($p<0.05$). Co- Control, Vd- *V. dahliae*.

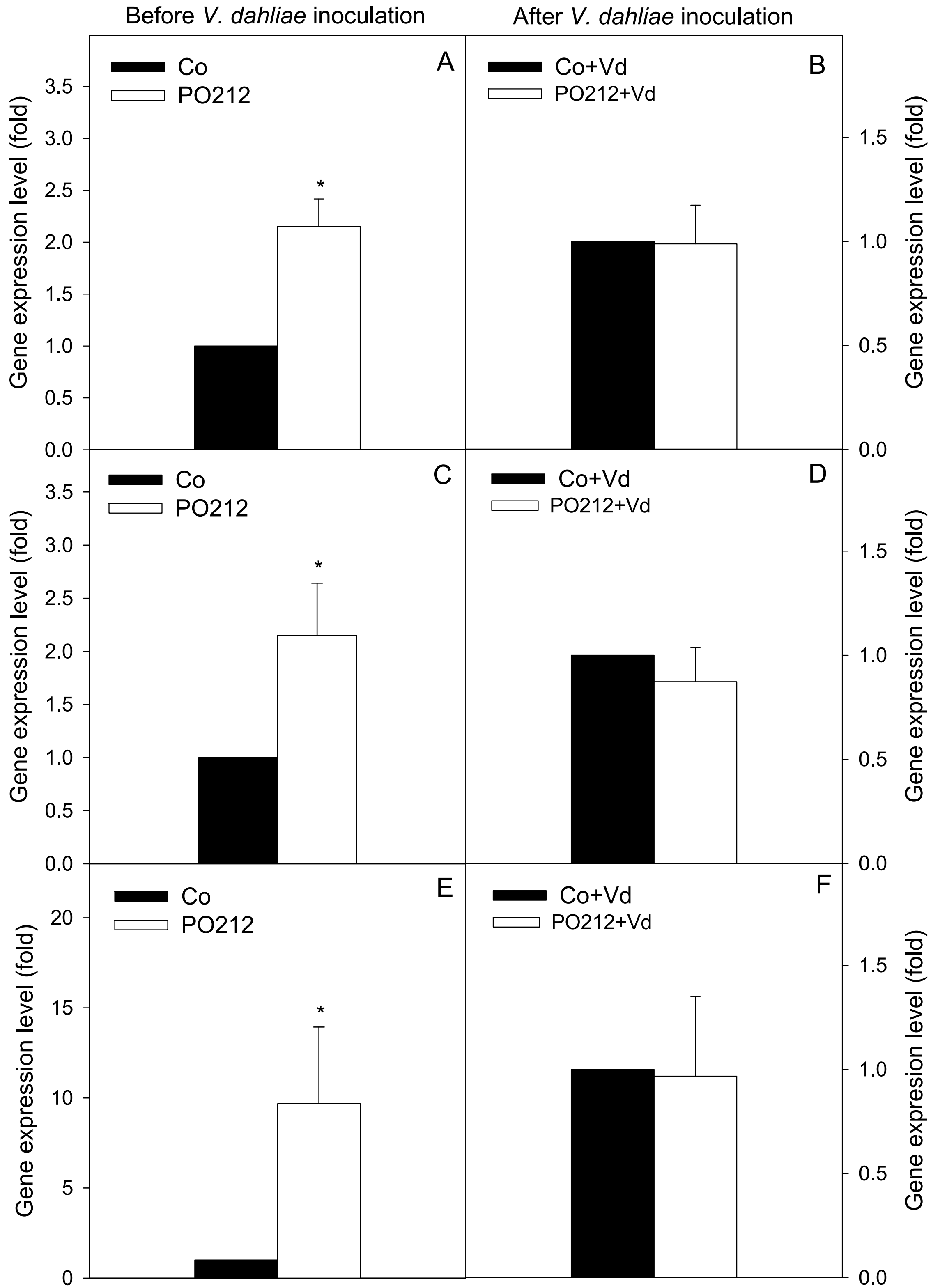

Figure 1.8. Effect of PO212 on relative expression in pepper roots of three peroxidase genes (*CaPO1*, *CaPO2* and *CanPOD*) before and after inoculation with *V. dahliae*. (A) *CaPO1* expression before inoculation (n=3). (B) *CaPO1* expression 24 hours after inoculation of pepper

roots with *V. dahliae* (n=6). (C) *CaPO2* expression before inoculation (n=4). (D) *CaPO2* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=4). (E) *CanPOD* expression before inoculation (n=4). (F) *CanPOD* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=53). Data are means ± SE. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

Discussion

PO212 has been reported previously to protect different crops against different diseases, namely: tomato against *F. oxysporum* f. sp. *lycopersici* (De Cal *et al.*, 1997), *V. dahliae* (Larena *et al.*, 2003b), *Botrytis cinerea*, *Phytophthora parasitica* and *P. infestans* (Sabuquillo *et al.*, 2005), strawberry against *Podosphaera aphanis* (De Cal *et al.*, 2008) and melon and watermelon against *Fusarium oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *niveum* respectively (De Cal *et al.*, 2009). Here we demonstrated that PO212 also protects pepper against *V. dahliae*. The biocontrol effectiveness of PO212 against *Verticillium* spp. and *Fusarium oxysporum* f. sp. *lycopersici* in tomato is based on the application of conidia of the BCA (Pascual *et al.*, 2000) in contact with the roots (De Cal *et al.*, 1997, 2000). In the present work the effectiveness of PO212 in pepper against *V. dahliae* was also based on conidia application to the roots before the inoculation with the pathogen. However, in pepper the period between PO212-treatment and pathogen challenge was shorter (48 h) than in tomato (7 days). We still have to try different time points and methods of PO212-treatment to optimize the outcome, but the protection achieved with the present method of application was noteworthy. In fact, the biomass of the pathogen was reduced in the plants treated with PO212, an additional proof of the effectiveness of the treatment and an evidence of the phenomenon of induced resistance (inhibition of the growth of the pathogen by plant defenses) rather than a phenomenon of tolerance (just suppression of symptoms) (Bos & Parlevliet,

1995). Also, PO212 was able to increase RWC in inoculated-pepper plants in comparison with non-induced plants subsequently inoculated with *V. dahliae*. *V. dahliae* is able to colonize water-conducting tissues; therefore, PO212 alleviate the effect caused by *V. dahliae* in terms water transport across the plant. Moreover, the data of plant defense components in PO212-treated plants also point to induced resistance.

Regarding our data on several growth parameters, we can concluded that PO212 does not act as PGPF. PO212 showed to increase stem lenght, fresh weight and dry weight in inoculated-pepper plants in comparison with non-induced plants subsequently inoculated with *V. dahliae*. However, none of these growth parameters was higher in PO212-treated plants challenged with *V. dahliae* compared with control plants. Moreover, mock-inoculated PO212-induced plants showed similar growth values to the mock-inoculated control plants before inoculation. All these data prove that PO212 is not a PGPF in pepper. Our results are in contrast with those obtained by Khan *et al.* (2014), which observed a plant growth promotion effect in pepper when plants were induced with *Penicillium resedanum* strain LK6.

Other many *Penicillium* strains are able to promote growth of differen plant species, being potential PGPFs (Murali *et al.*, 2013; Shimizu *et al.*, 2013; Hossain *et al.*, 2014; Murali & Amruthesh, 2015; Sreevidya *et al.*, 2015; Boughalleb-M'Hamdi *et al.*, 2018). PO212 did not promote pepper growth in our work, but De Cal *et al.* (1997) showed that PO212 is a potential PGPF in tomato. Boughalleb-M'Hamdi *et al.* (2018) found that two *Penicillium* strains did not enhance shoot and root dry weights in melon seedlings after inoculation with *F. oxysporum* f. sp. *melonis*. However, these two *Penicillium* strains promoted shoot and root dry weights in watermelon seedlings after inoculation with *M. phaseolina*. Therefore, the same *Penicillium* strain can act as PGPF or simply as inducer of resistance depending on the host plant and the target pathogen.

In the study of Carrero-Carrón *et al.* (2016), *Trichoderma asperellum* strain T25 promoted growth of olive plants against *V. dahliae*, but not *T. asperellum* strain Bt3. Thus, PO212 act as PGPF in tomato (De Cal *et al.*, 1997), but did not promote pepper growth.

Induced resistance leads to the biosynthesis of various signaling compounds and the consequent activation of plant immune-related genes, including those encoding PR proteins such as β-1,3-glucanases (PR-2) and chitinases (PR-3); these proteins hydrolyze β-1,3-glucane and chitin from fungal cell wall respectively, compromising the pathogen viability and releasing oligosaccharides that act as signals for the subsequent defense reaction (van Loon *et al.*, 2006). In the present work β-1,3-glucanase and chitinase increased significantly at the enzyme and gene level in PO212-treated plants. Therefore, PO212 induced β-1,3-glucanase and chitinase are expected to degrade *V. dahliae* cell wall and prevent both fungal penetration and further colonization. Murali *et al.* (2013) also observed a high expression of β-1,3-glucanase genes in plants induced with a *Penicillium chrysogenum* strain. Later, Murali & Amruthesh (2015) observed an increased chitinase activity in pearl millet plants induced with *P. oxalicum* UOM strain 16 after inoculation with the oomycete *Sclerospora graminicola*. On the other hand, the *CaBPR1* gene, encoding a PR-1 protein, was more expressed in PO212-treated plants. *PR-1* was also up-regulated in the study of Murali & Amruthesh (2015). PR-1 proteins are involved in sterol sequestering, therefore affecting the permeability of the membranes of plant pathogens (Gamir *et al.*, 2016). In pepper, *CaBPR1* accumulates early in incompatible interactions (Kim & Hwang, 2000; Hong & Hwang, 2002), as expected according to its role in plant defense. In the present work, the *CaSC1* gene also increased its expression in pepper plants induced with PO212; this gene codes for a 5-epi-aristolochene synthase/sesquiterpene cyclase, which is involved in isoprenoid

pathway and participates in the synthesis of the antimicrobial sesquiterpene phytoalexin capsidiol, which inhibits fungal growth (Chmielowska *et al.*, 2010). In pepper, the enhancement of *CaSC1* expression has been previously observed in the resistance induced by *F. oxysporum* f. sp. *lycopersici* (Silvar *et al.*, 2009), the response to copper stress associated to *V. dahliae* resistance (Chmielowska *et al.*, 2010), and the resistance to *V. dahliae* induced by Fo47, a *Fusarium* isolate used as BCA (Veloso & Díaz, 2012).

A significant increase in peroxidase activity was observed in PO212-induced pepper plants both before and after inoculation with *V. dahliae*. Moreover, the expression of three peroxidase genes, *CaPO1*, *CaPO2* and *CanPOD*, also increased in pepper roots induced with PO212, but after inoculation no differences were observed. Murali *et al.* (2013) also observed a high expression of peroxidase genes in plants treated with a strain of *Penicillium* sp. Peroxidases (and also laccases) catalyze the oxidative polymerization of phenolic monolignols (phenylpropanoid pathway) to produce lignin for cell wall-resistance after pathogen attack (Almagro *et al.*, 2009; Shigeto & Tsutsumi, 2016; Tobimatsu & Schutz, 2019). In fact, lignification is one of the mechanisms by which the plant restricts the growth of *V. dahliae* hyphae in the xylem (Gayoso *et al.*, 2010), and has been proposed as a mechanism of induced resistance against other pathogens of pepper (García *et al.*, 2018). We detected a significant increase in lignin deposition 24 hours after inoculation with *V. dahliae* in plants elicited with PO212, and this correlates with a significant increase in peroxidase activity. This result contrasts with the previously obtained in tomato, where PO212 did not induce lignin staining of the tissues (De Cal *et al*, 2000). An explanation is that the stain procedures employed are not fully specific for lignin (e.g., toluidine blue stains lignin and other poly-aromatic compounds as tannins or suberin) or did not stain all types of lignin (e.g., phloroglucinol only stains cinnamaldehyde end-groups of lignin) (Mitra & Loqué, 2014). This explains

why lignification staining assay in pepper also showed no differences in lignin between PO212-treated and inoculated plants and un-treated and inoculated plants. On the other hand, lignification has been reported to be induced by other BCAs. *Pythium oligandrum* induces lignin deposition to prevent the spread and propagation of the pathogen (Rekanovic *et al.*, 2007). The cell filtrate of *Penicillium simplicissimum* GP17-2 induces lignification in cucumber after inoculation with *Colletotrichum orbiculare* (Shimizu *et al.*, 2013).

We also observed a significant decrease in phenolic compounds in PO212-treated plants both before and after inoculation. Such phenolic decrease could reflect a great consumption of these compounds by peroxidases to synthesize lignin. However, peroxidases are also involved in ROS (reactive oxygen species) generation (Shigeto & Tsutsumi, 2016). Resistant plant cells experience an oxidative burst caused by the production of ROS after pathogen attack (Almagro *et al.*, 2009). ROS generation leads to a highly toxic environment for pathogens and they also can be used as signals in plant defense (Lehman *et al.*, 2015). The observed decrease in phenolics in PO212-treated plants can be explained because phenolic content also reflects plant redox status, that is, the less phenolic compounds there are the less reducing power exists in the cells. The increase of ROS (oxidative compounds) can lead to a decrease in phenolics (reducing compounds) in plants treated with PO212. Such a redox status is part of the signaling in some inducing responses as Systemic Acquired Resistance (SAR) (Veloso *et al.*, 2014). However, the signaling processes triggered by PO212 in pepper will be studied in the following chapters of this thesis (Chapters 2 and 3).

The overall picture is that PO212 induces the reinforcement of the cell wall by increasing lignin biosynthesis, mediated by peroxidases, at the expenses of soluble phenolic consumption (Fig. 1.9).

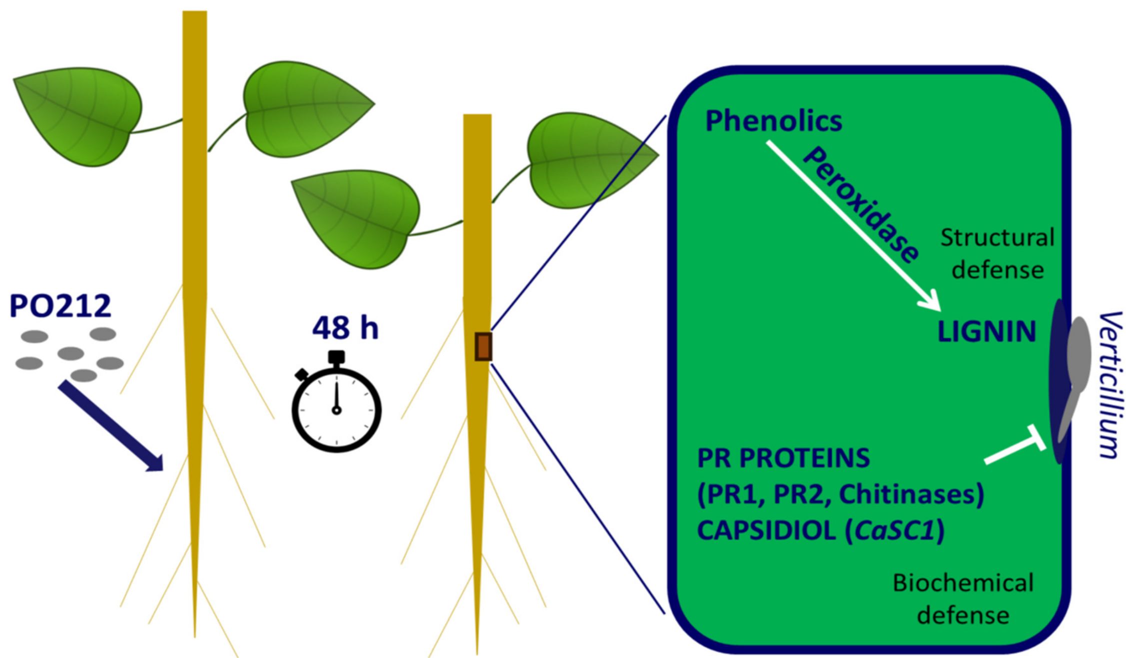

Figure 1.9. Model depicting a proposed mechanism for PO212-induced resistance to *V. dahliae*. PO212 is applied to the roots and 48h later a resistance response is observed, consisting of an increase in PR protein and gene expression, a *CaSC1* increase (presumably accompanied of capsidiol biosynthesis) and a lignin accumulation in the cell wall driven by peroxidases at expenses of soluble phenolic consumption. Both structural (lignin) and biochemical (PR proteins and phytoalexins) defenses make difficult to *V. dahliae* to enter the root cells and to further colonize the root tissues.

In summary, PO212 induces resistance in pepper against *V. dahliae* by reducing significantly *Verticillium* biomass and symptoms. We propose a model in which PO212 triggers phenolic compounds consumption by peroxidases to produce lignin deposition as a reinforcement of the plant cell wall. Lignin acts as a physical barrier, working together with chemical barriers (PR proteins and the phytoalexin capsidiol) also induced by PO212 to delay *V. dahliae* penetration and colonization of pepper roots.

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Chapter 2. Elicitation of defense responses in *Capsicum annuum* cell cultures with PO212

Introduction

In vitro plant cell/tissue/organ culture allows massive production of plant secondary metabolites under a controlled environment. Many chemicals are produced using *in vitro* plant cultures, namely drugs, cosmetics, perfumes and agrochemicals (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018). Plant cell cultures are typically grown as suspension cultured cells (SCC) or as callus. The use of SCC allows to separate the culture medium from the cells without cell disruption, in order to recover the free extracellular proteins and metabolites (Belchí-Navarro *et al.*, 2013; Miras-Moreno *et al.*, 2016). Some pathways in the plant cell culture remain inactive until they are activated using elicitors. Therefore, the exogenous application of elicitors in plant cell cultures is known as elicitation (Dörnenburg & Knorr, 1995; Narayani & Srivastava, 2017). Elicitation is usually used to increase secondary metabolite production. Besides increasing secondary metabolite production, elicitation allows to find new metabolites and obtain cells more metabolically competent and persistent, and activates different plant signaling pathways (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018).

When elicitors are perceived by the cells, a series of events takes place. First, signaling events occur, including the activation of the mitogen activated protein kinase (MAPK) cascade, Ca^{2+} influx, phosphorylation of proteins, extracellular alkalinization, cytoplasmic acidification, increase of nitric oxide (NO) and an oxidative burst through the generation of reactive oxygen species (ROS) such as H_2O_2 by NADPH oxidases or apoplastic peroxidases. Second, those signals can lead to the synthesis and accumulation of phytoalexins (e.g. capsidiol) and Pathogenesis-Related (PR) proteins (e.g. glucanases, chitinases) in the extracellular space, or the expression of defense-related genes involved in phytohormone signaling (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018).

According to Namdeo (2007), elicitors can be classified according to their origin in endogenous (derived from plant cells) or exogenous (non-derived from plant cells). Moreover, elicitors can be abiotic (derived from non-biological sources -physical and chemical agents, hormonal factors-) or biotic (derived from plants, fungi, yeasts, bacteria, herbivores) (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018).

There are many abiotic elicitors, such as jasmonic acid (JA), salicylic acid (SA), sodium acetate and copper sulphate (Narayani & Srivastava, 2017). On the other hand, several bacteria and yeasts have been used as biotic elicitors in different cell cultures (Gandi *et al.*, 2012; Chodisetti *et al.*, 2013), but fungi are the most common organisms used in *in vitro* systems. Fungal elicitors are generally derived from endophytes (e.g. saprobes, arbuscular mycorrhiza) or phytopathogens. Fungal (but also oomycete) cell wall components (e.g. chitin, glucan, oligosaccharides, hydrolysates), proteins, their mycelium or their filtrate have been used as elicitors in several *in vitro* plant cultures (Shasmita *et al.*, 2018). When the host plant culture is cultivated with the living fungus, the association that occurs in the growth media allows a constant elicitation and a greater productivity (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018).

The elicitation with the genus *Penicillium* has been used to enhance the production of different substances in *in vitro* cell cultures (Vakil & Mendhulkar, 2013; Ahmed & Baig, 2014; Gai *et al.*, 2017). It is known that *Penicillium* sp. produce a wide range of polysaccharides (Ikotun, 1984) which can induce plant defense responses such as the expression of β-1,3-glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8, PR-11), which hydrolyze β-1,3-glucan and chitin respectively from fungal cell walls (van Loon & van Strien, 1999).

In this work, we tested if a conidial suspension from the soil-borne fungus *Penicillium rubens* (formerly *Penicillium oxalicum*) strain 212 (PO212) is able to elicit a plant defense response using Padron pepper cell cultures (*Capsicum annuum* L. var. *annuum* cv. Padron) as a model system. In order to check the elicitation we have determined β-1,3-glucanase, chitinase and peroxidase activities as well as phenolic compounds both in the extracellular medium and in the cellular fraction. The expression of several defense-related genes (e.g. *PR*-genes, hormone biosynthesis-related genes) was also analyzed. Peroxidases (PR-9) catalyze the oxidative polymerization of phenylpropanoids to produce lignin and cell wall-protein cross-linking, thus increasing cell wall reinforcement (Almagro *et al.*, 2009). Finally, as peroxidases are also involved in ROS production and consumption (Almagro *et al.*, 2009), we quantified H_2O_2 levels in pepper cell cultures elicited with PO212.

Materials and methods

Plant material

Calli of *Capsicum anuumm* L. var. *annuum* cv. Padron were established in our laboratory in 2015 from hypocotyl explants, and they were maintained at 25 ºC under a 16 h light/8 h dark photoperiod. These calli have been subcultured on Murashige and Skoog basal medium (MS) (Murashige & Skoog, 1962) supplemented with Morel vitamins (Morel & Wetmore, 1951), 30 $g·L^{-1}$ sucrose, 0.25 $g·L^{-1}$ casein hydrolysate, 3 $mg·L^{-1}$ 2,4-dichlorophenoxyacetic acid, 0.05 $mg·L^{-1}$ kinetinand 8 g/L agar, adjusting the pH to 5.8. They were sub-cultured on MS medium every 30 days. Pepper SCC were established from friable calli in 250 mL flasks containing 100 mL of MS medium without agar. Cultures were maintained at 25 ºC in darkness on a rotary shaker (150 rpm) and subcultured every 21 days by diluting 1:1 (v/v) in sterile fresh MS medium.

Fungal material

Penicillium rubens 212 ATCC 201888 (PO212) was cultured on Petri dishes which contained potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 25 ºC.

Elicitation of pepper suspension cell cultures

Elicitation experiments were carried out using 10 day-old pepper SCC. In order to obtain conidia, PO212 strain was grown for seven days in PDA medium and then, the inoculum was obtained by gently flooding the dishes with sterile distilled water (Díaz *et al.*, 2005). The conidial suspension was adjusted to 10^6 conidia·mL^{-1}. Then, the conidial suspension was gently dislodged in sterile nutrient solution (Hoagland & Arnon, 1950) and remained stirring one hour at 150 rpm.

The experiments were performed by introducing 3 g of fresh weight (FW) of pepper cells into 100 mL flasks which contained 10 mL of sterile fresh MS medium. Then, 10 mL of nutrient solution were added to control SCC, while 10 mL of the PO212-conidial suspension were added to PO212-treated SCC. All cell cultures were incubated at 25 ºC in darkness on a rotary shaker (150 rpm) for 6 and 24 hours. After treatments, cells were separated from the culture medium under a gentle vacuum. Thus, two kinds of samples were obtained: cellular fraction and extracellular medium. All these operations were performed under aseptic conditions.

Extraction and determination of enzyme activities

Cellular fraction and extracellular medium were sampled 6 and 24 h after PO212-treatment. The samples were frozen in liquid nitrogen and stored at -80 ºC. For enzyme extraction, the samples of extracellular medium were desalted through a PD-10 Sephadex G-25 column (GE Healthcare). The eluate was used for determining the enzyme activities and the protein content. The samples of cellular fraction were

homogenized at 4 ºC in 50 mM Tris HCl pH 7.5 buffer with 1 M KCl and adding 5 mg of polyvinylpolypyrrolidone (PVPP) for each 500 mg of fresh tissue. The extract obtained was centrifuged at 12857 xg and 4 ºC for 20 min. The supernatant was collected and desalted through a PD-10 Sephadex G-25 column (GE Healthcare). The eluate was used for determining the enzyme activities and the protein content.

The Stoscheck method, based on Brilliant Blue Coomasie G-250, was used to determine the total amount of proteins (Stoscheck, 1990).

Chitinase and glucanase activities were measured as described in Chapter 1.

Chitinase activity was quantified using glycol-chitin as substrate according to García *et al.* (2015). Glycol-chitin was synthesized as described by Trudel & Asselin (1989). Chitin was obtained by acetylation of glycol-chitosan as described by Molano *et al.* (1979) with some modifications. For chitinase activity measurement, the reaction mixture consisted of 1% glycol-chitin, 100 mM sodium acetate pH 5.0 and the sample. The mixture was incubated for 10 min at 37 ºC and then kept in ice for other 10 min. Afterwards 15 mM potassium ferricyanide in 0.5 M sodium carbonate was added and then boiled for 15 min. Finally, the absorbance was measured at 420 nm. A calibration curve was made with N-acetyl-glucosamine and the enzyme activity in the samples was expressed as mU per mL for extracellular medium samples and mU per mg of protein for cellular fraction samples.

β-1,3-glucanase activity was quantified following the procedure described by García *et al.* (2015) for chitinase, but in this case laminarin was used as substrate. The reaction mixture consisted of 1% laminarin, 100 mM sodium acetate pH 5.0 and the sample. The mixture was incubated for 10 min at 37 ºC and then kept in ice for other 10 min. Afterwards 15 mM potassium ferricyanide in 0.5 M sodium carbonate was added and

then boiled for 15 min. Finally, the absorbance was measured at 420 nm. A calibration curve was made with N-acetyl-glucosamine and the enzyme activity in the samples was expressed as mU per mL for extracellular medium samples and mU per mg of protein for cellular fraction samples.

Peroxidase activity was determined according to Ferrer *et al.* (1990). The enzyme activity in the samples was expressed as mU per mL for extracellular medium and mU per mg of protein for cellular fraction samples (Díaz *et al.*, 2005).

Determination of phenolic compounds and lignin

Cellular fraction and extracellular medium were sampled 6 and 24 h after PO212-treatment. The samples were frozen in liquid nitrogen and stored at -80 ºC. Soluble phenolics were extracted with 95% methanol (MeOH) as described by García *et al.* (2015) for samples of cellular fraction. Samples of extracellular medium were directly used to quantify total soluble phenolic compounds. The phenolic content for both the extracellular medium and the cellular fraction was determined using Folin-Ciocalteau reagent according to García *et al.* (2015). The homogenate containing the cell wall was stored in fresh MeOH at room temperature and extracted as previously described (Díaz & Merino, 1998). Lignin was determined by the acetyl bromide/acetic acid method according to García *et al.* (2015).

Gene expression study

The samples of cellular fraction were collected 6 and 24 hours after PO212-treatment and frozen in liquid nitrogen and stored at -80 ºC. The protocol described in Silvar *et al.* (2008, 2009) was followed to process the samples. We analyzed the expression of different genes involved in defense: a basic PR-1 protein (*CaBPR1*), a sesquiterpene cyclase (*CaSC1*), a β-1,3-glucanase (*CaBGLU*), and a chitinase (*CaCHI2*), two

peroxidases (*CaPO1* and *CaPO2*), a phenylalanine ammonia-lyase (*CaPAL1*), two ethylene-biosynthesis genes (*CaACS3* and *CaACO*), a jasmonate-biosynthesis gene (*CaAOS*) and a salicylic acid-biosynthesis gene (*CaICS*). The primers that were used are listed in Table 2.1. The actin gene (AY572427) was used as housekeeping gene (Veloso & Díaz, 2012). The extraction and reverse transcription were carried out following the protocol of the Bio- Rad Aurum-Total RNA Mini kit and iScript-cDNA Synthesis Kit, respectively. The cDNA samples were analyzed with the Biorad iCyclerTM iQ System following the protocol described by Veloso & Díaz (2012). The PCR reactions consisted of Biorad 1× iQ SYBR Green Supermix, 0.3 M of each primer and 2.5 L of cDNA for a 50 L end reactionvolume. The PCR program started with 2 min denaturation step at 95 °C followed by 40 cycles of amplification (95 ºC for 20 s, 58 ºC for 25 s and 72 ºC for 50 s) and finished with an elongation step of 5 min at 72 ºC. The data analysis was carried out with the Biorad Optical System Software 3.0. The efficiency was calculated and the obtained Ct values were processed by the Pfaffl Method (Pfaffl, 2001) to obtain the relative expression values.

Table 2.1. Primers used to quantify the target genes by real time qPCR.

Gene	Accession number	Reference	Name	Sequence	Amplicon
CaBPR1	AF053343	Gayoso *et al.* (2007)	*PR1FW* *PR1RV*	5'GTTGTGCTAGGGTTCGGTGT3' 5'CAAGCAATTATTTAAACGATCCA3'	**301 bp**
CaSC1	AF061285	Silvar *et al.* (2008)	*CaSCFW* *CaSCRV*	5'GCCTCCTGCTTCTGAATACC3' 5'TTAATATCCTTCCATCCCGACTC3'	**312 bp**
CaBGLU	AF227953	Silvar *et al.* (2008)	*GLUFW* *GLURV*	5'ACAGGCACATCTTCACTTACC3' 5'CGAGCAAAGGCGAATTTATCC3'	**226 bp**
CaCHI2	AF091235	Silvar *et al.* (2009)	*CHIFW* *CHIRV*	5'AAGACTTAGTGAACAACCCTGATAAAG3' 5'CTATCTGCTGGTGATGGCTTCC3'	**148 bp**

CaPO1	AF442386	García *et al.* (2015)	*CAPO1FW* *CAPO1RV*	5'ACACTGGAAGCGTGAACAAT 3' 5'CAGCTTGCGCTAACATGAAC 3'	**333 bp**
CaPO2	DQ489711	García *et al.* (2018b)	*CAPO2FW* *CAPO2RV*	5'TAGCACTAGAAGACGTCGGT 3' 5'TAATCATGGCAGCAGCGAAA 3'	**233 bp**
CaPAL1	KF279696	García *et al.* (2018a)	*CaPAL1FW* *CaPAL1RV*	5'GTGGCACGATCACTGCCTCG3' 5'TGGTCCGTGAACTCGGGCTT3'	**319 bp**
CaACO	AJ011109	Carballeira (2010)	*CaACOFW* *CaACORV*	5'CGCCACTCCATTGTG3' 5'TAGATTACTGCATCGCTTCC3'	**152 bp**
CaACS3	X82265	García (2018)	*CaACS3FW* *CaACS3RV*	5'TCTGCTTGCGTCAATGTTGTCTG3' 5'TCCTCCACAGTTCCAATTCAGCA3'	**215 bp**
CaAOS	DQ832720	García (2018)	*CaAOSFW* *CaAOSRV*	5'TGTCTACGAATCTCTCCGCA3' 5'GGGACAAATTCTTCAGCCCT3'	**183 bp**
CaICS	AY743431	García (2018)	*CaICSF2* *CaICSR2*	5'GCCAGAACTCATGTGCCGGG 3' 5'AATGCTGAGGCGGTCCCGAT 3'	**273 bp**
CaACT	AY572427	Silvar *et al.* (2008)	*CaACTFW* *CaACTRV*	5'ATCCCTCCACCTCTTCACTCTC3' 5'GCCTTAACCATTCCTGTTCCATTATC3'	**128 bp**

Quantification of extracellular hydrogen peroxide

After PO212-conidial suspension treatment, samples of cellular fraction were separated from the extracellular medium by filtration at different times to measure hydrogen peroxide (H_2O_2). H_2O_2 production was monitorized after elicitation with PO212 in the extracellular medium using the xylenol orange assay. The xylenol orange reagent was freshly prepared and remained stable for 6 to 8 h. One milliliter of solution A [98 mg $FeSO_4$ dissolved in 1.332 mL H_2SO_4] was added to 100 mL of solution B [9.5 mg xylenol orange and 1.82 g sorbitol]. The extracellular medium was mixed with 50 mM phosphate buffer pH 5.7 (1:1). The reaction consisted of 100 µL of the mixture with 1 mL of xylenol orange. The mixture was incubated for 30 min at room temperature

before measuring the A560. The blank contained 100 μL of 50 mM phosphate buffer pH 5.7 and 1 mL of xylenol orange. H_2O_2 concentration in each sample (μmol/mL) was calculated. H_2O_2 production was measured at 5, 8, 24 and 29 hours after elicitation of pepper SCC with PO212.

Statistical analysis

All statistical analyses were performed using STATGRAPHICS 5.1 for Windows. The tests employed, the statistically significant differences and the significance level found in each case are reported in the results section and shown in the figures. Conover test was employed as a post-hoc test after Kruskal-Wallis test, using Microsoft Excel.

Results

Effect of PO212 on enzyme activities

β-1,3-glucanase activity in the extracellular medium showed a significant increase 6 hours after elicitation with PO212 in comparison with control SCC but 24 hours after elicitation, there were no differences between both treatments (Fig. 2.1A). In the cellular fraction, β-1,3-glucanase only showed significant differences 24 hours after elicitation in the PO212 treatment in comparison with control SCC (Fig. 2.1B). Moreover, glucanase activity in the cellular fraction increased significantly in PO212 SCC 24 hours after elicitation in comparison with PO212 SCC at 6 hours (Fig. 2.1B). In the extracellular medium it was no increase of chitinase activity as a response to PO212, but 24 hours samples showed more chitinase than the 6 hours samples, irrespective to the treatment (Fig. 2.1C). This fits with the idea of the enzyme being firstly synthetized in the cell and later on secreted to the medium. However, in the cellular fraction, there was a significant increase of chitinase activity in PO212-SCC in the two analyzed time points in comparison with the control SCC (Fig. 2.1D).

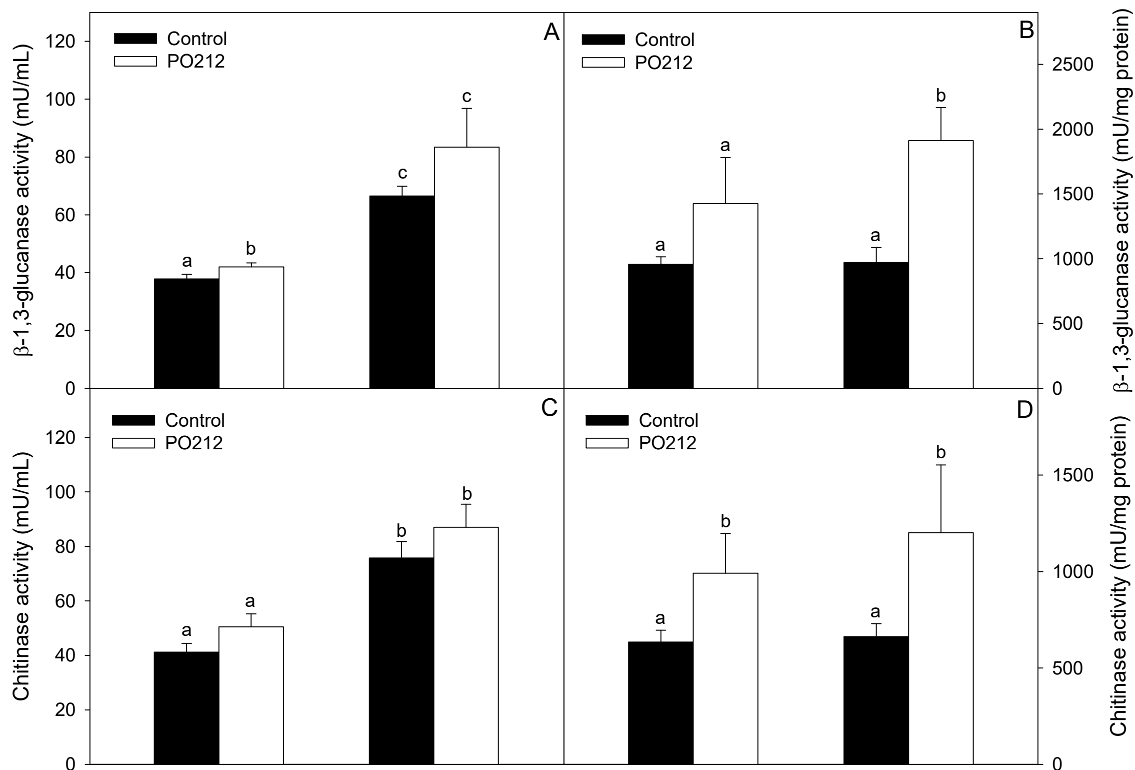

Figure 2.1. Induction of β-1,3-glucanase and chitinase activities by PO212 in pepper cell cultures. (A) β-1,3-glucanase activity in the extracellular medium 6 and 24 hours after elicitation. (B) β-1,3-glucanase activity and in the cellular fraction 6 and 24 hours after elicitation. (C) Chitinase activity in the extracellular medium 6 and 24 hours after elicitation. (D) Chitinase activity in the cellular fraction 6 and 24 hours after elicitation. Data are means ± SE of three independent experiments. In (A) and (C), different letters indicate statistical differences in ($p < 0.05$) in a Kruskal-Wallis test. In (B) and (D), different letters indicate statistical differences ($p < 0.1$) in a Kruskal-Wallis test test.

A significant decrease in peroxidase activity was observed in the extracellular medium of pepper SCC elicited with PO212 24 hours after elicitation in comparison with both treatments at 6 hours after elicitation (Fig. 2.2A). Surprisingly, an increase in intracellular peroxidase activity was observed in PO212-elicited SSC as compared to control 6 hours after PO212 treatment (Fig. 2.2B), but 24 hours after PO212 elicitation,

cellular fractions of both treatments showed no differences between them in peroxidase activity (Fig. 2.2B). On the other hand, peroxidase activity in cellular fraction of control SCC significantly increased over time (Fig. 2.2B). It seems that PO212 increases the speed of the changes in the peroxidase activity. In the extracellular medium, the activity tends to decrease but it decreases faster in the PO212 treated cultures (Fig. 2.2A).

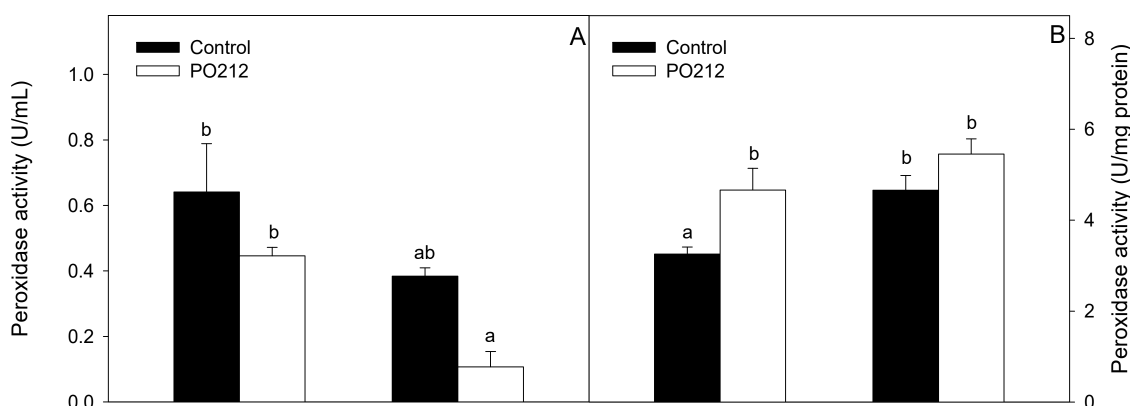

Figure 2.2. Peroxidase activity in elicited pepper cell cultures. (A) Peroxidase activity in the extracellular medium 6 and 24 hours after elicitation. (B) Peroxidase activity in the cellular fraction 6 and 24 hours after elicitation. Data are means ± SE of three independent experiments. In (A), different letters indicate statistical differences ($p < 0.05$) in a Kruskall-Wallis test. In (B), different letters indicate statistical differences ($p < 0.1$) in a Kruskall-Wallis test.

Effect of PO212 on soluble phenolics and lignin

No differences in phenolic compounds were observed in the extracellular medium between control SCC and PO212-SCC (Fig. 2.3A). In the cellular fraction, no significant differences were observed between both treatments neither 6 nor 24 hours after elicitation, but phenolic compounds increased significantly in both control SCC and PO212-SCC 24 hours after elicitation if compared with 6 hours samples (Fig. 2.3B).

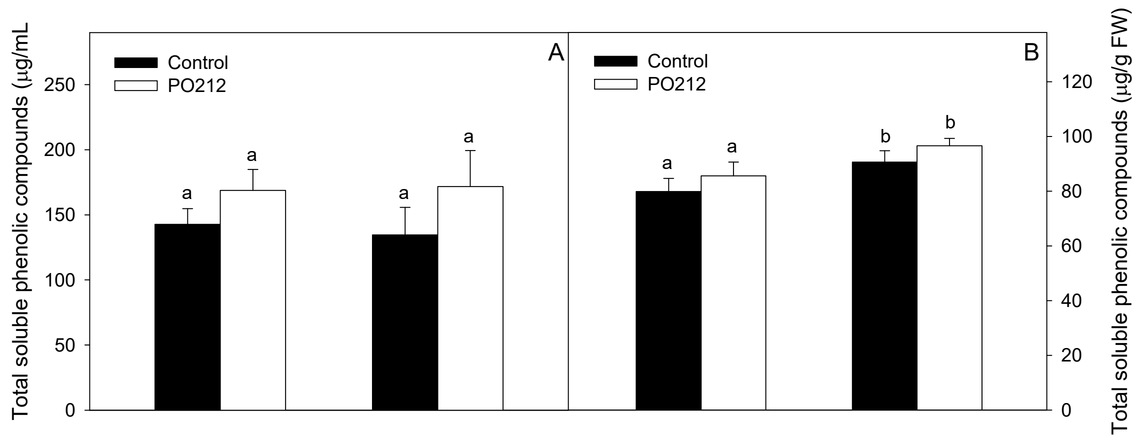

Figure 2.3. Effect of PO212 treatment on total soluble phenolic compounds in pepper cell cultures. (A) Phenolic compounds in the extracellular medium 6 and 24 hours after elicitation. (B) Phenolic compounds in the cellular fraction 6 and 24 hours after elicitation. Data are means ± SE of four independent experiments. Different letters indicate statistical differences ($p < 0.1$) in a Kruskall-Wallis test.

Regarding to lignification, cell suspensions generally produce slight amounts of this polymer or do not show any lignin deposition. As peroxidases catalyze the oxidative polymerization of phenylpropanoids to produce lignin and cell wall-protein cross-linking, and Egea *et al.* (2001) found lignin deposits in *C. annuum* SCC 24 hours after elicitation with the oomycete *Phytophthora capsici*, in this study lignification was also studied to check if PO212 can also induce lignification at early time points. However, no lignin was detected in pepper SCC at any time (6 and 24 hours after elicitation) and any treatment (control and PO212).

Effect of PO212 on gene expression

A significant increase in gene expression of three PR-proteins was observed after PO212 elicitation (Fig. 2.4). 6 and 24 hours after elicitation, there was a significant up-regulation of the basic PR1 gene *CaBPR1* (Fig. 2.4A), the PR-2 *CaBGLU*, (Fig. 2.4B) and the PR-3 *CaCHI2* (Fig. 2.4C). *CaBPR1* and *CaCHI2* expression was significantly

higher 24 hours after elicitation in pepper SCC treated with PO212 in comparison with PO212-SCC at 6 hours (Fig. 2.4A and C respectively). Moreover, *CaBPR1* and *CaBGLU* expression was significantly higher in control SCC 24 hours after elicitation in comparison with control SCC at 6 hours (Fig. 2.4A and B).

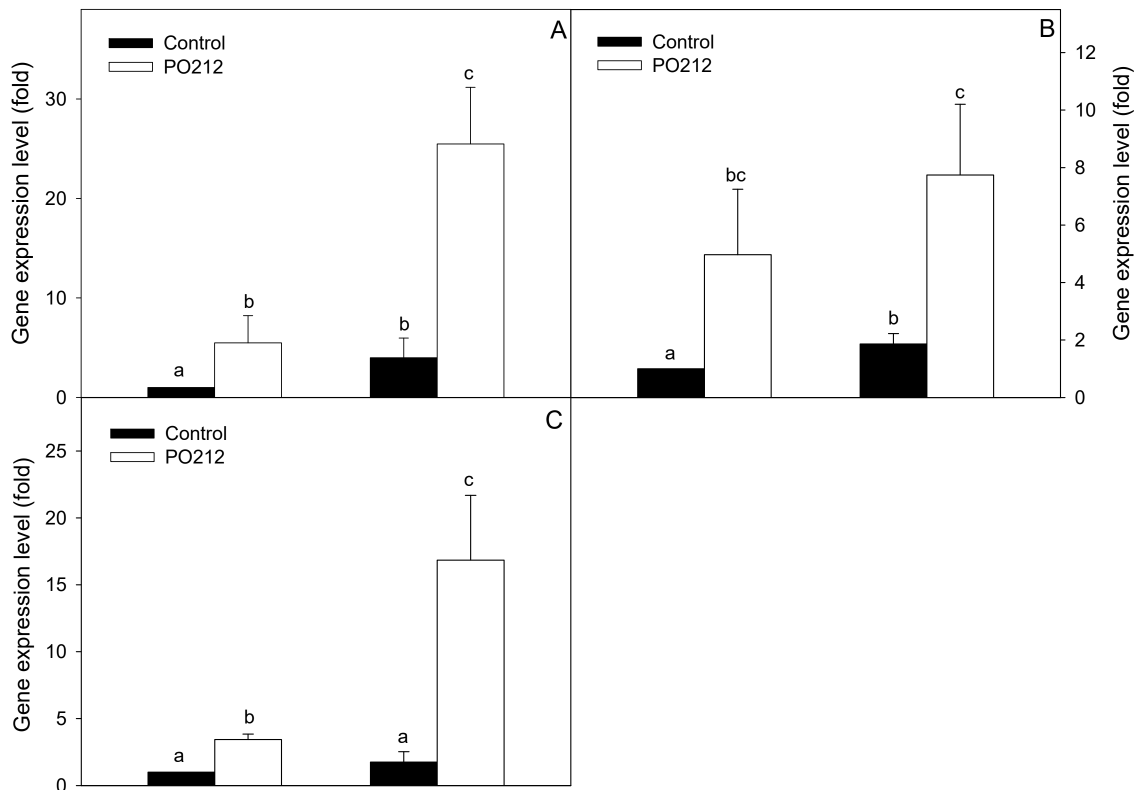

Figure 2.4. Relative gene expression in pepper cell cultures of three PR-proteins (*CaBPR1*, *CaBGLU* and *CaCHI2*) after elicitation with PO212. (A) *CaBPR1* expression 6 and 24 hours after elicitation (n=3). (B) *CaBGLU* expression 6 and 24 hours after elicitation (n=4). (C) *CaCHI2* expression 6 and 24 hours after elicitation (n=3). Data are means ± SE. Different letters indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test.

The two peroxidase genes showed different gene expression patterns after elicitation with PO212 (Fig. 2.5). *CaPO1* expression showed no differences between treatments 6 and 24 hours after elicitation. 24 hours after elicitation, this gene was up-regulated in

both treatments in comparison with 6 hours expression (Fig. 2.5A). *CaPO2*, however, was significantly up-regulated both 6 and 24 hours after elicitation with PO212 in pepper SCC (Fig. 2.5B).

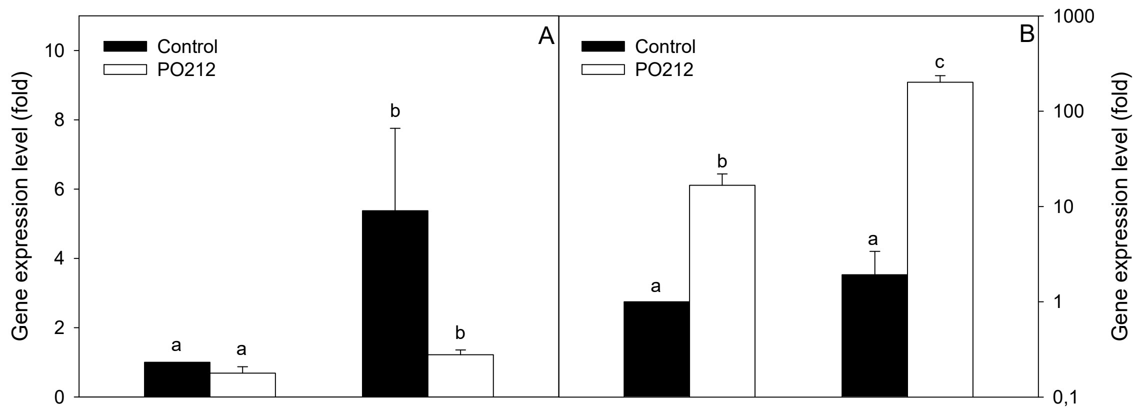

Figure 2.5. Relative gene expression in pepper cell cultures of two peroxidase genes (*CaPO1* and *CaPO2*) after elicitation with PO212. (A) *CaPO1* expression 6 and 24 hours after elicitation (n=3). (B) *CaPO2* expression 6 and 24 hours after elicitation (n=3). Data are means ± SE. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskall-Wallis test.

A significant increase in gene expression of a phenylalanine ammonia lyase gene and a sesquiterpene cyclase gene was observed after PO212 elicitation (Fig. 2.6). 6 hours after elicitation, there was a significant up-regulation of *CaPAL1* (Fig. 2.6A) and *CaSC1* (Fig. 2.6B) by PO212. 24 hours after elicitation, *CaPAL1* gene expression remained significantly higher in comparison with control SCC, and the gene expression of *CaSC1* in PO212 treatment was greater than 6 hours after elicitation (Fig. 2.6B). Moreover, control SCC showed a significant decrease in the expression of both genes 24 hours after elicitation in comparison with control SCC at 6 hours (Fig. 2.6A and B).

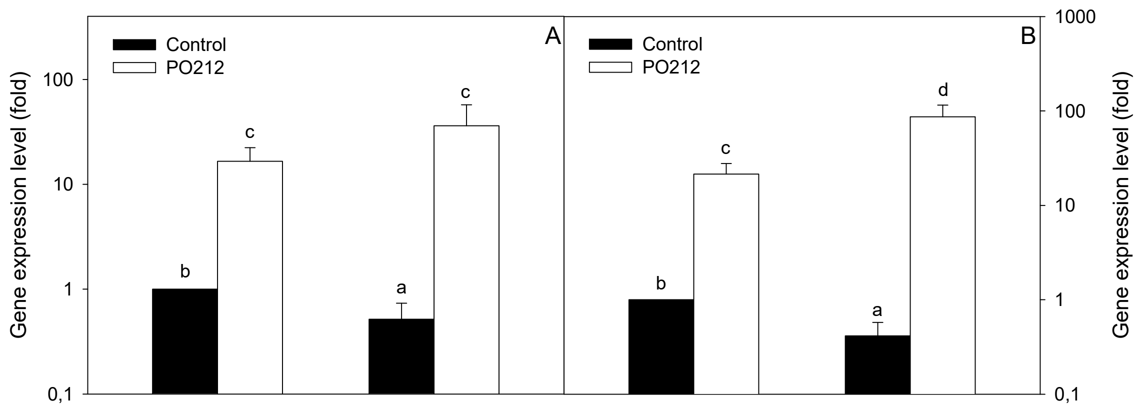

Figure 2.6. Relative gene expression in pepper cell cultures of a phenylalanine ammonia lyase gene (*CaPAL1*) and a sesquiterpene cyclase gene (*CaSC1*) after elicitation with PO212. (A) *CaPAL1* expression 6 and 24 hours after elicitation (n=3). (B) *CaSC1* expression 6 and 24 hours after elicitation (n=4). Data are means ± SE. Different letters indicate statistical differences ($p < 0.05$) in a Kruskall-Wallis test.

Finally, the phytohormone-related genes were also up-regulated after elicitation with PO212 (Fig. 2.7). Two genes involved in ethylene biosynthesis, *CaACS3* (ACC synthase) and *CaACO* (ACC oxidase), were up-regulated in pepper SCC elicited with PO212 both 6 and 24 hours after elicitation in comparison with control SCC (Fig. 2.7A and B, respectively). *CaACO* gene expression was down-regulated in control SCC 24 hours after elicitation in comparison with control SCC at 6 hours (Fig. 2.7B). *CaAOS*, an allene oxide synthase gene involved in jasmonate biosynthesis, was up-regulated 6 hours after elicitation in comparison with control SCC (Fig. 2.7C), but 24 hours after elicitation control and PO212 treatment were not significantly different. The expression of *CaICS*, an isochorismate synthase (ICS) involved in SA biosynthesis, was not detected at any time point. These data point to a possible role of both ethylene and jasmonic acid in the defense elicited by PO212 in pepper. Even though CAICS was not detectable, salicylic acid involvement cannot be ruled out, because PAL constitutes an

alternative SA biosynthetic pathway and *CaPA1L1* was up-regulated by PO212 (Fig. 2.6A).

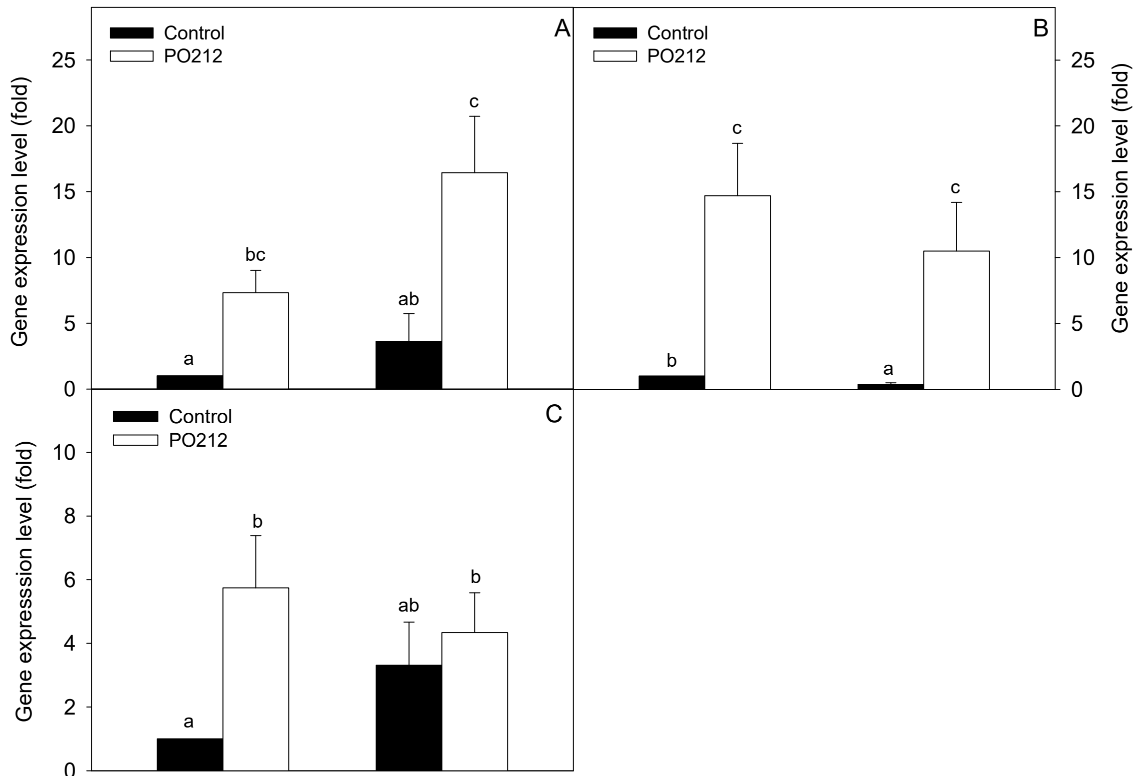

Figure 2.7. Relative gene expression in pepper cell cultures of three phytohormone-related genes (*CaACS3*, *CaACO* and *CaAOS*) after elicitation with PO212. (A) *CaACS3* expression 6 and 24 hours after elicitation (n=4). (B) *CaACO* expression 6 and 24 hours after elicitation (n=3). (C) *CaAOS* expression 6 and 24 hours after elicitation (n=4). Data are means ± SE. Different letters indicate statistical differences ($p < 0.05$) in a Kruskall-Wallis test.

Hydrogen peroxide quantification in pepper cell cultures elicited with PO212

Hydrogen peroxide levels showed differences over time after PO212 elicitation (Fig. 2.8). 5 hours after elicitation with PO212, there was a significant increase in H_2O_2 levels, and, a peak was observed in PO212 SCC 8 hours after PO212 application (Fig. 2.8). At 24 hours after elicitation, H_2O_2 levels in SCC treated with PO212 were reduce to the control SCC levels and sustained at 29 hours (Fig. 2.8). H_2O_2 levels remained stable in control SCC over time (Fig. 2.8). Therefore, there were differences in H_2O_2 only at early time points (5 and 8 hours after elicitation), which fits with the idea of H_2O_2 being an early signaling molecule.

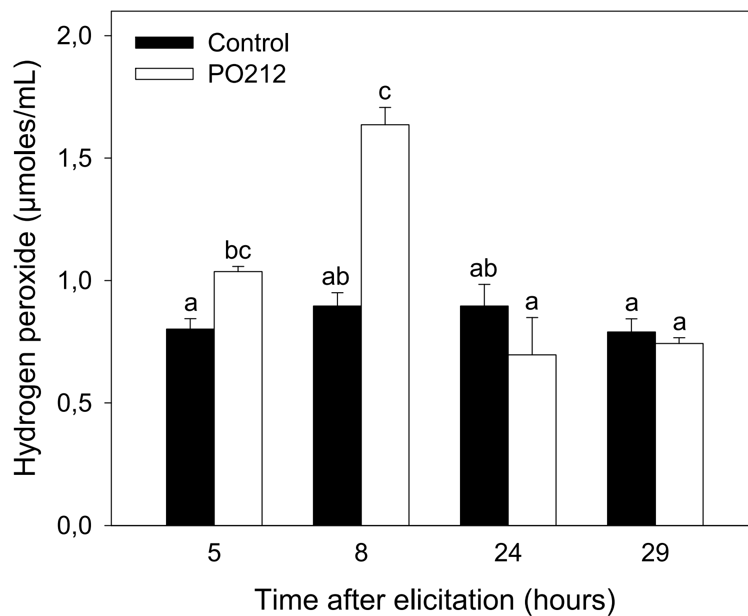

Figure 2.8. H_2O_2 in extracellular medium of elicited pepper SCC with PO212. Data are means ± SE of three independent experiments. Different letters indicate statistical differences ($p < 0.05$) in a Kruskall-Wallis test.

Discussion

In vitro plant culture has many advantages in comparison with differentiated plants: it is easily manipulated, with a higher rate of metabolism and the biosynthetic cycles compressed into shorter time periods, enhancing thus productivity (Narayani & Srivastava, 2017). Elicitation of plant cell cultures can induce defense responses that resemble those triggered by pathogen attack, such as PR-proteins, phytoalexins and phenolic compounds, which might accumulate in the extracellular space (García-Pérez *et al.*, 1998; Egea *et al.*, 2001; Gómez-Vásquez *et al.*, 2004; Ma, 2008; Sabater-Jara *et al.*, 2010, 2011; Briceño *et al.*, 2012; Lima *et al.*, 2012; Belchí-Navarro *et al.*, 2013; Tashackori *et al.*, 2018).

In this work, a significant increase in β-1,3-glucanase and chitinase activities was observed in Padron pepper cell cultures after elicitation with PO212. Moreover, the level of expression of three *PR*-genes, namely *CaBPR1*, *CaBGLU* and *CaCHI2* significantly increased after PO212-elicitation. Increased glucanase activity was also observed in the studies of García-Pérez *et al.* (1998) and Nakazawa-Ueji *et al.* (2010) 24 hours after elicitation with the oomycete *Phytophthora capsici* in *C. annuum* and *C. chinense* SCC respectively. García-Pérez *et al.* (1998) also showed a significant increase in chitinase activity 24 hours after elicitation. The application of MeJA and cyclodextrins to pepper cell cultures also induced the expression of genes that encode chitinases and β-1,3-glucanases (Sabater-Jara *et al.*, 2010). Induction of PR-proteins and *PR*-genes after abiotic/biotic elicitation of SCC was reported in many other studies (Egea *et al.*, 2001; Sabater-Jara *et al.*, 2011; Briceño *et al.*, 2012; Lima *et al.*, 2012; Belchí-Navarro *et al.*, 2013). Therefore, elicitation of pepper SCC with PO212 conidia increased β-1,3-glucanases and chitinases that can break down the cell wall components

of microbial agents, thus enhancing resistance against pathogens. In addition, the highest *CaBPR1* expression level in pepper SCC treated with PO212 could also inhibit pathogen growth due to changes in the permeability of pathogen membranes by sequestering sterol from them (Gamir *et al.*, 2017). However, enzymatic activities in pepper cell cultures elicited with PO212 did not increase as much as the expression of genes encoding for those enzymes. Genes encoding for glucanases and chitinases are highly expressed after PO212 elicitation but the proteins themselves may be inactive until contact with certain stimuli such as pathogen attack that trigger their activation (post-transcriptional regulation).

Peroxidases are reported to be induced after exposure to different elicitors (Egea *et al.*, 2001; Gómez-Vásquez *et al.*, 2004; Chakraborty *et al.*, 2009; Sabater-Jara *et al.*, 2010; Gao *et al.*, 2011; Belchí-Navarro *et al.*, 2013; Simic *et al.*, 2015). In our study, peroxidase activity in culture media of PO212-elicited SCC progressively decreased in comparison with control SCC. However, peroxidase activity in cells showed an increase after PO212 conidial suspension application. These results correlated with the observation of a significant high expression of the extracellular peroxidase gene pepper *CaPO2* and a decrease (although it was not significant) in *CaPO1* gene expression in PO212-SCC. Based on these results, we could assume that peroxidases acting in the cells and in the culture media of PO212-elicited pepper SCC are different. In this work we only analyzed the expression of two important peroxidase genes involved in plant defense, but there are at least 13 putative peroxidase genes in *Capsicum annuum* homologous to peroxidase genes in *Arabidopsis thaliana* (https://solgenomics.net/), and some of them may be acting in PO212-elicited SCC.

Phenols are one of the main types of secondary metabolites synthetized by the phenylpropanoid pathway through the phenylalanine ammonia lyase (PAL), and their

elicitation has been observed in a large number of studies using different plant cell cultures and both abiotic and biotic/fungal elicitors (Gómez-Vásquez *et al.*, 2004; Hano *et al.*, 2006; Chakraborty *et al.,* 2009; Gao *et al.*, 2011; Lima *et al.*, 2012; Tahsili *et al.*, 2013; Simic *et al.*, 2015; Miras-Moreno *et al.*, 2016; Tashackori *et al.*, 2018). In our study, we found an increase in phenolic compounds in cells of SCC elicited with PO212, but no differences were observed in the culture media. Both polyphenol oxidases and peroxidases catalyze the oxidation of phenols into highly reactive quinones, which are more toxic to pathogens and could lead to ROS production (Prasannath, 2017). According to War *et al.* (2012), it would be possible that in the culture media quinones were formed by oxidation of phenols mostly by polyphenol oxidases, leading thus to the subsequent increase in H_2O_2 observed in PO212-elicited SCC.

PAL catalyzes the monoxidative deamination of phenylalanine to trans-cinnamic acid (which is a precursor of phytoalexins and lignin) and directs the carbon flow from the shikimate pathway to the various branches of the phenypropanoid pathwhay (Dempsey & Klessig, 2017). In our study we found a significant increase in *CaPAL1* gene expression in pepper SCC elicited with PO212. Our results are in agreement with those obtained by Hano *et al.* (2006), Giberti *et al.* (2012) and Lima *et al.* (2012), which observed an up-regulation of PAL expression after treatment with fungal elicitors at times similar to ours. Up-regulation of *PAL* genes and an increased PAL activity were observed in many studies by fungal (among others) elicitors (Gómez-Vásquez *et al.*, 2004; Hano *et al.*, 2006; Chakraborty *et al.*, 2009; Gao *et al.*, 2011; Giberti *et al.*, 2012; Tahsili *et al.*, 2013; Simic *et al.*, 2015; Sarkate *et al.*, 2018; Tashackori *et al.*, 2018). Therefore, phenolic compounds produced in cells after PO212 treatment probably are

formed from phenylalanine via the shikimate/phenylpropanoid pathway due to the increase in *CaPAL1*-gene expression.

However, differences in magnitude of total phenolic compounds and *CaPAL1*-gene expression were observed in pepper cell cultures elicited with PO212. An explanation for these results is the possible existence of a bottleneck in shikimic acid pathway in which the enzyme shikimate dehydrogenase limits the throughput of PAL leading thus to a lower production of phenolic compounds in comparison with the high levels of *CaPAL1* expression observed after PO212-elicitation.

On the other hand, after PO212-treatment there was an increase in gene expression of *CASC1*, a sesquiterpene cyclase involved in the synthesis of the phytoalexin capsidiol. In pepper, phytoalexins are cyclic sesquiterpenes that are biosynthetized as a consequence of the elicitation with fungi which increase sesquiterpene cyclase activity and reduce squalene synthase activity promoting the formation of defense compound. Accumulation of extracellular sesquiterpenoids upon addition of fungal (and oomycete) elicitors to the cell cultures was previously reported. Brooks *et al.* (1986) observed the accumulation of the sesquiterpene capsidiol in suspended callus cultures of *C. annuum* after elicitation with the saprophytic fungus *Trichoderma viride*. García-Pérez *et al.* (1998) showed a significant increase in capsidiol after treatment of pepper SCC with *Phytophthora capsici*. Liu *et al.* (1999) observed that cotton SCC treated with a *Verticillium dahliae* elicitor increased the expression of a sesquiterpene cyclase. Forlani *et al.* (2011) found an up-regulation of a sesquiterpene alcohol in rice cell cultures elicited with *Magnaporthe oryzae*. Synthesis of phytoalexins/sesquiterpenes was also observed in other studies using abiotic elicitors (Chávez-Moctezuma & Lozoya-Gloria, 1996; Ma, 2008; Sabater-Jara *et al.*, 2010; Almagro *et al.*, 2012). We also studied the expression level of two genes involved in ET biosynthesis, *CaACS3* and *CaACO*, and

we observed a significant increase in the expression of both genes after PO212-elicitation. A gene involved in JA biosynthesis, *CaAOS*, showed a significant increase 6 hours after elicitation, but no differences between control SCC and PO212 SCC were observed 24 hours after PO212 application. These results point to an important role of both ethylene and jasmonic acid in the response induced by PO212 in pepper SCC. A gene involved in SA biosynthesis, *CaICS*, was not expressed at all in pepper SCC. There are two main pathways of SA biosynthesis in plants: the isochorismate pathway through the isochorismate synthase (ICS) and the phenylpropanoid pathway by the PAL (Dempsey & Klessig, 2017). The strong up-regulation of *CaPAL1* gene observed in pepper SCC elicited with PO212 may be related with the synthesis of SA; Tashackori *et al.* (2018) observed that fungal elicitation produces SA trough PAL. Therefore, we cannot exclude the involvement of SA in pepper SCC elicited by PO212. Moreover, increased SA levels were observed in elicited SCC in several studies (Li *et al.*, 2016; Sarkate *et al.*, 2018; Tashackori *et al.*, 2018).

On the other hand, it is well known that *CAPO2* is required for early H_2O_2 generation and is able to induce hypersensitive cell death, PR genes, and Systemic Acquired Resistance (SAR) (Choi *et al.*, 2007). Increased H_2O_2 (and ROS) after elicitation has been reported in many studies (Gómez-Vásquez *et al.*, 2004; Nakazawa-Ueji *et al.*, 2010; Forlani *et al.*, 2011; Almagro *et al.*, 2012; Lima *et al.*, 2012; Kushwaha *et al.*, 2019). In our study, we observed a high expression of the extracellular peroxidase gene *CaPO2* in PO212-treatment both 6 and 24 hours after elicitation. The up-regulation of *CaPO2* correlated with a significant increase in H_2O_2 production 5 and 8 hours after elicitation with PO212, leading thus to an oxidative burst as a defensive response as a result of fungal recognition in elicited SCC. However, 24 hours after PO212 application, H_2O_2 levels were reduced in pepper SCC and were maintained similar to control SCC.

We hypothesize that the augmented peroxidase activity observed after PO212 treatment in SCC cells is related with the increased level of *CAPO2* after PO212 elicitation, and, accordingly, it could lead to the release of ROS such as H_2O_2. Further, as we mentioned previously, there are other peroxidases different from *CaPO1* and *CaPO2* that could be responsible for the increased peroxidase activity in SCC cells after PO212 elicitation and they could be consuming H_2O_2, which would explain the decrease to basal levels of this ROS 24 hours after elicitation. Choi *et al.* (2007) also demonstrated that the expression levels of *CABPR1*-pepper gene depend on *CAPO2* gene expression, and this is in agreement with our results, since we observed a significant increase in *CABPR1* expression after PO212 elicitation.

The success and efficiency of elicitation depends on the plant cell line, the elicitor(s) selected, concentration, time of addition and time of exposure to the elicitor and the composition of the medium (Narayani & Srivastava, 2017; Shasmita *et al.*, 2018). Sometimes the combination of two or more elicitors induces a stronger defense response in comparison with the use of a single elicitor (Xu *et al.*, 2015); however, there are cases in which the combination of various elicitors had an antagonistic effect on the levels of certain secondary metabolites (Belchí-Navarro *et al.*, 2013).

Suspension-cultured cells of *Capsicum annuum* L. var. *annuum* cv. Padron were able to produce β-1,3-glucanases and chitinases and enhance *PR*-gene expression when the PO212-based conidia suspension was applied, in addition to the up-regulation of *CaSC1* presumably accompanied of the biosynthesis of the phytoalexin capsidiol. Other signals such as H_2O_2 production orchestrated the defense response induced by PO212. Our results show that a strong defense response was induced by PO212 in pepper cell cultures, and such a response seems to be regulated by both ET/JA and SA signaling

pathways. This response can be the reason that made the pepper plants stronger against *V. dahliae* after PO212 elicitation.

This work opens up new possibilities for the use of PO212 or their elicitors to modulate phytochemical levels in horticultural plants as pepper. The assay of different timing and concentrations for the application of PO212 will probably help to optimize the method of elicitation of plant defenses.

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Chapter 3. Plant signaling involved in the resistance induced by PO212 in pepper plants and study of direct interactions between PO212 and *Verticillium dahliae*

Introduction

Induced resistance is a promising strategy to control plant diseases and avoid excessive usage of chemicals that affect environment and human health. Many microorganisms have been used to induce resistance against a wide spectrum of pathogens in several crops, including non-pathogenic strains, saprobes, plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) (Thuerig *et al.*, 2006; Elsharkawy *et al.*, 2012; Song *et al.*, 2015; Niu *et al.*, 2016; Hossain *et al.*, 2017; Shahzad *et al.*, 2017; Jogaiah *et al.*, 2018).

In terms of biocontrol against *V. dahliae*, many BCAs have been used in different plant species (Deketelaere *et al.*, 2017). For example, PGPR such as *Pseudomonas* fluorescens PICF7 in olive (Gómez-Lama Cabanás *et al.*, 2017) or *Paenibacillus alvei* K165 in *Arabidopsis thaliana* (Tjamos *et al.*, 2005) and eggplant (Angelopoulou *et al.*, 2014), non-pathogenic strains such as *Fusarium oxysporum* F2 in eggplant (Pantelides *et al.*, 2009; Gizi *et al.*, 2011; Angelopoulou *et al.*, 2014) or *Fusarium oxysporum* Fo47 in pepper (Veloso & Díaz, 2012; Veloso *et al.*, 2015), and PGPF such as *Trichoderma harzianum* in olive (Carrero-Carrón *et al.*, 2018), *Piriformospora indica* in *Arabidopsis thaliana* (Sun *et al.*, 2014), or *Penicillium simplicissimum* in cotton (Yuan *et al.*, 2017).

As mentioned above, one of the mechanisms of action of beneficial microorganisms to control phytopathogens is induced resistance, which is regulated by plant hormone signaling pathways that can interact antagonistically or synergistically. The phytohormones salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), ethylene (ET), gibberellic acid (GA), auxin indolacetic acid (IAA), brassinosteroids (BR) and cytokinins (CT) are important modulators of plant defenses against pathogens (Berens *et al.*, 2017). In the case of *V. dahliae*, these phytohormones can act as positive and

negative regulators of disease resistance depending on plant-pathogen interaction or plant-BCA-pathogen interaction (Tjamos *et al.*, 2005; Sun *et al.*, 2014; Veloso *et al.*, 2015; Zhang *et al.*, 2016; Fousia *et al.*, 2018; Scholz *et al.*, 2018).

SA, JA and ET are the central players in resistance against pathogens. Generally, SA is involved in the resistance against biotrophs and the biotrophic phase of hemibiotrophs, and JA and ET are involved in the resistance against necrotrophs and herbivores, being JA and ET synergists of each other, and both are also antagonists of SA (Glazebrook, 2005). However, in several cases, there is a cross-talk between SA, JA and ET which provides an effective protection against pathogens that enter in contact with the plant (Salas-Marina *et al.*, 2011). The concentration and timing of these hormones as well as their interaction with each other provide the plant with a widespread network to develop the stimuli and respond appropriately to different stresses (Pieterse *et al.*, 2012). PGPR and PGPF are usually related to the JA/ET signaling pathway, but there are studies in which induced resistance triggered by BCAs against pathogens is related with JA/ET and SA pathways (Salas-Marina *et al.*, 2011).

SA is a naturally occurring phenolic compound that can be synthetized in plastids by the isochorismate synthase (ICS) pathway and/or the phenylalanine ammonia-lyase (PAL) pathway (Dempsey & Klessig, 2017), and it was recently reported that both pathways are required for resistance against pathogens (Shine *et al.*, 2016). SA has several inactive derivatives that regulate its active levels in cytoplasm (Dempsey & Klessig, 2017). Salicylic acid β-glucoside (SAG) is the major non-volatile conjugate of SA and the most common storage form. SAG is generated via glucosylation of SA at its hydroxyl group, and is transported from the cytosol to the vacuole, where subsequently is accumulated and hydrolyzed to release SA after pathogen attack (Dempsey & Klessig, 2017). Other important SA-conjugated is MeSA, which is a volatile ester

generated via methylation of SA and serves as an endogenous mobile signal to trigger SAR (Dempsey & Klessig, 2017).

NPR1 is the major regulator of SA, which interacts with TGA transcription factors that are involved in the activation of PR genes. Downstream of NPR1, WRKY transcription factors are also activated during pathogen attack, playing important roles in the regulation of SA-dependent defense responses. Both NPR1 and WRKY transcription factors are points of crosstalk between SA and JA signaling pathways (Bari & Jones, 2009). The activation of the JA/ET-responsive gene PDF1.2 (Plant Defensin1.2) in Arabidopsis is also an evidence of the antagonistic interaction between SA and JA/ET pathway (Takahashi *et al.*, 2004; Ndamukong *et al.*, 2007).

JA is an oxylipin synthetized in plastids from α-linoleic acid by the octadecanoid pathway. The precursor of JA, cis-12-oxo-phytodienoic acid (OPDA), moves to peroxisomes where is reduced to JA. OPDA can regulate the expression of a separate subset of genes in which are involved the enzymes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Wasternack & Hause, 2013). There are four main JA-signaling components: coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) Jasmonate insensitive 1/MYC2 (JIN1/MYC2), and jasmonate ZIM-domain (JAZ) proteins (Bari & Jones, 2009). JA is conjugated with the amino acid isoleucine by JAR1, which encodes a JA amino acid synthetase, to form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), which is the bioactive JA molecule perceived by plants. During pathogen attack, JA-Ile is perceived by COI1, which encodes an F-box protein, triggering the degradation of the JAZ proteins and leading to the transcriptional activation of JA-responsive genes. In the absence of JA-Ile, JAZ proteins can interact with JIN1/MYC2, which encodes a transcription factor involved in the transcriptional

regulation of some JA-responsive genes, thus blocking the expression of JA-responsive genes (Bari & Jones, 2009; Hickman *et al.*, 2017).

ET is a gaseous hormone synthetized from methionine involving the enzymes 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (Booker & DeLong, 2015). ET is perceived by a group of several receptors, e.g. in Arabidopsis there are five receptors, namely ETR1 (Ethylene Response 1), ERS1 (Ethylene Response Sensor 1), ETR2 (Ethylene Response 2), ERS2 (Ethylene Response Sensor 2) and EIN4 (Ethylene Insensitive 4). In absence of ET, these receptors inhibit the protein EIN2 (Ethylene Insensitive 2) through CTR1 (Constitutive Triple Response 1) (Gallie, 2015). In presence of ET, EIN2 suffers the proteolytic release of the EIN2 C-end, which migrates to the nucleus inhibiting EIN3 (Ethylene Insensitive 3) and EIL1 (EIN3-like) degradation. EIN3 and EIL1 are consequently stabilized and activate the transcription factors that trigger the ET-response genes (Gallie, 2015). EIN3 and ELI1, along with JAZ proteins, can modulate positively or negatively the crosstalk between ET and JA. Moreover, the Ethylene Response Factor1 (ERF1) can be activated by ET or JA and synergistically by both hormones, being a key element for the integration of both signals in the defense response (Bar & Jones, 2009).

Besides phytohormones, reactive oxygen species (ROS) are also important modulators of plant defense responses. ROS can act as toxic compounds that directly kill the pathogen or as signaling molecules that activate plant defenses (Lehman *et al.*, 2015). Production of ROS such as superoxide anion ($O^{\cdot}_2{}^-$), hydroxyl radical (·OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) is one of the earliest plant responses to pathogen invasion (Lehman *et al.*, 2015; Camejo *et al.*, 2016; Ali *et al.*, 2018). Generally, ROS levels are balanced between production and degradation, but, when

plants are attacked by pathogens, ROS increase quickly in the apoplast, leading to an oxidative burst. Depending on the plant-pathogen interaction, ROS production takes place in the apoplast, but also in chloroplasts, mitochondria and peroxisomes (Lehman *et al.*, 2015; Ali *et al.*, 2018).

Biphasic ROS in plants responding to pathogen infection is well known. Firstly, ROS are accumulated within minutes after pathogen recognition in one place. Secondly, ROS are produced hours after pathogen recognition in several locations, leading generally to the establishment of plant defenses and HR (Camejo *et al.*, 2016).

ROS overproduction affects cell process negatively by oxidizing proteins, carbohydrates, lipids, DNA and/or RNA. Therefore, plant cells are able to activate antioxidant enzymes such as peroxidase (POX), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), polyphenol oxidase (PPO), superoxide dismutase (SOD), and/or catalase (CAT) to prevent ROS accumulation and cell toxicity (Lehman *et al.*, 2015; Camejo *et al.*, 2016; Ali *et al.*, 2018). These enzymes regulate the cellular redox potential under stress conditions and their activity depends on the level of stress perception by plants. NADPH oxidases are the first players in triggering ROS oxidative burst in the apoplast after pathogen attack, but cell wall peroxidases (apoplastic peroxidases), lipoxygenases and/or oxalate oxidases are also activated during plant-pathogen interaction as ROS sources (Lehman *et al.*, 2015; Camejo *et al.*, 2016; Ali *et al.*, 2018).

Deposition of lignin/callose as structural barriers to reinforce plant cell wall, and synthesis of ROS (such as H_2O_2), phytoalexins and PR-proteins are the main events during plant-*V. dahliae* interaction (Chmielowska *et al.*, 2010; Gayoso *et al.*, 2010; Veloso & Díaz, 2012). It has been previously demonstrated a protective effect of PO212

against *Verticillium dahliae* (Chapter 1). In fact, induction of lignification and genes involved in phytoalexin production and PR-proteins has been observed in pepper treated with PO212 in previous studies (Chapter 1). In order to further understand the mechanism orchestrating the responses that lead to resistance caused by the biocontrol agent PO212 in pepper, the involvement of plant hormones has been examined by measuring endogenous levels, and, regarding the importance of ROS in plant signaling, H_2O_2 levels were also analyzed. Several genes involved in phytohormone biosynthesis were also analyzed in pepper roots induced with PO212 both before and after inoculation with *V. dahliae*: *CaPAL1* and *CaICS* (two genes involved in salicylic acid biosynthesis), *CaACO* and *CaACS3* (two genes involved in ethylene biosynthesis) and *CaAOS* (a gene involved in jasmonic biosynthesis). Moreover, the involvement of ET, SA, JA and ABA was also examined by using tomato mutants impaired in different steps of these phytohormone signaling routes. Tomato mutans are available and characterized long time ago because is a model solanaceous plant, whereas pepper mutants are not available and it is difficult to obtain transformed plants with solanaceous crop.

The mode of action of fungal BCAs can be divided into two main mechanisms: direct antagonism through competition for space or nutrients (infection sites), mycoparasitism (phytopathogen cell walls are degraded by degrading enzymes produced from biocontrol fungi) and/or antibiosis (production of antimicrobial compounds as inhibitors of phytopathogen growth), or indirect antagonism through plant growth promotion and activation of the host plant defenses (induced resistance). Sometimes only one mechanism acts to control a disease, but, at other times, multiple mechanisms operate simultaneously (Ghorbanpour *et al.*, 2018). It is important to understand the mechanism(s) employed by these strains to colonize the root surface because during this

colonization many interactions take place with the plant and other root microorganisms, and these interactions will determine the mechanism by which biocontrol will be achieved. We previously demonstrated that induced resistance is one of the mechanisms of action of PO212 in pepper against *V. dahliae* (Chapter 1). However, other mechanisms of action may be operating in this tripartite interaction. Therefore, we finally conducted a microscopical study of PO212_inGFP9 (Villarino *et al.*, 2018) root colonization to observe the behavior of this BCA in pepper plants and check other potencial modes of action that may be operating in the protection conferred by PO212 in pepper.

Materials and methods

Plant material

Pepper

Capsicum annuum L. cv. Padron seeds harvested in previous years in our greenhouse were stored at 4ºC and low humidity conditions. Before sowing, seeds were disinfected in 0.1% (v/v) commercial bleach for 15 min, washed and stored overnight in darkness. Subsequently seeds were sown in sterile vermiculite, and watered with nutrient solution (Hoagland & Arnon, 1950). Plants were grown in a growth chamber with a photoperiod of 16 h light at 25ºC and 8 h darkness at 18ºC during 24 days.

In the case of seedlings used in microscopy assays, details are described in other section (see below).

Tomato

Wild type tomato (*Solanum lycopersicum* L.) cv. Pearson and a mutant of tomato that blocks ethylene perception, *Never ripe* (*Nr*), were kindly provided by Dr. Jan van Kan (Wageningen Universty, The Netherlands). Wild type tomato cv. Castlemart and a

mutant of tomato, *Defenseless-1* (*def-1*), which is deficient in wound- and systemin-induced JA accumulation and expression of downstream target genes, were kindly provided by Victor Flors (University Jaume I, Spain). Wild type tomato cv. Money Maker and a mutant of tomato, *NahG*, which is deficient in salicylic acid synthesis, were kindly provided by Victor Flors (University Jaume I, Spain). Wild type tomato cv. Rheinlands Rhum and a mutant of tomato, *Sitiens* (*sit*), which is deficient in functional enzyme activity at the final step in abscisic acid biosynthesis, were kindly provided by Victor Flors (University Jaume I, Spain).

Tomato seeds were sown in sterile vermiculite and watered with nutrient solution (Hoagland & Arnon, 1950). Plants were grown in a growth chamber with a photoperiod of 16 h light at 25ºC and 8 h darkness at 18ºC during 20 days.

Fungal material

Penicillium rubens 212 ATCC 201888 (PO212) was kindly provided by I. Larena (INIA, Madrid). Dried conidia were used in all the cases as it was described in Chapter 1. The *Verticillium dahliae* isolate Vd53 was cultured and obtained as described in Chapter 1. PO212_inGFP9 was kindly provided by I. Larena from INIA (Madrid), and was previously obtained as described by Villarino *et al.* (2018). Transformed strains were maintained on PDA DIFCO medium and incubated at 25 ºC in the dark.

PO212 treatment method

For both pepper and tomato plants, PO212 conidia were gently dislodged in nutrient solution (Hoagland & Arnon, 1950), and the conidial suspension was applied to the roots of the plants as it was described in Chapter 1. Plants were incubated in the growth chamber with a photoperiod of 16 h light at 25ºC and 8 h darkness at 18ºC throughout the experiments.

Inoculation with *Verticillium dahliae*

Inoculum preparation for pepper plants

For pepper plants, the inoculum was obtained as described by Díaz *et al.* (2005). The concentration of the inoculum was adjusted to 10^6 conidia·mL^{-1}.

Inoculum preparation for tomato plants

For tomato plants, the inoculum was also obtained as described by Díaz *et al.* (2005), and the concentration of the inoculum was adjusted to $2x10^6$ conidia·mL^{-1}.

Pepper-inoculation treatment

Pepper plants treated with the PO212-conidial suspension or sterile nutrient solution were transferred to sterile flasks with fresh sterile nutrient solution where roots were dipped during 45 minutes (mock inoculation) or in a *V. dahliae*-conidial suspension in sterile nutrient solution during 45 minutes (*V. dahliae*-inoculation) following the protocol described in Veloso & Díaz (2012). After pathogen inoculation, pepper plants were transplanted to a mixture of soil/perlite (3:1, v/v) previously autoclaved. Control plants without PO212 treatment were included in the assays. Independent experiments were carried out with 14 plants per treatment.

Tomato-inoculation treatment

Tomato plants were treated with PO212 and inoculated as described above for pepper plants, except that the time of inoculation was 2 hours instead of 45 minutes. The number of wilted leaves was recorded at 7, 14 and 21 days post-inoculation (dpi). Several independent experiments were carried out in all the cases. The number of experiments and the number plants per treatment in each experiment is indicated in the legend of the figures.

Gene expression study

Roots were sampled 48 h after PO212-treatment (just before inoculation) and 24 h after inoculation with *V. dahliae*. The samples (six roots per sample) were frozen with liquid nitrogen and stored at -80 ºC. The protocol described in Veloso & Díaz (2012) was followed to process the samples. We analyzed the expression of different genes involved in phytohormone biosynthesis. The primers that were used are listed in Table 3.1.

Table 3.1. Primers used to quantify the target genes by real time qPCR.

Gene	Accession number	Reference	Primer		
			Name	**Sequence**	**Amplicon**
CaPAL1	KF279696	García *et al.* (2018)	*CaPAL1FW* *CaPAL1RV*	5'GTGGCACGATCACTGCCTCG3' 5'TGGTCCGTGAACTCGGGCTT3'	**319 bp**
CaACO	AJ011109	Carballeira (2010)	*CaACOFW* *CaACORV*	5'CGCCACTCCATTGTG3' 5'TAGATTACTGCATCGCTTCC3'	**152 bp**
CaACS3	X82265	García (2018)	*CaACS3FW* *CaACS3RV*	5'TCTGCTTGCGTCAATGTTGTCTG3' 5'TCCTCCACAGTTCCAATTCAGCA3'	**215 bp**
CaAOS	DQ832720	García (2018)	*CaAOSFW* *CaAOSRV*	5'TGTCTACGAATCTCTCCGCA3' 5'GGGACAAATTCTTCAGCCCT3'	**183 bp**
CaICS	AY743431	García (2018)	*CaICSF2* *CaICSR2*	5'GCCAGAACTCATGTGCCGGG 3' 5'AATGCTGAGGCGGTCCCGAT 3'	**273 bp**
CaACT	AY572427	Silvar *et al.* (2008)	*CaACTFW* *CaACTRV*	5'ATCCCTCCACCTCTTCACTCTC3' 5'GCCTTAACCATTCCTGTTCCATTATC3'	**128 bp**

The actin gene was used as housekeeping gene (Veloso & Díaz, 2012). The RNA extraction and the reverse transcription were carried out following the protocol of the Bio-Rad Aurum-Total RNA Mini kit and iScript-cDNA Synthesis Kit, respectively. An extensive description of qPCR reactions and data analysis were previously described in Veloso and Díaz (2012). At least two independent experiments were carried out.

Phytohormone quantification in pepper plants

Different plant hormones were quantified in pepper roots treated with PO212 both before and after inoculation with *V. dahliae*. Pepper roots from 7 plants were collected before inoculation (0 hours), and 8 and 24 hours after inoculation with *V. dahliae* and immediately frozen in liquid nitrogenand lyophilized. Then, they were homogenized and 50 mg were placed in a tube to be analyzed according to Sánchez-Bel *et al.* (2018) and García (2018). Samples were analyzed by Victoria Pastor and Victor Flors (Metabolic Integration and Cell Signaling Group, Universitat Jaume I, Castellón, Spain). Salicylic acid (SA), 4-hydroxybenzoic acid (4-HBA), jasmonic acid (JA) and abscisic acid (ABA) were analyzed in Acquity Ultraperformance Liquid Chromatography System (UPLC) coupled to triple quadrupole mass spectrometry (TQD) as described by Sánchez-Bel *et al.* (2018). For the hormone extraction, 1 mL of Milli-Qwater and internal standards containing a pool ofabscisic acid-d6 (ABA-d6), salicylic acid-d5 (SA-d5), indole acetic acid-d5 (IAA-d5), dehydrojasmonic acid (hJA) and JA-Ile-d6 was added to each sample and incubated for 1 h in ice. Five glass beads (2mm of diameter) were added to each sample and then they were homogenized in a shaker for 3 min 30 Hz. Samples were centrifuged at 15493.24 xg at 4 °C for 40 min and the supernatant was recovered and its pH was adjusted to 2.6 with 30% acetic acid. After that, a liquid-liquid extraction was performed twice with 1.5 mL of diethyl ether. Both organic phases were gathered to fresh tube after a centrifugation of 3 min at 3689.4 xg and 4 °C. Then the supernatant was evaporated in a Speedvac. The precipitated was resuspended in 1 mL of 10% methanol and filtered through a 0.22 µm RC membrane filter. A 20 µL of this solution was then directly injected into the HPLC system. Samples were injected in a Kinetex C18 analytical column with a 5µm particle size, 2.1 100 mm (Phenomenex) for component separation. The conditions of UPLC

and mass spectrometry are described in Sánchez-Bel *et al.* (2018). Masslynx NT v.4.1 (Waters) software was used to process the data. The hormone quantity in the samples was expressed as ng per g of dry weight.

H_2O_2 quantification in pepper plants

H_2O_2 production was monitored using the xylenol orange assay (García, 2018). The xylenol orange reagent was freshly prepared and remained stable for 6 to 8 h. One milliliter of solution [98 mg $FeSO_4$ dissolved in 1.332 mL H_2SO_4] was added to 100 mL of 125 mM xylenol orange (9.5 mg) and 100 mM sorbitol (1.82 g). Samples were homogenized in liquid N_2 in a mortar with 0.2N HCl and the extracts were centrifuged at 10000 xg during 5 minutes at 4 ºC. The supernatants were mixed with 50 mM phosphate buffer pH 5.7 and 0.2 N NaOH (1:1). The reaction consisted of 100 µL of the mixture with 1 mL of xylenol orange. The reaction was incubated for 30 min at room temperature before measuring the A560. The blank contained 100 µL of a mixture of HCl 0.2N, 50 mM phosphate buffer pH 5.7 and 0.2N NaOH and 1 mL of xylenol orange. We collected samples at 2, 4, 6 and 8 hours after PO212-treatment, and 0, 0.1, 2, 4, 6 and 8 hours after inoculation with *V. dahliae*. The H_2O_2 quantity in the samples was expressed as nmoles per g of fresh weight.

Microscopic assay and sample preparation in pepper plants

Padron pepper seeds were disinfected in 10% (v/v) commercial bleach for 30 min, washed with sterile tap water and incubated for 5 to 7 days in high humidity conditions in Petri dishes. Germinated seeds were put on Petri dishes with Hoagland agar medium (Hoagland & Arnon, 1950) and incubated for 7 days. The resulting plants were inoculated with PO212_inGFP9 according to Veloso *et al.* (2016). Pepper roots were inoculated using a single 0.5 µl droplet of a 6 x 10^6 conidia per mL suspension, 1 cm

apart from the root neck (Fig. 3.1). Then, plants were incubated in a growth chamber with a photoperiod of 16 h light at 25ºC and 8 h darkness at 18ºC. Plants were prepared for microscopic observation once a day for a week after the induction with PO212_inGFP9. Two cm pieces of pepper root were cut for superficial observation. The root piece was mounted in a microscope slide with Citifluor AF1™ antifading mounting medium, and two cm root pieces were then sliced with a diamond blade and mounted on the same mounting medium for transversal examination. Observation of the fluorescence marker GFP was carried out on a Nikon A1 Confocal laser microscope system, and images were acquired and processed with the software NIS-Elements Advanced Research 3.00 SP6.

Figure 3.1. Inoculation of pepper plants with PO212-GFP.

Statistical analysis

All statistical analyses were performed using STATGRAPHICS 5.1 for Windows. The tests employed and the significance level are indicated in each figure. If possible, parametric tests were preferred, but if data did not fit normality of variance test even with transformations, non-parametric tests were used instead. Statistically significant differences are indicated in the figures.

Results

The protection of PO212 against *Verticillium* in tomato is broken in ABA, JA and SA tomato mutants

Treatment with PO212 before inoculation with *V. dahliae* protected all wild type tomato cultivars against this pathogen (Fig. 3.2, 3.3, 3.4 and 3.5).

Wild type tomato cv. Pearson treated with PO212 and challenged with *V. dahliae* showed a significant reduction of symptoms from the second week after inoculation to the third week after inoculation (Fig. 3.2A). The wilting began at the second week in all the plants inoculated with *V. dahliae* but the plants induced with PO212 showed reduced wilt symptoms compared with the non-induced. Tomato *Never ripe* mutants (*Nr*) treated with PO212 also showed a significant reduction in *Verticillium* symptoms from the second week after inoculation in comparison with non-induced and inoculated *Nr* plants (Fig. 3.2B).

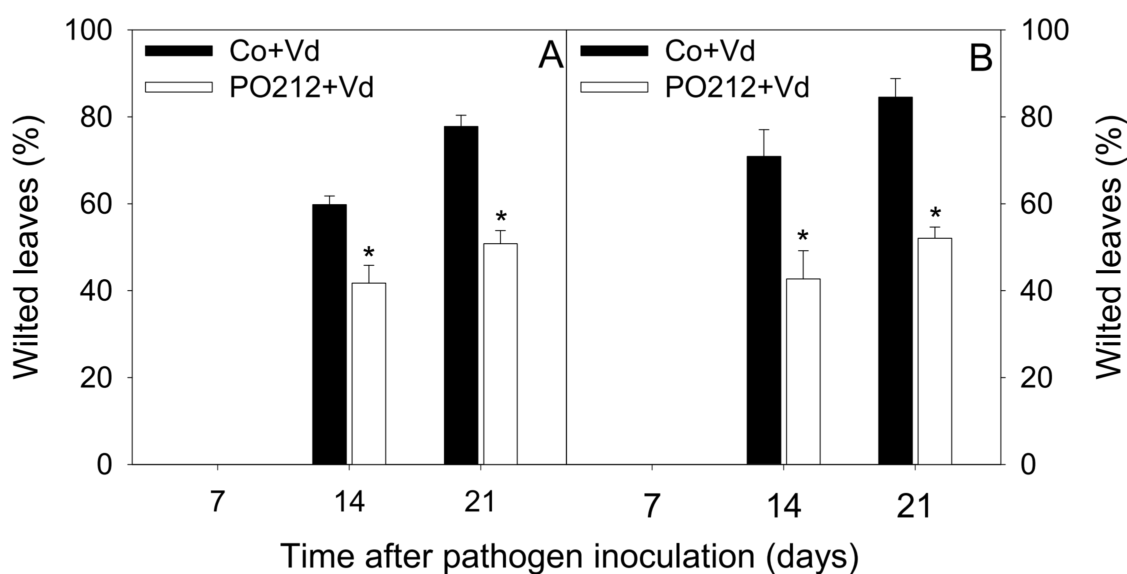


Figure 3.2. PO212 confers protection to verticillium wilt in both wild type and *Never ripe,* a tomato mutant insensitive to ethylene. (A) Percentage of wilted leaves recorded after inoculation of wild type tomato cv. Pearson roots with *V. dahliae*. (B) Percentage of wilted leaves recorded after inoculation of *Never ripe* with *V. dahliae*. Data are means ± SE of three independent experiments with five plants per treatment and experiment (n=15). Asterisks indicate statistical differences respect to the control ($p < 0.05$) in a Mann-Whitney test. Co- Control, Vd- *V. dahliae*.

Wild type tomato cv. Rheinlands Rhum treated with PO212 and challenged with *V. dahliae* showed a significant reduction of symptoms from the first week after inoculation to the second week after inoculation (Fig. 3.3A). The wilting began at the first week in all the plants inoculated with *V. dahliae* but the plants induced with PO212 showed reduced wilt symptoms compared with the non-induced. *Sitiens* (*sit*) tomato mutants treated with PO212 showed a similar percentage of wilted leaves as the non-induced and inoculated *sit* plants one and two weeks after pathogen inoculation (Fig. 3.3B).

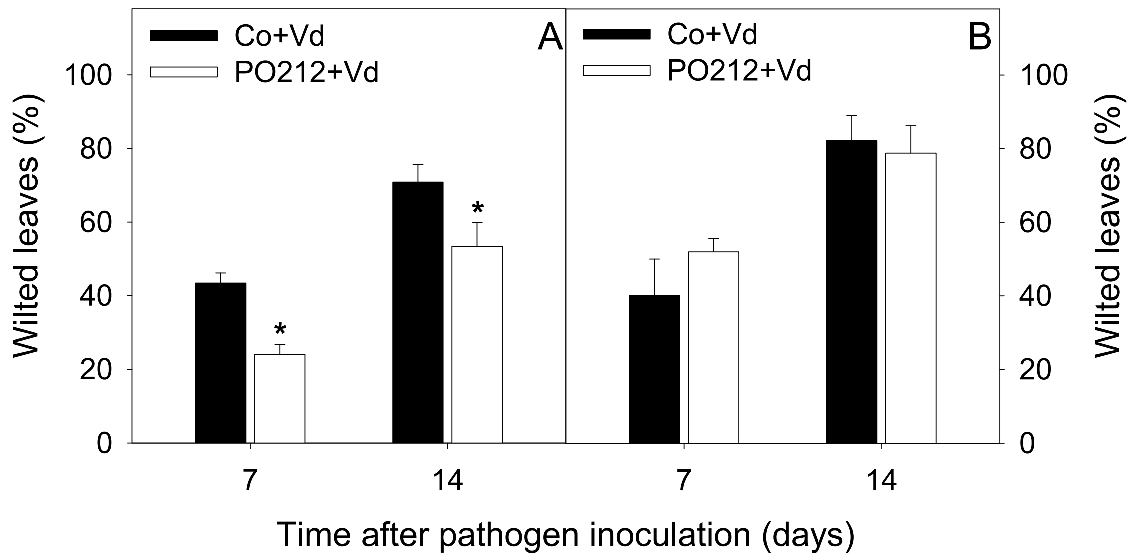


Figure 3.3. PO212 confers protection to verticillium wilt in wild type tomato, but does not protect *Sitiens*, a tomato mutant deficient in abscisic acid production. (A) Percentage of wilted leaves recorded after inoculation of wild type tomato cv. Rheinlands Rhum tomato with *V. dahliae*. (B) Percentage of wilted leaves recorded after inoculation of S*itiens* tomato mutant with *V. dahliae*. Data are means ± SE of two independent experiments with four plants per treatment and experiment (n=8). Asterisks indicate statistical differences respect to the control ($p < 0.05$) in a Mann-Whitney test. Co- Control, Vd- *V. dahliae*.

Wild type tomato cv. Castlemart treated with PO212 and challenged with *V. dahliae* showed a significant reduction of symptoms from the first week after inoculation to the second week after inoculation (Fig. 3.4A). The wilting began at the first week in all the plants inoculated with *V. dahliae* but the plants induced with PO212 showed reduced wilt symptoms compared with the non-induced. No differences in percentage of wilted leaves were observed in *Defenseless-1* (*def-1*) tomato mutants (impaired in JA biosynthesis) between control and PO212-treated plants (Fig. 3.4B).

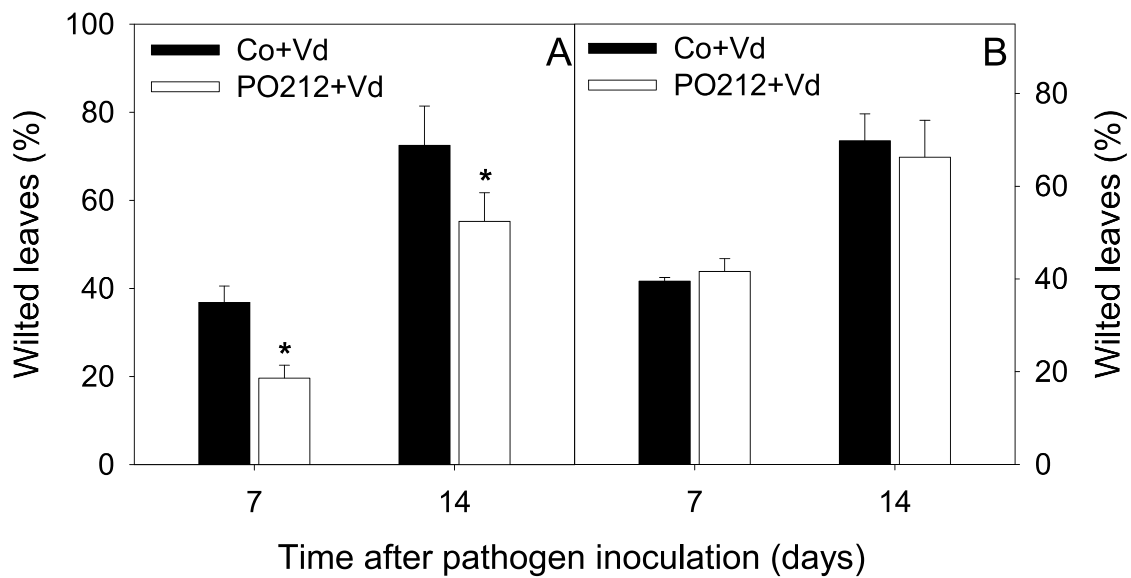


Figure 3.4. PO212 confers protection to verticillium wilt in wild type tomato, but does not protect *defenseless-1*, a tomato mutant impaired in jasmonic acid production. (A) Percentage of wilted leaves recorded after inoculation of wild type tomato cv. Castlemart tomato with *V. dahliae*. (B) Percentage of wilted leaves recorded after inoculation of *defenseless-1* tomato mutant roots with *V. dahliae*. Data are means ± SE of two independent experiments with seven plants per treatment and experiment (n=14). Asterisks indicate statistical differences respect to the control ($p < 0.05$) in a Mann-Whitney test. Co- Control, Vd- *V. dahliae*.

Wild type tomato cv. Money Maker treated with PO212 and challenged with *V. dahliae* showed a significant reduction of symptoms at the second week after inoculation (Fig. 3.5A). The wilting began at the second week in all the plants inoculated with *V. dahliae* but the plants induced with PO212 showed reduced wilt symptoms compared with the non-induced. No differences in percentage of wilted leaves were observed in *NahG* tomato mutants between control and PO212-treated plants (Fig. 3.5B).

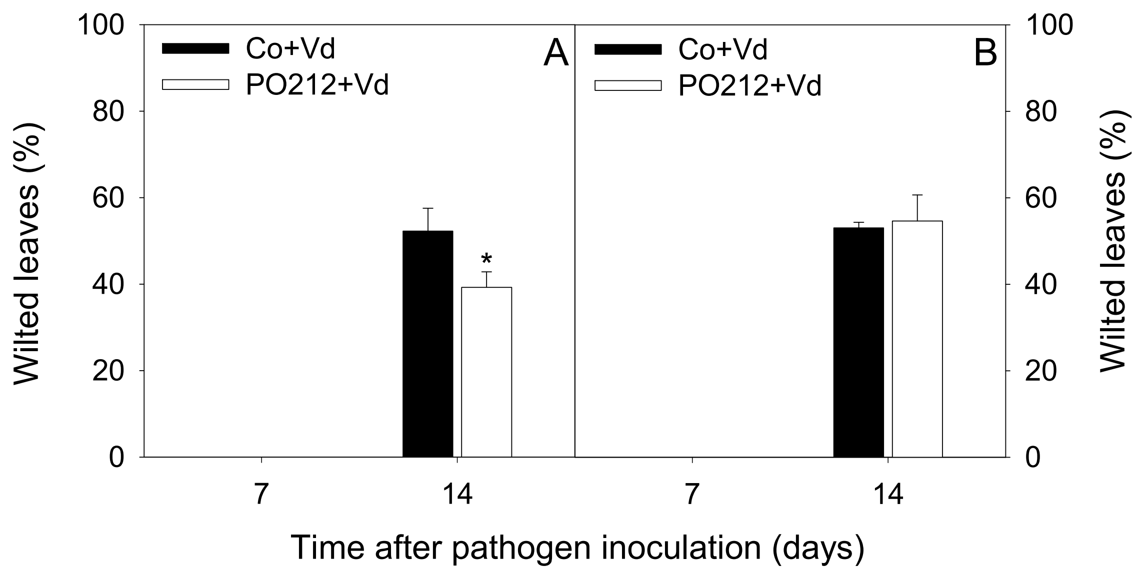


Figure 3.5. PO212 confers protection to verticillium wilt in wild type tomato, but does not protect *NahG*, a tomato mutant with low levels of salicylic acid. (A) Percentage of wilted leaves recorded after inoculation of wild type tomato cv. Money Maker tomato with *V. dahliae*. (B) Percentage of wilted leaves recorded after inoculation of *NahG* tomato mutant with *V. dahliae*. Data are means ± SE of two independent experiments with ten (Wild type)/six (NahG) plants per treatment and experiment (n=20/12). Asterisks indicate statistical differences respect to the control ($p < 0.05$) in a Mann-Whitney test. Co- Control, Vd- *V. dahliae*.

PO212 increases gene expression of hormone biosynthetic genes in pepper

A phenylalanine ammonia lyase gene, *CaPAL1*, was significantly up-regulated in pepper roots treated with PO212 before inoculation with *V. dahliae* (Fig. 3.6A). *CaPAL1* is involved in the biosynthesis of salycilic acid. However, this gene was significantly down-regulated after inoculation in PO212-treated plants (Fig. 3.6B).

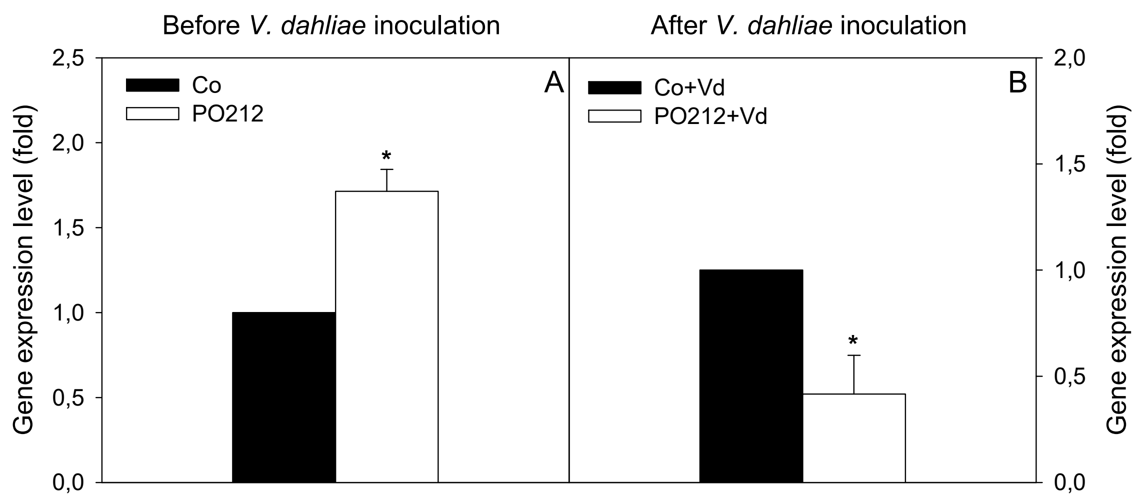
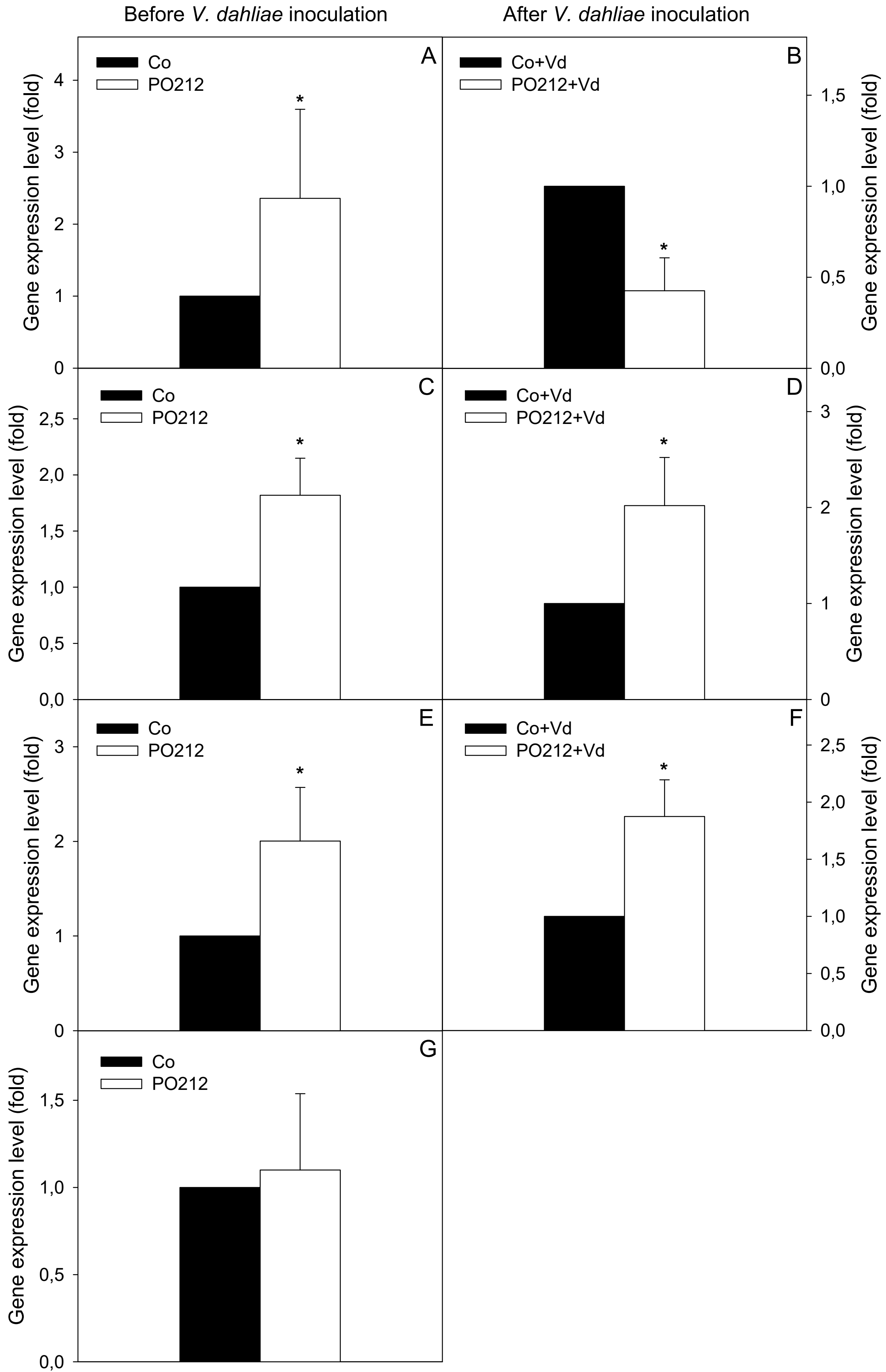


Figure 3.6. Effect of PO212 on relative expression of *CaPAL1* in pepper roots before and after inoculation with *V. dahliae*. (A) *CaPAL1* expression before inoculation (n=3). (B) *CaPAL1* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=4). Data are means ± SE. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

The expression of two ethylene-related genes *(CaACO and CaACS3)* was induced significantly in pepper roots treated with PO212 before inoculation with *V. dahliae* (Fig. 3.7A, C). However, only *CaACS3* gene was significantly up-regulated after *V.dahliae* inoculation (Fig. 3.7D), whereas *CaACO* was significantly down-regulated (Fig. 3.7B). The jasmonate-related gene *CaAOS* was induced significantly both before and after inoculation with *V. dahliae* in PO212-treated roots (Fig. 3.7E, F). On the other hand, the other gene involved in salicylic acid biosynthesis, *CaICS*, was only expressed before inoculation with *V. dahliae*, and no differences in gene expression were observed between control plants and PO212-treated plants (Fig. 3.7G).



Before *V. dahliae* inoculation
After *V. dahliae* inoculation
A
B
C
D
E
F
G
Co
PO212
Co+Vd
PO212+Vd
Gene expression level (fold)
*

Figure 3.7. Effect of PO212 on relative expression of *CaACO*, *CaACS3*, *CaAOS* and *CaICS* genes in pepper roots before and after inoculation with *V. dahliae*. (A) *CaACO* expression before inoculation (n=3). (B) *CaACO* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=3). (C) *CaACS3* expression before inoculation (n=4). (D) *CaACS3* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=3). (E) *CaAOS* expression before inoculation (n=3). (F) *CaAOS* expression 24 hours after inoculation of pepper roots with *V. dahliae* (n=3). (G) *CaICS* expression before inoculation (n=4). Data are means ± SE. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

PO212 decreases SA levels, whereas increases the 4-HBA and ABA content

The internal levels of several plant hormones and a related compound (4-HBA) were measured in pepper roots treated with PO212 before and after pathogen inoculation.

SA levels significantly decresed in pepper plants induced with PO212 before inoculation (Fig. 3.8A). However, 8 and 24 hours after pathogen inoculation, no significant differences were observed between control plants and PO212-treated plants (Fig. 3.8B and C).

4-HBA levels significantly increased in pepper plants induced with PO212 before inoculation (Fig. 3.9A). However, 8 and 24 hours after pathogen inoculation, no significant differences were observed between control plants and PO212-treated plants (Fig. 3.9B and C).

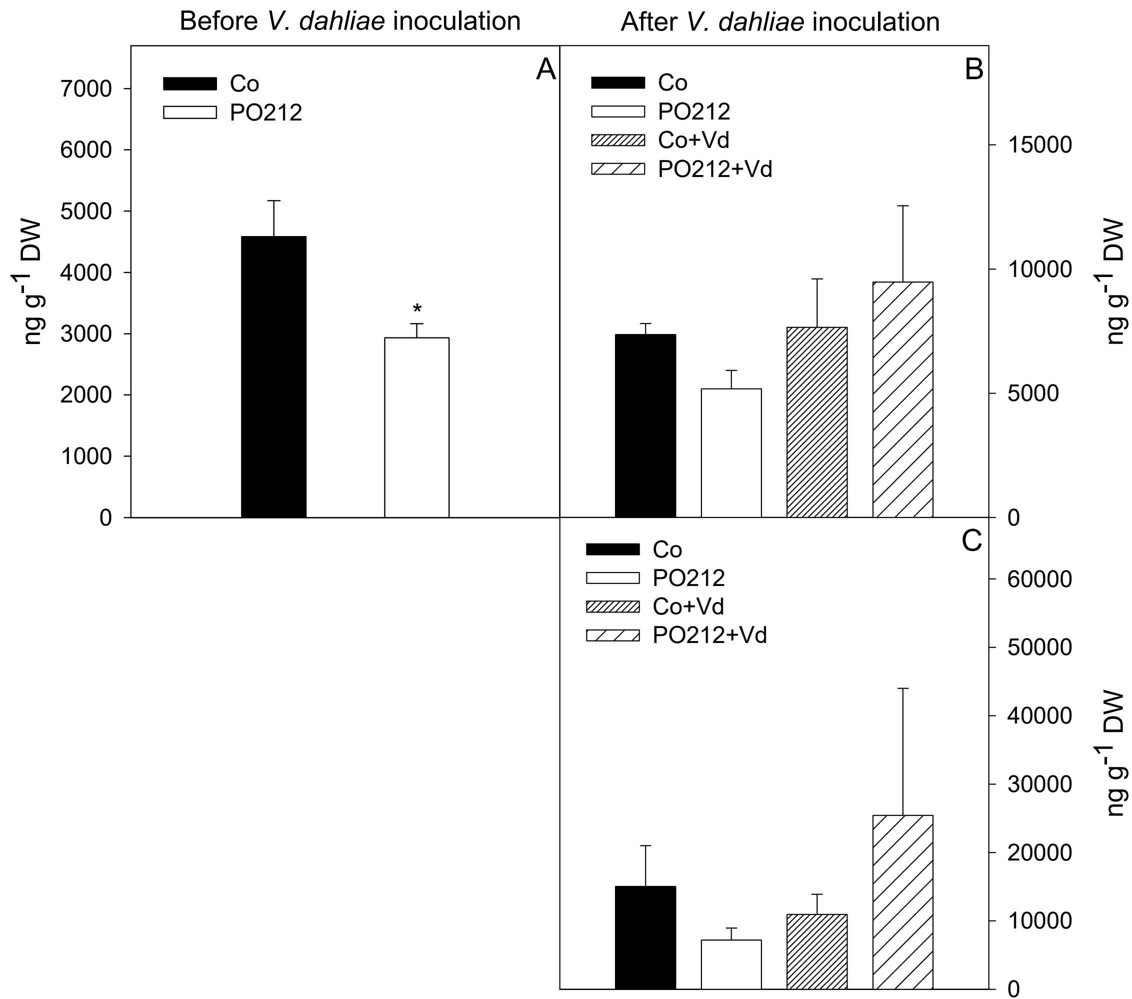


Figure 3.8. Salicylic acid (SA) levels measured in roots of pepper before and after inoculation of pepper roots with *V. dahliae*. (A) SA concentrations measured in roots before inoculation. (B) SA concentrations measured in roots 8 hours after inoculation with *V. dahliae*. C) SA concentrations measured in roots 24 hours after inoculation with *V. dahlia*. Data are the mean ± SE of three independent experiments. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

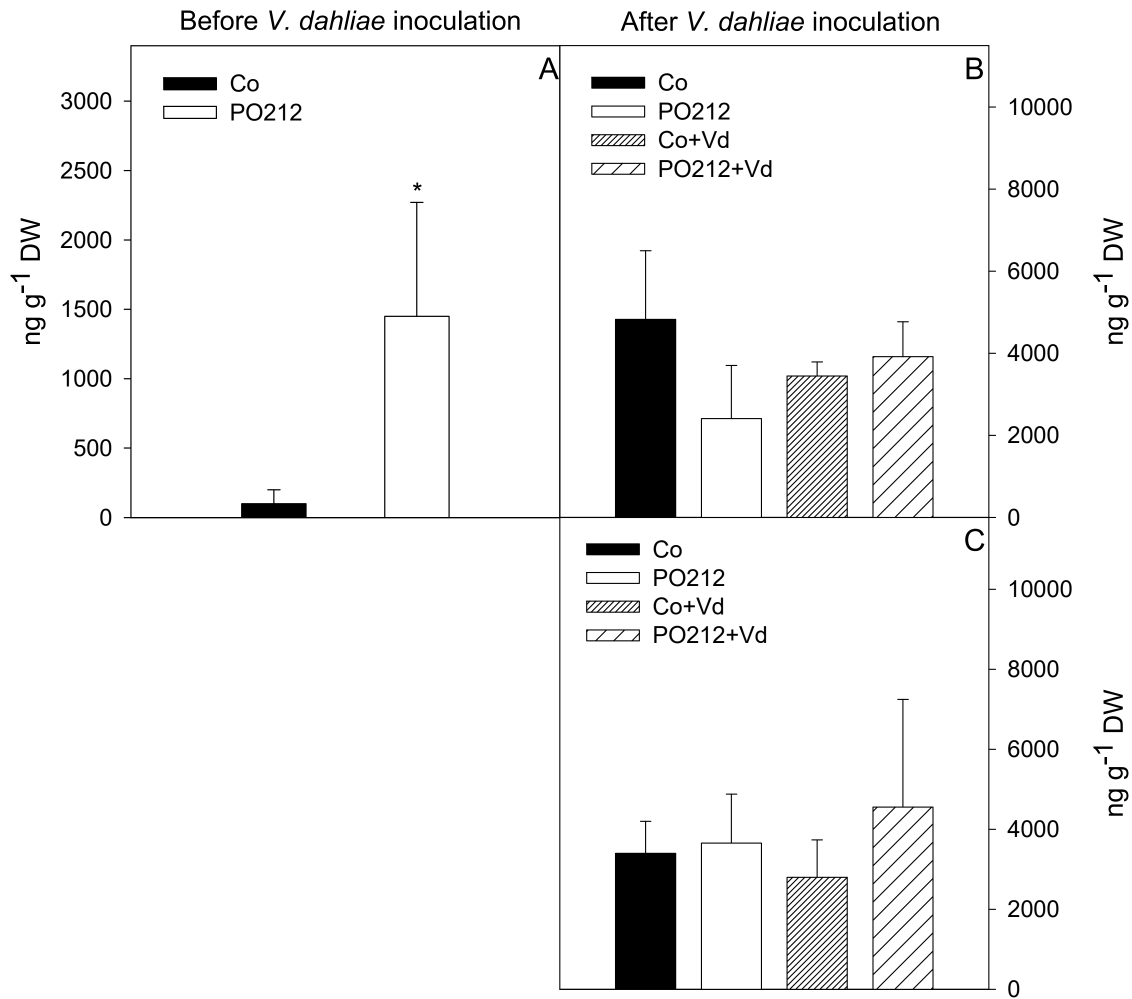


Figure 3.9. 4-hydroxybenzoic acid (4-HBA) levels measured in roots of pepper before and after inoculation of pepper roots with *V. dahliae*. (A) 4-HBA concentrations measured in roots before inoculation. (B) 4-HBA concentrations measured in roots 8 hours after inoculation with *V. dahliae*. C) 4-HBA concentrations measured in roots 24 hours after inoculation with *V. dahlia*. Data are the mean ± SE of three independent experiments. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

ABA levels significantly increased in pepper plants induced with PO212 before inoculation (Fig. 3.10A). However, 8 and 24 hours after pathogen inoculation, no significant differences were observed between control plants and PO212-treated plants (Fig. 3.10B and C).

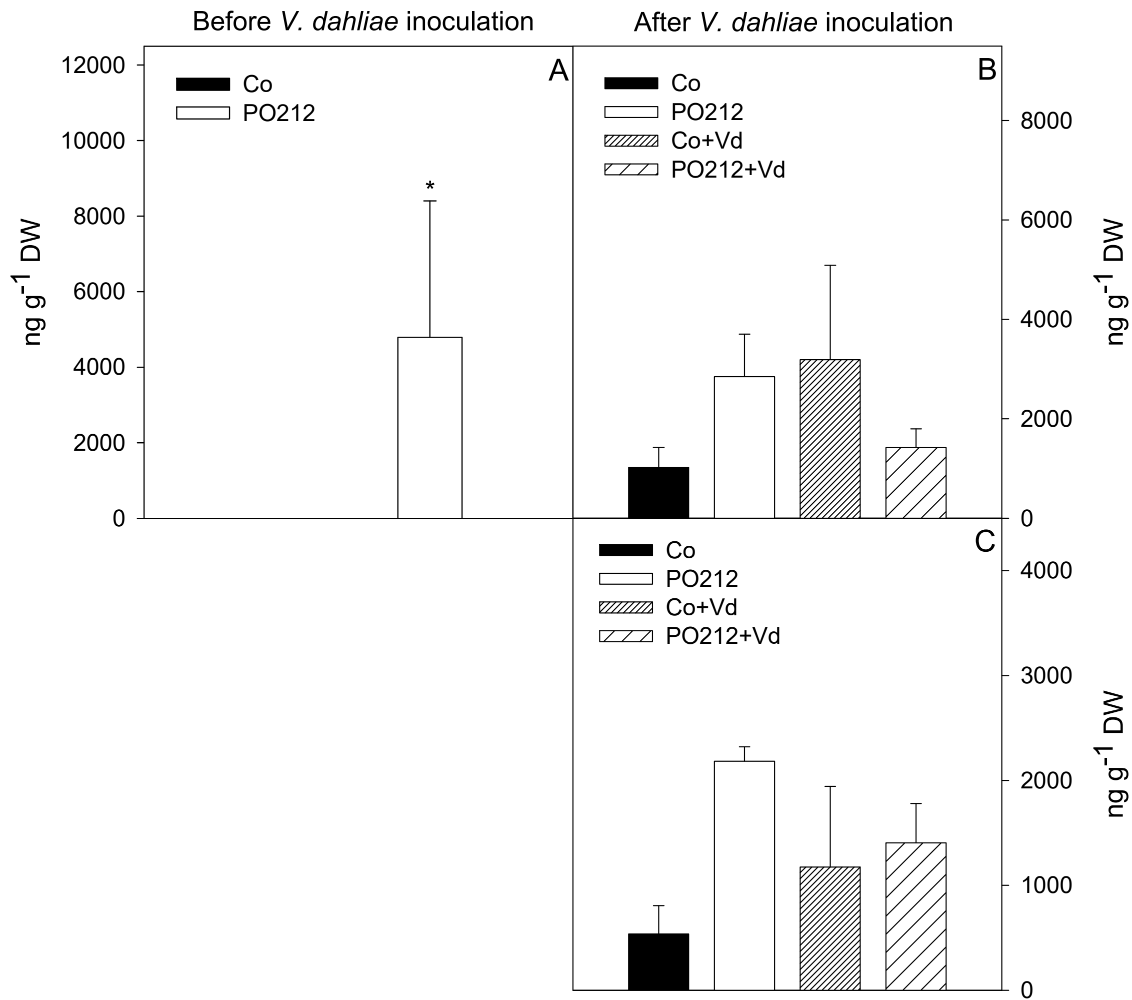


Figure 3.10. Abscisic acid (ABA) levels measured in roots of pepper before and after inoculation of pepper roots with *V. dahliae*. (A) ABA concentrations measured in roots before inoculation. (B) ABA concentrations measured in roots 8 hours after inoculation with *V. dahliae*. C) ABA concentrations measured in roots 24 hours after inoculation with *V. dahlia*. Data are the mean ± SE of three independent experiments. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

No significant differences in JA levels were observed between control plants and PO212-treated plants both before and after inoculation with *V. dahliae* (Fig. 3.11).

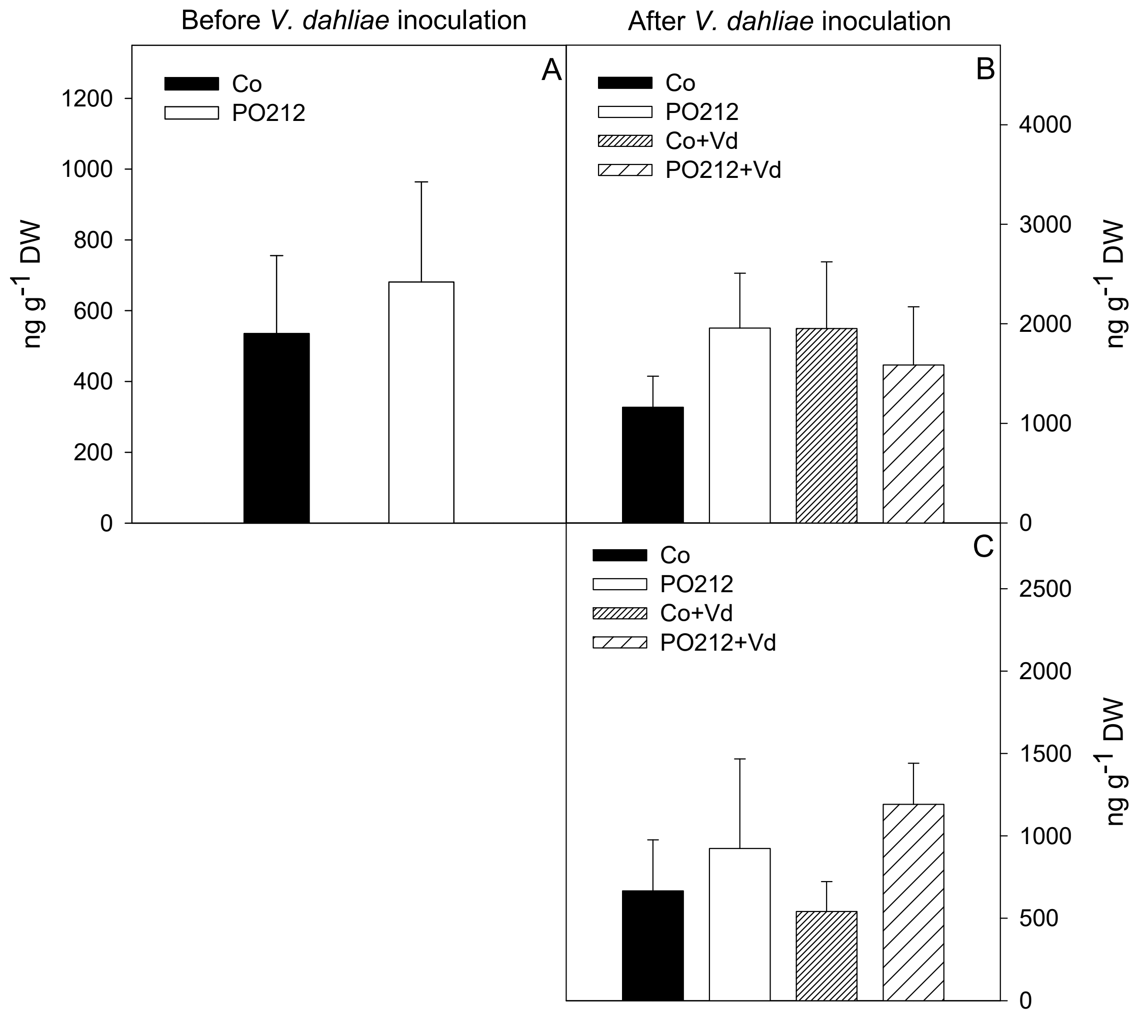


Figure 3.11. Jasmonic acid (JA) measured in roots of pepper before and after inoculation of pepper roots with *V. dahliae*. (A) JA concentrations measured in roots before inoculation. (B) JA concentrations measured in roots 8 hours after inoculation with *V. dahliae*. C) JA concentrations measured in roots 24 hours after inoculation with *V. dahlia*. Data are the mean ± SE of three independent experiments. Asterisks indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test. Co- Control, Vd- *V. dahliae*.

PO212 increases H_2O_2 production in pepper roots

H_2O_2 acts as an early messenger in the immune response. To determine its participation in the defense response induced by PO212, H_2O_2 was measured during the first 8 hours after induction and also during the first 8 hours after inoculation with *V. dahliae*.

There was a significant increase in H_2O_2 in pepper roots 2 hours after induction with PO212 (Fig. 3.12). However, no significant differences between both treatments were observed at none of the following times analyzed (4, 6, 8 and 10 hours) (Fig. 3.12).

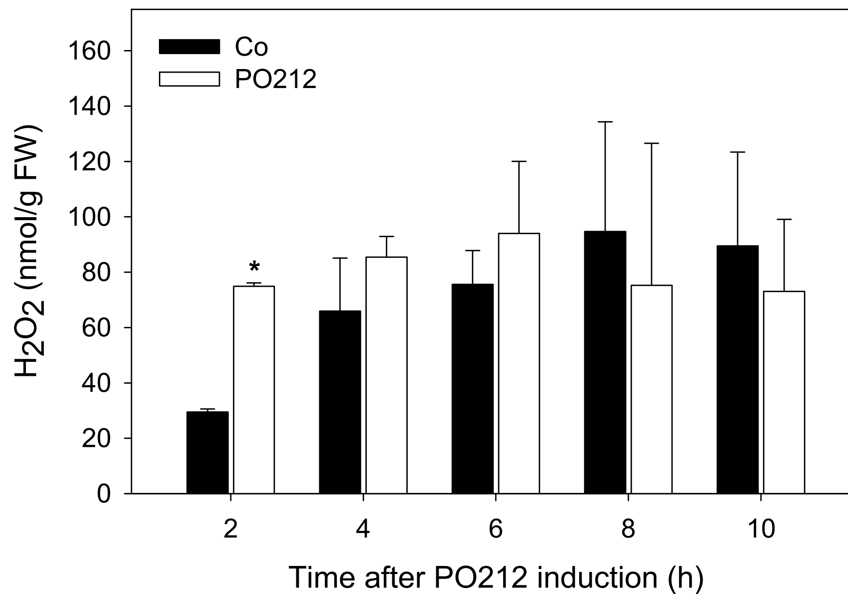


Figure 3.12. Hydrogen peroxide levels after induction of pepper plants with PO212. Data are the mean ± SE of two independent experiments. Asterisks indicate statistical differences ($p < 0.05$) in Student's t-Test. Co- Control, Vd- *V. dahliae*.

There was a significant increase in H_2O_2 in pepper roots treated with PO212 after inoculation with *V. dahliae* (Fig. 3.13). Before pathogen inoculation (0 hours), no significant differences in H_2O_2 levels were observed between plants treated with PO212 and its respective control (Fig. 3.13). Shortly after *Verticillium* inoculation (0.1 hours),

these levels were maintained in all treatments (Fig. 3.13). However, 2 hours after pathogen inoculation, there was a significant increase in H_2O_2 in pepper roots treated with PO212 and inoculated with the pathogen in comparison with the control (non-induced and non-inoculated) (Fig. 3.13). 4 hours after inoculation, all treatments showed similar levels of H_2O_2 (Fig. 3.13). Surprisingly, 6 hours after *V. dahliae* inoculation, H_2O_2 levels in pepper plants only inoculated with *V. dahliae* showed a significant decrease in comparison with pepper plants induced with PO212 either inoculated or not with the pathogen, and also with the control treatment (Fig. 3.13). Later on, 8 hours after inoculation, H_2O_2 levels in pepper plants treated with PO212 and inoculated with *V. dahliae* remained signicantly higher in comparison with control plants either inoculated or not with the pathogen (Fig. 3.13).

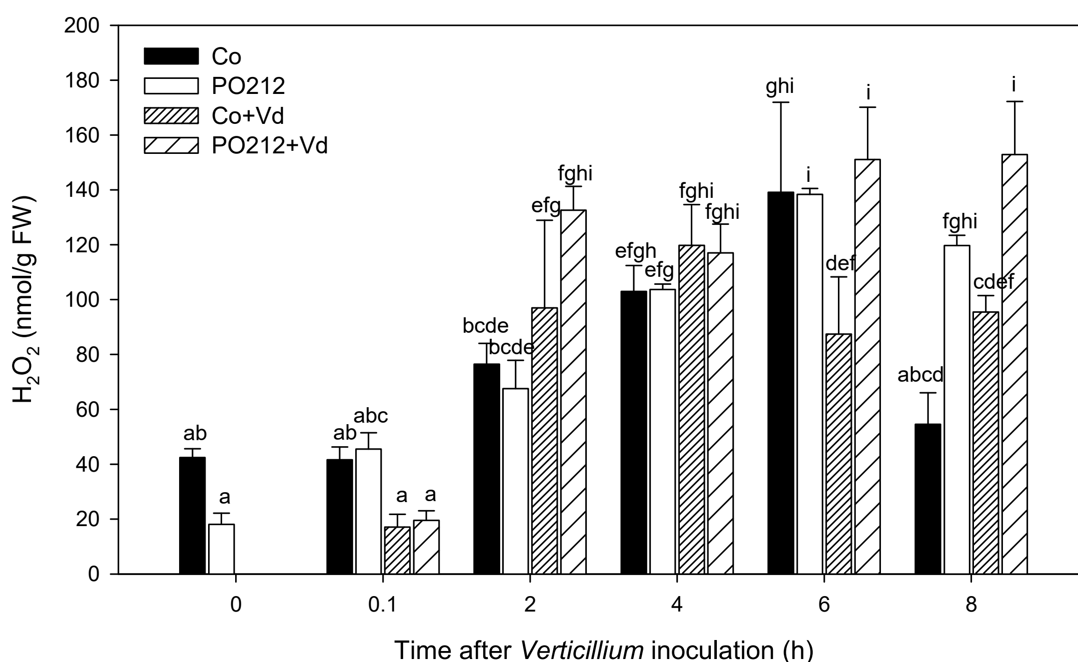


Figure 3.13. Hydrogen peroxide levels after inoculation of pepper plants with *V. dahliae*. Data are the mean ± SE of two independent experiments. Different letters indicate statistical

differences ($p < 0.05$) in a Kruskal-Wallis test followed by a post-hoc Conover test. Co- Control, Vd- *V. dahliae*.

PO212 colonizes only the surface of pepper roots

We followed the growth of the BCA for 7 days, and we observed that PO212 did not penetrate into the roots, but grew around the pepper root surface (Fig. 3.14). Thick masses of conidia and hyphae were observed in pepper roots treated with PO212 at early time points after induction (Fig. 3.14A-H). 24 hours after induction, PO212-GFP conidia colonized the root surface (Fig. 3.14A, B), and 48 hours after inoculation, a dense mass of PO212 hyphae grew around the root hairs (Fig. 3.14C, D). 72 hours after induction, PO212-GFP kept colonizing the root surface (Fig. 3.14E, F), and some hyphae enter in contact with the cortical cells (Fig. 3.14G, H). 5 days after induction, pepper plants seemed to have less colonization by PO212 due to the lower fluorescence observed (Fig. 3.14I, J), and 7 days after induction, the same trend was observed (Fig. 3.14K, L). However, PO212 hyphae growing around the roots were still observed (Fig. 3.14I-L). No penetration attempts by PO212 were observed in pepper roots where the biocontrol agent was applied.

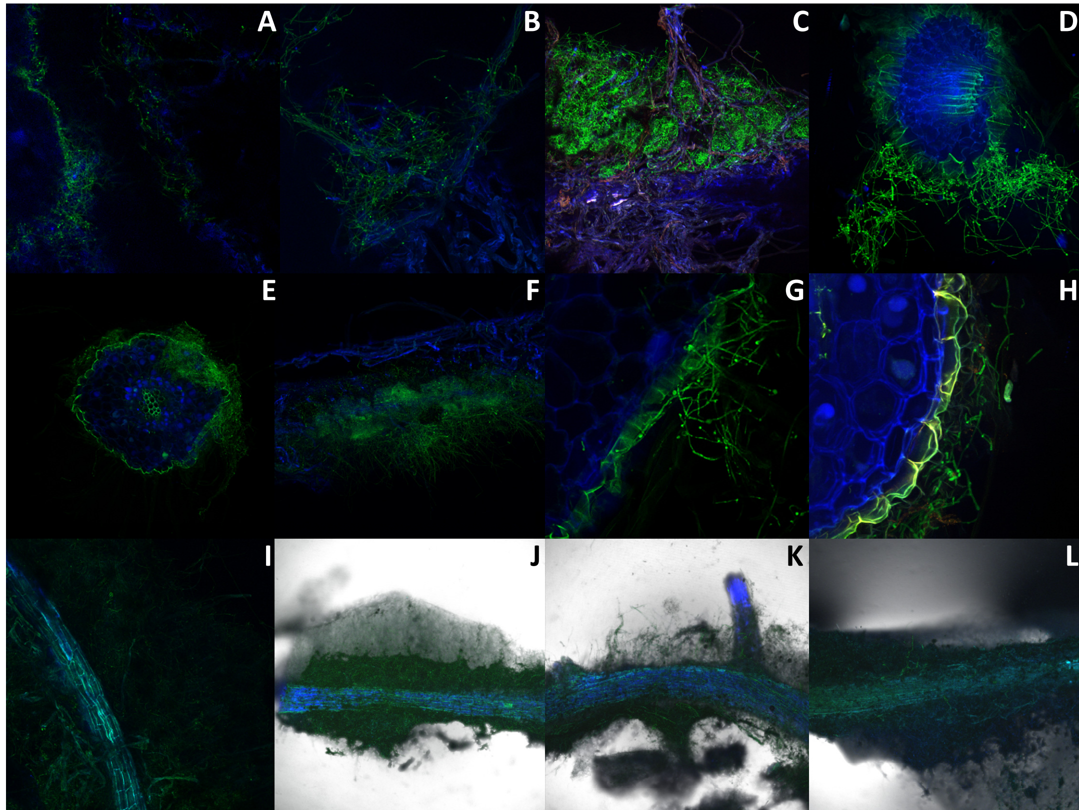


Figure 3.14. Roots of *Capsicum annuum* colonized by PO212. Colonization of the root surface by PO212 24 (A: 10x; B: 20x) 48 (C, D: 20x), 72 (E, F: 10x, G, H: 20x), 120 (I: 10x; J: 4x) and 144 (K, L: 4x) hours after induction. PO212-GFP conidia around root surface 24 hours after induction (A, B). Hyphae of PO212-GFP growing on the root surface and 48 (C, D) and 72 (E, F) hours after induction. PO212-GFP colonizing root hairs of *C. annuum* (G, H) 72 hours after induction. (I, J) (K, L).

Discussion

Several BCAs have been tested to reduce Verticillium wilt disease through different mechanisms of action involving antibiosis, parasitism, competitionfor space, infection sites and nutrients, secretion of cell-wall degrading enzymes and induction of resistance (Deketelaere *et al.*, 2017). Sometimes a single BCA can trigger more than one of these mechanisms in response to *V. dahliae* infection (Deketelaere *et al.*, 2017). In Chapter 1, the soil-borne fungus *Penicillium rubens* strain 212 (PO212) was able to protect pepper

plants against the pathogen *V. dahliae*. To better study the mode of action of this biocontrol agent, in the present chapter the protective capacity of PO212 in pepper was compared to the response observed in tomato induced with PO212, both *Solanaceae* members. We used tomato mutants deficient in hormone signaling or accumulation (there are no pepper mutants currently available) and quantified both phytohormone levels and gene expression in pepper plants induced with PO212 in order to gain more information about the defense response induced by this BCA.

In the experiments reported here, four wild type tomato cultivars treated with PO212 displayed a significant reduction in *Verticillium*-wilted leaves. We used different concentrations of conidia for PO212 (10^6 conidia per mL) and the pathogen *V. dahliae* (2×10^6 conidia per mL) in tomato plants to achieve protection by reducing the symptoms produced by the disease (wilt of leaves). The reason why we used a higher pathogen concentration in tomato than in pepper is due to the higher basal resistance of tomato to our *Verticillium* strain. Tomato mutants impaired in the, ABA, SA and JA signaling and/or accumulation, namely *sitiens* (*sit*), *NahG* and *defenseless1* (*def1*) respectively, were not protected against *V. dahliae* after treatment with PO212. This implies that the protective effect of PO212 relies on SA, JA and ABA in tomato.

In pepper, we observed that the gene responsible for SA synthesis, *CaICS,* was only expressed in pepper before inoculation with *V. dahliae*, showing no differences between control and PO212-treated plants. However, SA can be synthetized by both ICS and PAL pathways, and it was suggested that *CaPAL1* acts as a positive regulator of SA-defense signaling against biotic stress (Kim & Hwang, 2014). *CaPAL1* gene expression was induced in pepper roots treated with PO212 before pathogen inoculation, indicating that SA might be involved in the response induced by PO212 at the root level in pepper.

Intriguinly, we also observed that PO212 treatment significantly increased the levels of 4-HBA and decreased at the same time the levels of SA increased in pepper roots, as in the study of García (2018) when pepper leaves were induced with the capsinoid vanillyl nonanoate. 4-HBA, initially classified as a biologically inactive compound (Chen *et al.*, 1993), has a structure which is similar to SA structure and it is involved in the synthesis of ubiquinone, shikonin and as a component of lignin (Okrent *et al.*, 2009). Other studies also suggested a role of 4-HBA in plant defense (Smith-Becker *et al.*, 1998; Tan *et al.*, 2004). In fact, 4-HBA has been proposed as a regulator in SA synthesis through PBS3, a member of GH3 family which acts upstream of SA in early steps of the defense response. After *V. dahliae* inoculation, pepper plants treated with PO212 showed a slight increase in the levels of SA (although it was not significant). It would be possible that 8 hours after *V. dahliae* inoculation, PBS3 led to the conjugation of 4-HBA with glutamic acid thus priming SA biosynthesis (Okrent *et al.*, 2009) and reducing (in a slight way) 4-HBA levels. Thus, pepper plants induced with PO212 would accumulate 4-HBA as SA reservoir to subsequently turn it into SA against *V. dahliae* attack.

Therefore, the increase in 4-HBA observed in pepper roots treated with PO212 and the up-regulation of *CaPAL1* gene in these plants point to the participation of the SA-pathway in the defense response induced by PO212 in pepper. Also, *NahG* tomato mutant showed no response to PO212, which constitutes other evidence that SA-pathway play a role in the response induced by PO212 in both pepper and tomato.

As we mentioned above, JA-impaired mutant *def1* were not protected against *V. dahliae* after treatment with PO212, so the protective effect of PO212 also relies on JA in tomato. In pepper, the expression of *CaAOS* gene, which codes for allene oxide synthase that acts on the substrate released by the enzyme LOX, was up-regulated both before and after inoculation with *V. dahliae* in PO212-treated plants. Moreover, pepper

plants induced with PO212 showed a slight increase (although it was not significant) in JA levels before inoculation with *V. dahliae*. These data point to the involvement of JA-signaling pathway in the PO212-induced defense mechanism. The formation of JA-Ile (the JA active form) is necessary for the activation of JA-responsive genes. During a stress, the formation of JA-Ile is tightly regulated allowing only the formation of the minimum necessary amounts to activate JA-responsive genes (Wasternack & Hause, 2013). It would be interesting to measure the content of JA-Ile in pepper plants treated with PO212 to further understand why JA levels did not showed differences after inoculation with *V. dahliae* in pepper plants treated with PO212 but the expression of *CaAOS* gene was up-regulated both before and after inoculation with *V. dahliae*.

Scholz *et al.* (2018) suggested that in the first 24 hours, *V. dahliae* starts quite early to reprogram its primary metabolism and adapts amino acid and sugar metabolism to being inside a host (Scholz *et al.*, 2018). They observed that the contents of SA, JA and JA-Ile in *Arabidopsis* leaves were not significantly induced by the fungus 24 hours after inoculation (Scholz *et al.*, 2018), but genes related with JA and SA biosynthesis and ROS production were up-regulated in *Arabidopsis* plants inoculated with *V. dahliae*. Therefore, changes in the gene expression profiles occur before the fungus induces changes in the plant phytohormone levels, and this would explain our reduced phytohormone levels in pepper plants treated with PO212 8 and 24 hours after inoculation with *V. dahliae* and the up-regulation of genes involved in JA and SA bioynthesis. Moreover, these authors demonstrated that *V. dahliae* stimulates the host JA functions in order to promote host cell death during the later necrotrophic phase, due to the strong retardation of disease symptom development in host plants impaired in jasmonate mutants. Complete or strong inhibition of JA functions in the mutants may favor SA accumulation and/or SA signaling function which, in turn, may prolong the

biotrophic phase and thus retard necrosis and disease development (Scholz *et al.*, 2018). In our study, *def-1* tomato mutant treated with PO212 showed no resistance to *V. dahliae*, and jasmonate mutants showed the same disease severity in comparison with WT tomato, so JA signaling pathway would be necessary in the PO212-induced resistance against *V. dahliae* in tomato. In pepper, based on our results in phytohormone quantification and phytohormone-related gene expression, JA-signaling would be also necessary in the PO212-induced resistance against *V. dahliae*.

Moreover, JA is antagonistic to SA, which explains our significant increase in the expression of one gene (*CaAOS*) related with JA biosynthesis after inoculation with *V. dahliae* and the significant decrease in *CaPAL1* gene expression and the non expression of *CaICS* gene after *V. dahliae* inoculation. That is, *V. dahliae* would enhance JA-related gene expression in order to supress SA-signaling mechanism. In the study of Yuan *et al.* (2017), *P. simplicissimum* increased PAL and peroxidase gene expression in plants after inoculation with *V. dahliae*. Peroxidase genes showed no differences in their expression in pepper plants treated with PO212 after inoculation with *V. dahliae* (Chapter 1), and this can also be related with the down-regulation of *CaPAL1* observed in our study.

ABA functions in many plant developmental processes, but also plays an important role in plant defense against pathogens. In our work, ABA-impaired mutant *sit* showed lower resistance to *V. dahliae* than tomato wild-type plants, and ABA levels showed a significant increase in pepper plants treated with PO212. These data show that ABA works in both tomato and pepper in the defense responde triggered by PO212. It is necessary to highlight that ABA was not detected in control pepper plants before *V. dahliae* inoculation; control plants would not need ABA production since they were not under abiotic/biotic stress.

Hieno *et al.* (2016) observed that *P. simplicissimum* strain GP17-2 induced the expression of *MYB44* against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana*, a gene which enhances ABA signaling and consequently closes stomata. Moreover, they found that *myb44* mutant plants induced with *Penicillium* failed to restrict stomatal reopening and showed lower resistance to the bacterial pathogen than wild-type plants. Therefore, the enhanced expression of *MYB44* helped to prevent the penetration of the pathogen through stomata. It is well known that ABA interacts with several phytohormones, including JA (Ku *et al.*, 2018). JA also closes the stomata under stress, but the effects of ABA and JA are opposite in the case of evoking wounding response (Ku *et al.*, 2018).

Bae *et al.* (2016) observed that a *Trichoderma* extract led to significant up-regulation of ABA levels in pepper leaves both before and after inoculation with the oomycete *Phytophthora capsici*, which was accompanied by a significant increase in JA contents. In our work, the significant increase in ABA levels observed in pepper plants treated with PO212 coincides with a significant increase in JA levels before inoculation with *V. dahliae*.

After pathogen inoculation, ABA levels showed no significant differences between pepper plants treated with PO212 and their respective controls. It has been described that ABA is a negative regulator of SA (Kusajima *et al.*, 2017). In fact, 8 hours after inoculation, pepper roots treated with PO212 showed a slight decrease (although it was not significant) in ABA levels in comparison with control pepper plants challenged with the pathogen, which coincides with the slight increase in SA levels observed in pepper plants treated with PO212 at the same time after pathogen inoculation. Therefore, it is necessary a reduction in ABA to activate defenses by SA in the defense response induced by PO212 in pepper against *V. dahliae*.

Last, ET is another of the main phytohormones involved in plant defense against pathogens. In our work, ET-insensitive tomato, *Never-ripe* (*Nr*), was protected against *V. dahliae* after treatment with PO212. This implies that PO212 protective effect is not dependent on ET in tomato. In pepper, we studied two genes involved in ET synthesis (*CaACS* and *CaACO*) to know if ET was involved in PO212-induced resistance.

CaACS encodes the enzyme ACC synthase which is involved in the second step of ET synthesis, and *CaACO* encodes for the enzyme ACC oxidase, which catalyzes the last step of ET synthesis. ACS is considered the limiting step in the synthesis of this hormone. However, there are cases where ACO is the limiting step (Van de Poel *et al.*, 2012). We observed an up-regulation of *CaACO* and *CaACS3* gene expression after treatment with PO212, suggesting a role for ET in the response induced in pepper by PO212. The bioassays with tomato mutant *Nr* suggest that ET is not involved in the response induced by PO212 in tomato. *Nr* mutant possesses a mutation that impedes the binding of ET to the ET receptor NR, which constitutively inhibits the ET-responsive genes (Hackett *et al.*, 2000). Therefore, ET involvement in PO212 response depends on the host plant differing between pepper and tomato, as previously observed in the PhD thesis of Veloso (2011) using the protective strain Fo47 against *V. dahliae*. On the other hand, *CaACS3* also increased their expression in pepper plants induced with PO212 after inoculation with *V. dahliae*, but *CaACO* was down-regulated. We only have tested two genes related with ET biosynthesis, so other ET-biosynthetic genes may be involved in the pepper response induced by PO212 against *V. dahliae*. In fact, there are other *ACO* isoforms in the pepper genome (Aizat*et al.*, 2013).

All our results indicate that the PO212 signalling mechanism in pepper and tomato have some similarities and some differences The role of ET in PO212 response differs depending on the host plant but ABA, JA and SA have a role in both plants. Tomato,

unlike pepper, is a plant with a climacteric fruit. This means that ET is required for normal fruit ripening in tomato. This sensitivity to ethylene in tomato for developmental processeses like ripening might have displaced ethylene from some defense responses while pepper retains ethylene as a signaling component for this defense response. In pepper, *CaBPR1* is induced by SA, ET and JA, and *CaBGLU* is induced by JA and ET (Choi & Hwang, 2015). In a previous chapter (Chapter 1), both genes were up-regulated in pepper plants treated with PO212, which proves the involvement of these hormones in the defense response induced by PO212 in pepper.

In our work, PO212-induced resistance seems to require a complex crosstalk between SA, ET, JA, and ABA to modulate the adequate response that triggers pepper defenses against *V. dahliae*. The BCA *Penicillium simplicissimum* GP17-2 induced the expression of defense genes involved in the SA and JA/ET signaling pathways in *Arabidopsis* (Hossain *et al.*, 2007; Elsharkawy *et al.*, 2012). Also, Hossain *et al.* (2007) demonstrated that JA and ET signaling pathways are necessary in resistance against *Pseudomonas syringae* pv. tomato DC3000 using *jar1* and *ein2* mutants respectively, and that resistance partially depends of SA signaling by using *NahG* and *npr1* mutants. However, induced resistance by the same *Penicillium* strain against cucumber mosaic virus was independent of SA, JA and ET, using *Arabidopsis* mutants impaired in these hormones (Elsharkawy *et al.*, 2012). In the study of Hossain *et al.* (2017), the BCA *Penicillium viridicatum* GP15-1 did not modulate gene expression related with these hormonal signaling pathways, but pointed to the important role of ET in the induced resistance mechanism. Thuerig *et al.* (2006) also demonstrate that *Penicillium chrysogenum* induced resistance independently of JA/ET and SA signaling pathways. Therefore, different *Penicillium* strains stimulate different pathways to protect plants against pathogens.

A complex crosstalk between ET, SA, ABA and JA is necessary to modulate the adequate response that protects pepper plants induced with PO212 against *V. dahliae*. However, the response obtained could involve other phytohormones and/or the induction of new unknown pathways.

These plant hormones depend on the early signaling of ROS to be activated. Therefore, in this work the implication of H_2O_2 in PO212-defense response was also studied in pepper.

H_2O_2 is toxic to the pathogen and plays a role in plant defense by participating in cell wall reinforcement and also in signaling (Lehmann *et al.*, 2015). Increased H_2O_2 production confers enhanced disease resistance through the regulation of the expression of genes associated with plant defense mechanisms that are involved in lignification, cell wall crosslinking and direct killing of the pathogen. However, ROS production does not always favor plant resistance because the outcome of the pathogen infection will depend on the timing and intensity of the oxidative burst as well as the balance with other early signaling molecules such as nitric oxide. The place of production seems to be involved in an intricate interaction that determines the result of the oxidative burst. In pepper, ROS production associated with SAR is a consequence of the activity of NADPH oxidase and extracellular peroxidases (Choi & Hwang, 2015).

H_2O_2 accumulation during a defensive response at the root level is poorly studied. There are some cases where H_2O_2 accumulation in roots was observed (Lehmann *et al.*, 2015). In this work, we measured H_2O_2 production in pepper plants during the first 10 hours after induction with PO212. We observed a significant increase in H_2O_2 2 hours after induction, and then H_2O_2 levels remained equal to the control. This increase could correspond to the moment when pepper plants recognized the BCA, but then H_2O_2

levels stabilize since PO212 is not a pathogen and the plant does not need to create a toxic environment.

Moreover, an increase in H_2O_2 in the first hours after *V. dahliae* inoculation was observed in pepper roots, but it was faster and longer sustained in plants treated with PO212. This increase could correspond to the moment when the plant recognizes the attack of *V. dahliae* and generates an oxidative burst which results in an increase in defense response.

H_2O_2, at low concentrations, participates in the establishment of plant disease resistance via signal transduction with regards to, e.g., SA biosynthesis (Guo *et al.*, 2014). However, other phytohormones (e.g. JA) also activate ROS production (Ku *et al.*, 2018). In most cases, the HR adopts a PCD pathway to kill the pathogen directly or to create insulation for localization of the pathogen inside the dead cells, thus preventing disease from spreading (Gayoso *et al.*, 2010; Goyal & Mattoo, 2014). Shimizu *et al.* (2013) observed an accumulation of SA and H_2O_2 in cucumber plants treated with *Penicillium simplicissimum* GP17-2.

It has been observed that H_2O_2 affects the amount of JA in the plant (García, 2018). We found high H_2O_2 levels in PO212-treated plants after inoculation with *V. dahliae*. This would explain why pepper roots treated with PO212 and challenged with the pathogen showed no differences in JA levels. After PO212-treatment, H_2O_2 enhance its levels that would unleash JA biosynthesis but only a small portion would be used to activate JA-responsive genes. This increase in ROS is transient, and also the representation of JA in pepper roots, which suggest the dependence of both events.

Induced resistance triggered by beneficial microorganisms may involve a variety of signaling pathways, depending on the BCA, the target pathogen and the plant species. In our work, PO212 seems to rely on ROS, SA, JA and ET production in pepper roots.

In our study the root colonization observed in pepper roots by PO212 is similar to the pattern already described for the same BCA in tomato (Villarino *et al.*, 2018) and the fungal BCA Fo47 in pepper (Veloso *et al.*, 2016). Villarino *et al.* (2018) showed that PO212 efficiently colonizes tomato roots at the superficial level without enter inside the root tissues. In this study, PO212 remained in the most outer layers of the pepper root and a dense net of PO212 hyphae grew around the root hairs and attached closely to the superficial root cell walls 72 hours after induction. PO212 rapidly colonized the root surface, which may permit PO212 to reach the population density needed to stop the advance of *V. dahliae* even though the population density of PO212 was reduced with time. 72 hours after inoculation, hyphae of PO212 reached the root surface and attached closely to the superficial root cell walls. Hyphae of PO212 grew also randomly above the root surface as previously described by Veloso *et al.* (2016) with Fo47.

Other studies using the non-pathogenic *Fusarium oxysporum* strain F2 (Pantelides *et al.*, 2011; Gizi *et al.*, 2011) showed that the biocontrol agent colonizes preferentially the eggplant root surface excluding *V. dahliae* from the same ecological niche when it is applied by root drenching, but it can also compete with *V. dahliae* for nutrients and space inside vascular tissues when its growth is endophytical. Carrero-Carrón *et al.* (2018) observed that *Trichoderma harzianum* grew endophytically and reduced the *V. dahliae* growth within olive roots.

In the study of Scholz *et al.* (2018), *Arabidopsis* roots were heavily colonized superficially by the GFP-labeled *V. dahliae* 24 hours after inoculation, but did not

invade the vascular tissue at that time. We previously observed that PO212 reduced *V. dahliae* biomass in pepper roots 1 week after inoculation (Chapter 1). Therefore, in the first 48 hours, PO212-GFP could get ahead to the pathogen and compete for spaces with *V. dahliae* due to its potential to rapid colonize pepper root surface, leading thus to a reduction in pathogen biomass and delaying *V. dahliae* entry into the vascular system. In fact, competitiveness for resources was an antagonistic interaction between the biocontrol agent Fo47 and *V. dahliae* over the root surface of pepper plants in the study of Veloso *et al.* (2016), where Fo47 also reduced significantly the population density of *V. dahliae* present on the root surface. Pantelides *et al.* (2009) also showed that F2 reduced significantly the levels of *V. dahliae* in eggplant plant tissues.

Competition for nutrients and/or infection sites in the soil/roots could be one of the most efficient mechanisms of action to control *Verticillium* wilt disease (Deketelaere *et al.*, 2017). It is important to select BCAs that share the same ecological niche as *V. dahliae* and compete with the pathogen for infection sites, space and nutrients, such as non-pathogenic *Fusarium* strains (Deketelaere *et al.*, 2017). In our study, PO212 did not enter into the pepper roots but grew superficially around them, so PO212 could be acting by competing for infection sites or spaces with *V. dahliae* in pepper roots. Therefore, this biocontrol fungus would be a promising candidate to control Verticillium wilt in pepper due to its efficient root surface colonization. However, it is not well established that competition is the main antagonistic interaction in the inter-species biocontrol by *Penicillium* strains. Other mechanisms of action such as mycoparasitism and secretion of cell-wall degrading enzymes produced by PO212 (glucanases, chitinases) cannot be ruled out in the protection conferred by PO212 in pepper against *V. dahliae*. Further investigation is needed to fully understand the whole mode of action of PO212. The interaction between both fungi, *V. dahliae* and PO212, should be studied

in the future to further understand the interactions that take place between these fungi and also in the tripartite interaction. Concluding, PO212 has good perspectives as BCA but more research is needed to understand its effects on the plant.

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Chapter 4. Detection of suppressive and conducive soils from Galician pepper greenhouses and role of arbuscular mycorrhizal fungi in soil health

Introduction

The association between plants and arbuscular mycorrhizal fungi (AMF) is one of the most common beneficial plant-microbe interactions. AMF are well-characterized soil microorganisms of the monophyletic phylum Glomeromycota that form intimate associations with 92% of the plant families and comprise approximately 312 known species (Glomeromycota Species List, 2019; http://www.amf-phylogeny.com/). These obligate biotrophs transfer inorganic nutrients and water to the plant through their extraradical mycelium and receive carbohydrates in exchange (Smith & Smith, 2012). AMF are responsible of nutritional benefits for plants, such as improved nutrient uptake (specially phosphorus, but also nitrogen, potassium, calcium, copper, iron, magnesium, manganese, zinc and sulfur), improved plant growth (mainly in soils with low concentrations of available phosphorus), and improved yield and quality (Sharif & Claassen, 2011; Beltrano *et al.*, 2013; Delavaux *et al.*, 2017; Li *et al.*, 2018b). AMF also play a role in protection of plants against diseases, being able to colonize the habitats exposed to pathogens (Smith & Smith, 2012; Delavaux *et al.*, 2017; Li *et al.*, 2018b; Ghorbanpour*et al.*, 2018). In response to AMF colonization, plants can trigger a mechanism of enhanced defense known as mycorrhizal-induced resistance (MIR) (Sánchez-Bel *et al.*, 2016; Ghorbanpour*et al.*, 2018). MIR confers plant protection against pathogens and involves the expression of defense-related genes and enzymes, synthesis of plant secondary metabolites from phenylpropanoid pathway, and changes in several phytohormones (Garmendia *et al.*, 2006; López-Ráez *et al.*, 2010; Sánchez-Bel *et al.*, 2016). Other mechanism by which AMF can reduce the incidence of the disease in plants is the competition with pathogens for space, nutrients and infection sites (Smith & Smith, 2012; Ghorbanpour*et al.*, 2018). Moreover, AMF can modify the physical and chemical properties of the soil where they are growing, therefore affecting

all the microbial community in the soil (Smith & Smith, 2012; Delavaux *et al.*, 2017). The presence of AMF in the soil can make the soil suppressive to some diseases.

There are two types of soil based on the presence or absence of plant diseases: suppressive and conducive (non-suppressive) soils. Suppressive soils are soils where there is no disease even if the pathogen and the susceptible host plant are present. In these soils specific soil-borne plant pathogens do not establish or persist, establish but cause no damage, or cause lesser disease damage due to competitive interactions with the remaining soil microbial community (Baker & Cook, 1974). Suppressive soils can be detected by the observation that disease incidence remains low despite the presence of a susceptible host plant, climatic conditions favorable to disease expression and ample opportunity for the pathogen to have been introduced (Cook & Baker, 1983; Alabouvette *et al.*, 1999). Many microorganisms (e.g. pathogens, antagonists) remain in equilibrium in the soil, and this can determine its suppressiveness or conduciveness. Thus, Cook and Baker (1983) distinguished pathogen suppressive soils, where the inoculum is destroyed or does not survive, from disease-suppressive soils, where inoculum is present but does not induce disease. Microbial biomass, diversity, functions and activities are important indicators of soil suppressiveness (Alabouvette *et al.*, 1999; Bonilla *et al.*, 2012; Li *et al.*, 2018a; Orquera-Tornakian *et al.*, 2018). Moreover, physical and chemical factors (abiotic factors) such as pH, particle size distribution, mineral composition (e.g. nitrogen, hydrogen, sulfur, carbon, organic matter content, oligoelements), cation exchange capacity and also environmental conditions can directly or indirectly affect the microbial community or the interaction of the community with the pathogen and therefore the suppressiveness of the soil (Garbeva *et al.*, 2004; Ochiai *et al.*, 2008; Termorshuizen & Jeger, 2008; Bonilla *et al.*, 2012; Mahatma & Mahatma, 2015; Mitsuboshi *et al.*, 2018).

Historically, suppressiveness has been divided into two major categories: general and specific. General suppressiveness is the property of some soils to reduce the capability of a broad range of pathogens to produce disease by supressing their growth and/or activity (Cook & Baker, 1983). It is also termed as “general” or “nonspecific antagonism” (Rovira & Wildermuth, 1981; Hornby, 1983), or “biological buffering” (Huber & Watson, 1970). General suppression depends on soil composition, total soil microbiome biomass and activity (competition for available resources and/or spaces, antibiosis, production of cell-wall-degrading enzymes, parasitism and induced resistance) and soil management practices (Weller *et al.*, 2002; Stirling *et al.*, 2012; Jambhulkar *et al.*, 2015; Cha *et al.*, 2016). Therefore, there is not a specific microorganism responsible for the general suppression (Cook & Baker, 1983). General suppression is not transferable between soils (Cook & Rovira, 1976; Rovira & Wildermuth, 1981), and is generally recovered after sterilization due to the rapid colonization of microorganisms (Termorshuizen & Jeger, 2008), and can be augmented by addition of organic matter or increasing soil fertility, and the employment of certain farming practices (Rovira & Wildermuth, 1981; Ji *et al.*, 2012; Cretoiu *et al.*, 2013).

On the other hand, specific suppression is associated with the biomass and activity of individual or selected groups of microorganisms of the soil community against a particular pathogen by interfering with some stage of the pathogen life cycle. Specific suppression is more effective than general suppression, and, in many cases, is induced by monoculture (Weller *et al.*, 2002; Shrestha *et al.*, 2014; Cha *et al.*, 2016). In contrast to general suppression, specific suppression can be transferred to conducive soils by introducing an inoculum of 0.1–10% of suppressive soil into a conducive soil (Cook & Rovira, 1976; Weller *et al.*, 2002; Garbeva *et al.*, 2004; Durán *et al.*, 2017). Therefore, specific suppression is also termed as “transferable suppression”.

Generally, suppressive soils are a combination of general and specific suppression, and both may be affected differently by edaphic, climatic, and agronomic conditions (Rovira & Wildermuth, 1981; Postma *et al.*, 2008; Penton *et al.*, 2014; Avilés & Borrero, 2017). If suppressiveness is eliminated by biocides or harsher treatments as autoclaving, steam pasteurization or γ-irradiation, then the suppresiveness is due to biological factors, that means, the microbial populations occupying that ecosystem (Weller *et al.*, 2002; Mahatma & Mahatma, 2015).

Pepper crops are attacked by different pathogens that cause severe losses in terms of production. In Galicia, the soil-borne pathogens *Verticillium dahliae* and *Phytophthora capsici* are the most common vascular pathogens that cause wilting in Padron pepper greenhouses (but also in open field) (Pomar *et al.*, 2001). Pepper wilt ("Tristeza" disease) is caused by both pathogens and its symptoms are a consequence of the alteration of the plant hydric balance triggered by these pathogens. The main symptoms are the loss of turgor of the tissues, chlorosis in the aerial organs and the reduced global development of the plant (Palazón & Palazón, 1989).

The presence of AMF in soil communities and their benefitial contribution are well reported (Penton *et al.*, 2014; da Silva *et al.*, 2015; Li *et al.*, 2015; de Assis *et al.*, 2016; Crossay *et al.*, 2018; Siegel-Hertz *et al.*, 2018; Cruz-Paredes *et al.*, 2019). AMF are locally diverse and their community assemblages are influenced by plant and soil characteristics, and geographic and environmental conditions (da Silva *et al.*, 2015; de Assis *et al.*, 2016; Stürmer *et al.*, 2018). The conversion of natural ecosystems into agro-ecosystems may reduce AMF diversity (Oehl *et al.*, 2009). Moreover, the excessive use of synthetic fertilizers and pesticides and land use intensification are a handicap for using these fungi in agriculture and can significantly affect AMF richness and diversity (Hassan *et al.*, 2013). Therefore, it is of first importance to find a balance

between the use of chemicals and soil microorganisms in agriculture to achieve an effective productivity of crops, both economically and at the level of human and environmental health.

The analysis of AMF diversity in soils and crops in different farms allows to find which AMF have highest potential as biocontrol agents of plant pathogens. Pepper plants are able to easily form arbuscular mycorrhizal association, and it was demonstrated that AMF positively affect this crop (Garmendia *et al.*, 2006; Kaya *et al.*, 2009; Sharif & Claassen, 2011; Beltrano *et al.*, 2013). Therefore, in this work we first determined the morphological diversity and abundance of AMF associated with greenhouse pepper crops from Cambados and Ribadumia (Pontevedra, Galicia, NW Spain) with a record of either presence or absence of vascular wilt diseases in previous years. Second, we performed the molecular identification of the AMF present in these soils. Third, we carried out bioassays by growing plants in these soils, initially with *Trifolium pratense* L. and *Plantago lanceolata* L. (plant species which are commonly used to establish trap cultures and catch AMF) and later with Padron pepper plants, to monitor wilt symptoms and test if there are differences between both types of greenhouse soils in terms of suppressiveness/conduciveness. Finally, we quantified the biomass of both *Verticillium dahliae* and *Phytophthora capsici* present in pepper roots grown in greenhouse soils to prove that these vascular wilt pathogens infect pepper plants grown in these soils, and we also isolated the fungal microorganisms present in pepper stems to check the presence of other pathogens that can also cause pepper wilt. The aim is to be able to isolate these AMF to be used in the future as potential biocontrol agents in an integrated management strategy against Verticillium wilt in pepper (among other fungal diseases) through the combination of PO212 and AMF.

Materials and methods

Field sites and soil sampling

Soil samples were collected from 10 different greenhouses (named from I to X) where "Padrón" pepper (*Capsicum annuum* L. var. *annuum* cv. Padron) was grown in the municipalities of Cambados and Ribadumia (Pontevedra, Galicia, NW Spain) on February 2017 (Fig. 4.1). According to the farmers, each greenhouse had a different record for the presence or absence of pests and diseases in recent years, as well as different chemical treatments (Table 4.1). For this work, five greenhouses were selected due to previous presence of vascular wilts (named as I to V greenhouses) and five for not presenting vascular wilts in the recent years (named as VI to X greenhouses) (Table 4.1). The procedure for collecting soil samples consisted of removing the uppermost layer of soil (the first two centimeters of soil) and collecting soil cores with 8 cm in diameter and 10 cm in depth. For each greenhouse, 9 randomized samples were taken, which were thoroughly mixed in groups of three, resulting in three samples per greenhouse. For subsequent analyses, the three samples per greenhouse were mixed to form a single sample. Soils were immediately stored at 4 °C until further analysis.

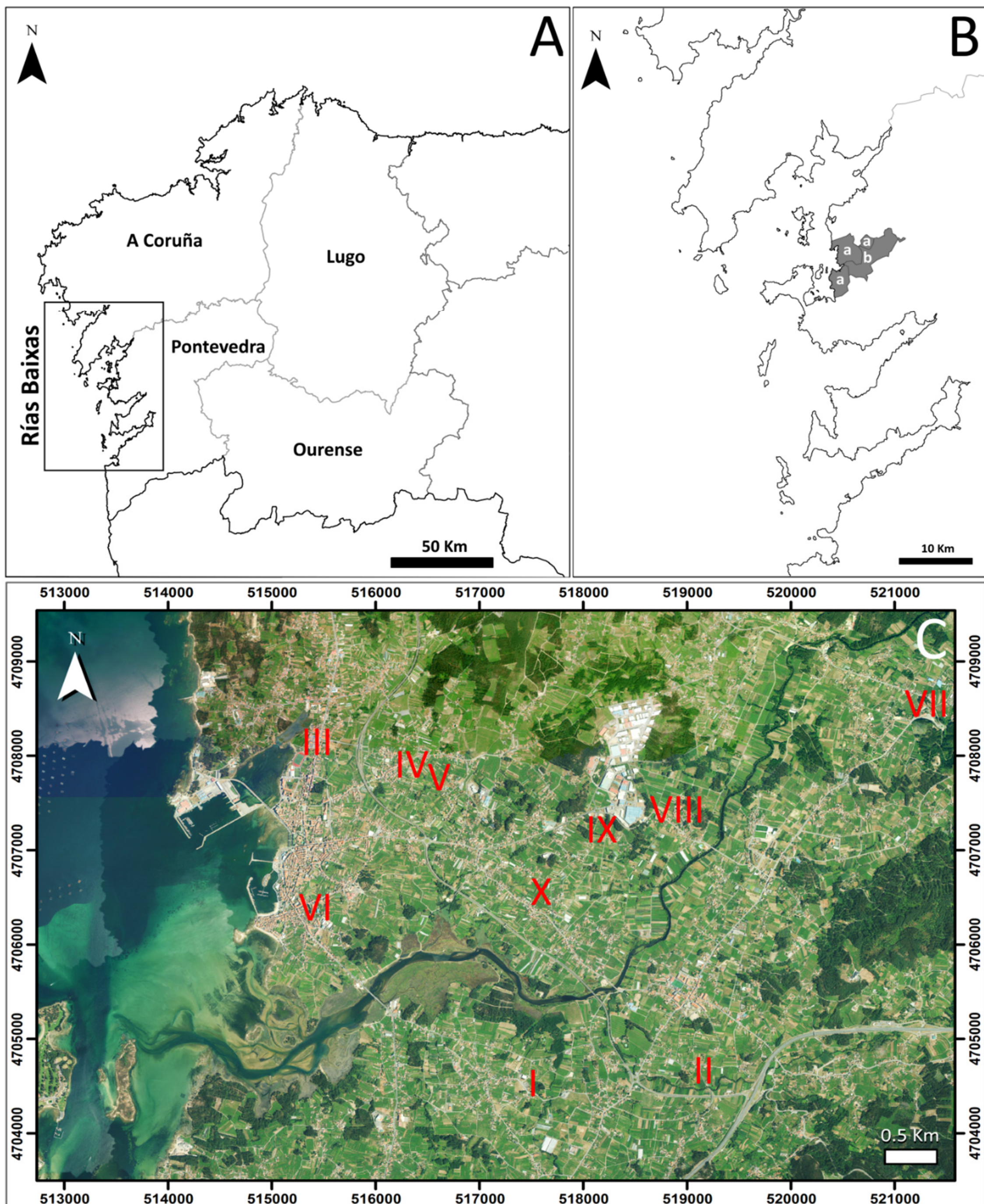

Figure 4.1. Geographic location of the sampling sites on Cambados and Ribadumia (Pontevedra, Galicia, NW Spain). (A) Map of Galicia pointing to "Rías Baixas", where Cambados and Ribadumia regions are located. (B) Map of "Rías Baixas" where a and b letters indicate Cambados and Ribadumia municipalities respectively (C) Orthophoto taken from PNOA ("Plan Nacional de Ortofotografía Aérea") and CNIG ("Centro Nacional de Información Geográfica") in 2017 with UTM (Universal Transverse Mercator) coordinates of each sampled greenhouse.

Table 4.1. Characteristics of the sampled greenhouses, in which Padron pepper crops were grown in the previous year (2016). According to the farmers and their technical advisor.

Greenhouse (municipality)	Reference name	Sampled surface (m2)	Previous record of pests and diseases	Treatments	Observations
Sisán (Ribadumia)	I	4000	Wilting (probably Phytophthora rot)	Previcur®, Proplant, sulfur, copper (no soil solarization)	No crops at the time of sampling
Barrantes (Ribadumia)	II	2400	Wilting	Terrazole® (no soil solarization, no chemical disinfestation)	Chard crops at the time of sampling
Foro (Corvillón, Cambados)	III	600	Wilting (probably Phytophthora rot)	Basamid®, Previcur®, Proplant (no soil solarization, no chemical disinfestation)	No crops at the time of sampling
Rodel 1 (Modia, Cambados)	IV	400	Drying (probably Phytophthora rot)	No soil solarization, no chemical disinfestation	No crops at the time of sampling
Rodel 2 (Modia, Cambados)	V	1200	Wilting	Previcur®, Proplant	Lettuce crops collection before sampling
Grenla (Cambados)	VI	2000	Powdery mildew, Thrips, Aphids	Previcur®, 80% sulfur, (no soil solarization, no chemical disinfestation)	Lettuce crops at the time of sampling
Abelenda (Ribadumia)	VII	800	Powdery mildew, Thrips, Aphids	Previcur®, Proplant, Plenum, 80% sulfur, (no soil solarization, no chemical disinfestation)	No crops at the time of sampling
Cabanelas (Ribadumia)	VIII	1000	Powdery mildew	Previcur®, Proplant, 80% sulfur, (no soil solarization, no chemical disinfestation)	Lettuce crops at the time of sampling
Fornos (Ribadumia)	IX	800	No	Previcur®, Proplant, 80% sulfur	No crops at the time of sampling
Telleira (Cambados)	X	2200	Thrips,Viral diseases	Previcur®, Proplant, Bayfidan®, 80% sulfur (no soil solarization)	Cabbage and turnip crops at the time of sampling

Determination of soil physicochemical properties

Three samples per greenhouse were thoroughly mixed to establish a single sample. Each soil sample was air-dried and 2 mm sieved. The pass-through was used to determine the physical-chemical characteristics of the soil. Elemental analysis of each soil was performed by the SAI Services (Servizos de Apoio á Investigación) of the University of A Coruña. Elemental analysis was carried out using the elemental analyzer FlashEA 1112 (Thermo Finnigan) with the following protocol: 200 mg of each soil were weighed in a tin (Sn) capsule and subjected to instant combustion in a quartz tube filled with tungsten trioxide (WO_3) and copper (Cu), and maintained at 1020º C. Then, the gas mixture generated (N_2, CO_2, H_2O y SO_2) was separated chromatographically and finally detected in a thermal conductivity detector. Soil pH and electrical conductivity (EC) were measured in 1:2.5 soil/deionized water suspensions.

AMF spore isolation

Isolation of AMF was carried out for the three samples of each greenhouse, so a total of 30 samples were analyzed. AMF spore isolation was performed by the wet sieving and decanting technique (Gerdemann & Nicolson, 1963) with some modifications. Each soil sample (15 g) was washed with distilled water (150 mL) and vigorously shaken. Soil suspension was sieved for three times through 1000, 500, and 354 μm sieves (corresponding to 18-, 35-, and 45-US meshes, respectively). The final suspension was let to settle down for two hours and, after decanting, the supernatant was passed through 300, 250 and 25 μm sieves (corresponding to 50-, 60-, and 500-US meshes, respectively). AMF spores were isolated from the last sieve and grouped according to their morphology (shape and size), color, and other characteristic features (e.g. spore-

wall properties) observed under a dissecting microscope. Isolated AMF spores were stored at 4 °C until DNA extraction.

Molecular identification of AMF

The molecular analysis was performed on each AMF morphotype. The selection of spores of each morphotype was carried out using the following strategy: (1) two spores were identified for each rare spore morphotype (*i.e.*, represented by fewer than 100 spores); (2) three spores were identified for all the morphotypes represented by more than 100 and less than 500 spores; (3) four spores were identified for those represented by more than 500 and less than 1000 spores; and (4) five spores were identified for those represented by at least 1000 spores. Total DNA was extracted from single spores using the REDExtract-N-Amp™ Plant PCR kit (Sigma-Aldrich, St. Louis, USA). DNA extraction from each spore was carried out by immersion in an extraction buffer (5 mL), freezing at -20 °C and then boiling (95 °C) for 10 minutes (thermal shock). Then, the mixture was centrifuged for 5 min at 16089 xg. After centrifugation, 4 μL of the supernatant were used as template for PCR. To enhance the final PCR yield, NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS4 (5'-CTT CCG TCA ATT CCT TTA AG-3') universal primers (White *et al.*, 1990) were used for a first amplification, followed by a first nested amplification with NS4 and AML1 (5'-ATC AAC TTT CGA TGG TAGGAT AGA-3') primers, and a second nested amplification with AML1 and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') primers (Lee *et al.*, 2008). First PCR was carried out using 10 μL of the REDExtract-N-Amp™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, USA), 4 μL of stored DNA, and 1 μL of each universal primer at 10 μM up to a final volume of 20 μL. The thermocycling program included an initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 40 °C for 1

min, 72 °C for 1 min and 30 s, and a final extension cycle at 72 °C for 10 min. The first PCR product was used as template DNA in the second PCR reaction (using NS4/AML1 primers), using Taq DNA Polymerase PCR kit (Sigma-Aldrich, St. Louis, USA). This second PCR was carried out using 5 µL of the Key buffer, 0.8 µL of dNTP mix at 10 mM (Sigma-Aldrich, St. Louis, USA), 4 µL of the first PCR product, 1 µL of NS4 and AML1 primers at 10 µM each, and 0.4 µL of Taq polymerase up to final volume of 50 µL. This PCR program included a 4 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 45 °C and 1 min extension at 72 °C, and a final extension cycle of 10 min at 72 °C. For getting further specificity, the product of this second PCR was used as template DNA in a third PCR reaction using AML1/AML2 primers. This third PCR was carried out using similar conditions to previous PCR, except that primer annealing occurred at 50 °C, instead of previous 45 ºC. The PCR products of the expected size (~800 bp) were obtained, as observed after a separation by electrophoresis on a 1% (v/v) agarose gel, followed by staining with RedSafe™ Nucleic Acid Staining Solution (*iNtRON Biotechnology*, South Korea) and visualization under UV light.

Amplification products were sequenced, using primer pair AML1-AML2, by Macrogen (Amsterdam, The Netherlands). Fungal sequences were blasted against UNITE (https://unite.ut.ee/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. The best BLAST hit was considered based on e-value, higher similarity identity and also on ecological considerations. Only the identifications corresponding to blasts with the lowest E-value (nearly 0.0) and high identity score (>97%) were accepted.

"Trap culture" establishment and symptom monitoring

The three samples per greenhouse were thoroughly mixed in one group to perform the bioassays. Trap cultures were established using *Plantago lanceolata* L. and *Trifolium pratense* L. seeds. 15 seeds of each plant species were sown in pots with 200 g of each soil sample mixed with 200 g of sterilized sand. Pots were watered every 2/3 days during 6 months. Subsequently, both percentage of seed germination and plant height were analyzed in each pot. The percentage of plants showing symptoms or not was also examined 6 months after trap culture establishment.

Root staining

6 months after trap culture establishment, *Trifolium pratense* and *Plantago lanceolata* roots were washed and cotton blue staining was used to observe microscopically the AMF structures (arbuscules, hyphae, spores or sporocarps) present in the roots. Plant roots were placed on the slide containing ethanol 70% and subsequently stained with cotton blue for 3 min. Tissues were washed with distilled water to remove the dye and fungal colonization was analyzed under a microscope.

Pepper bioassays

Seeds of *Capsicum annuum* L. cv. Padron were harvested in previous years in our greenhouse and stored at 4ºC and low humidity conditions. Before sowing, seeds were disinfected in 0.1% (v/v) commercial bleach for 15 min, washed and stored overnight in darkness. Subsequently seeds were sown in sterile vermiculite and watered. Plants were grown in vermiculite for 17 days in a growth chamber. Plants were then transferred to pepper greenhouse soils, using 250 g of soil in each pot. Two independent experiments were carried out with 4 plants per soil, being a total of 10 soils. The number of wilted leaves was recorded 28 days after trasplantation (dat). Stem length and total fresh

weight were also recorded at the end of the experiment (28 dat). Roots from these pepper plants were used to extract DNA and quantify *V. dahliae* and *P. capsici* biomass, while pepper stems were used to isolate fungal microbiome.

DNA extraction and pathogen quantification

Pepper roots from bioassays (four roots per sample) were stored at -20 ºC at the end of the experiment (28 dat). Samples were homogenized with liquid nitrogen, and *V. dahliae* and *P. capsici* biomass were quantified according to Gayoso *et al.* (2007) and García *et al.* (2018). The primers are listed in Table 4.2.

Table 4.2. Primers used in pathogen biomass quantification through real time qPCR.

			Name	**Sequence**	**Amplicon**
CaBPR1	*Capsicum annuum*	Gayoso *et al.* (2007)	*PR1FW* *PR1RV*	5'GTTGTGCTAGGGTTCGGTGT 3' 5'CAAGCAATTATTTAAACGATCCA 3'	**301 bp**
P450 (Chr3g12080)	*Verticillium dahliae*	Lois *et al.* 2019 (submitted)	*VDS1* *VDS2*	5'AGCATTCAGTTCAGAAGACGGA 3' 5'CCGAAATACTCCAGTAGAAGG 3'	**517 bp**
ITS regions	*Phytophthora capsici*	Silvar *et al.* (2005)	*CAPFW* *CAPRV1*	5'TTTAGTTGGGGGTCTTGTACC 3' 5'CCTCCACAACCAGCAACA 3'	**452 bp**

Plant DNA quantification was carried out with the primers for *CaBPR1* and the method described in Gayoso *et al.* (2007) and García *et al.* (2018). Pathogen colonization was calculated as the ratio of pathogen DNA to plant DNA. Two independent experiments were carried out per soil.

Isolation of fungal microbiome

Stems from pepper bioassays (4 stems per sample) were disinfected in 5% (v/v) commercial bleach for 5 min, 70% (v/v) ethanol for 1 min, washed and placed in PDA

(Potato Dextrose Agar) medium. Every day during 21 days, fungal growth was monitored in all pepper stem samples from both types of greenhouses. Fungal strains were isolated individually by putting each fungal mycelium that was visually or morphologically different in a single Petri dish with PDA medium.

Statistical analysis

All statistical analyses were performed using STATGRAPHICS 5.1 for Windows, except statistical analysis for diversity indexes, which were performed using the Community Analysis Package 5.0 (CAP5) software. The tests that were applied and statistically significant differences are reported in the results section and shown in the figures.

Results

Similar physicochemical properties of the soil among diseased and disease-free greenhouses

Physical–chemical properties of each soil are summarized in Table 4.3. No remarkable differences in pH, nitrogen (N), carbon (C), hydrogen (H) or sulfur (S) levels were observed between I-V and VI-X greenhouses. Electrical conductivity (EC) measures the ability of a material to allow the transport of an electric charge. EC values showed variations within the same group of greenhouses, but not marked differences were observed between both types of soils (I-V and VI-X). Only IX and X soils had higher EC values in comparison with the remaining soils.

Table 4.3. Physicochemical characteristics of each greenhouse soil. EC: electrical conductivity; N: nitrogen; C: carbon; H: hydrogen; S: sulfur.

Greenhouse	pH	EC (μS/cm)	N (%)	C (%)	H (%)	S (%)
I	6.17 (slightly acid)	0.404	0.26	3.13	0.85	< 0.05
II	7.27 (neutral)	0.660	0.17	2.13	0.61	< 0.05
III	6.73 (neutral)	0.527	0.16	1.64	0.39	< 0.05
IV	5.99 (moderately acid)	0.210	0.26	2.74	0.52	< 0.05
V	6.29 (slightly acid)	0.711	0.22	2.20	0.48	< 0.05
VI	5.17 (strongly acid)	0.246	0.15	1.64	0.52	< 0.05
VII	7.42 (slightly alkaline)	0.351	0.19	2.15	0.99	< 0.05
VIII	5.47 (strongly acid)	0.618	0.23	2.67	1.08	< 0.05
IX	5.23 (strongly acid)	0.884	0.28	3.23	1.15	< 0.05
X	5.85 (moderately acid)	0.978	0.32	3.43	0.93	0.05

AMF diversity differs between soil of diseased and disease-free greenhouses

From the 30 samples collected in pepper greenhouses of Cambados and Ribadumia, a total of 13460 spores were counted under the dissecting microscope (Table 4.4). The total number of spores obtained from greenhouses without wilt diseases was 8924, and the total number of spores obtained from greenhouses with wilt diseases was 4536 (Table 4.4). Moreover, a total of 18 spore morphotypes (A to R) were distinguished in samples from different greenhouses, and the number of these spore morphotypes was the same in both types of greenhouses (17 morphotypes) (Table 4.4; Fig. 4.2 and 4.3). K morpthotype was exclusive to greenhouses without wilt diseases, and L morphotype was exclusive to greenhouses with wilt diseases (Table 4.4; Fig. 4.2).

Table 4.4. Number of spores in each sampled pepper greenhouse (from I to X). The numbers 1, 2 and 3 correspond to the three samples collected for each greenhouse.

Greenhouse	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	Total
I 1	57	19	9	0	22	10	8	18	11	13	0	15	18	10	10	6	8	6	240
I 2	68	30	11	0	23	27	6	19	20	15	0	19	14	9	11	7	12	14	305
I 3	55	37	4	0	33	26	4	17	16	29	0	16	21	12	9	3	16	11	309
II 2	75	44	4	0	41	29	8	20	15	23	0	17	11	9	13	6	11	9	335
II 2	43	24	7	0	17	17	10	12	13	18	0	22	8	15	14	3	9	8	240
II 3	66	52	8	0	25	22	7	22	17	24	0	12	21	11	17	3	16	16	339
III 1	81	47	9	0	37	21	7	20	14	17	0	16	10	17	12	5	10	8	331
III 2	60	37	11	1	22	12	5	18	18	14	0	23	10	9	13	1	10	11	275
III 3	68	46	18	2	31	24	6	29	20	13	0	28	21	12	11	1	25	18	373
IV 1	64	30	5	0	18	10	11	25	9	12	0	12	17	11	7	6	12	6	255
IV 2	77	32	15	0	35	22	7	23	17	21	0	12	11	8	15	2	19	17	333
IV 3	40	27	19	0	33	18	4	30	21	17	0	22	30	19	12	1	16	9	318
V 1	72	34	12	0	31	15	9	26	18	13	0	15	7	8	13	8	9	8	298
V 2	53	37	9	0	25	19	6	20	18	12	0	19	15	12	9	5	14	11	284
V 3	47	28	15	2	19	13	8	34	26	9	0	13	21	11	17	2	21	15	301
Total	**926**	**524**	**156**	**5**	**412**	**285**	**106**	**333**	**253**	**250**	**0**	**261**	**235**	**173**	**183**	**59**	**208**	**167**	**4536**
VI 1	102	59	10	15	51	28	7	30	25	32	0	0	13	8	8	2	29	16	435
VI 2	205	48	5	8	103	24	13	21	18	23	2	0	6	4	3	1	10	8	502
VI 3	165	51	7	9	68	25	10	24	19	22	2	0	5	4	4	1	12	11	439
VII 2	170	54	10	5	66	32	11	38	28	26	1	0	3	6	4	3	19	13	489
VII 2	163	60	12	12	35	20	14	40	26	36	0	0	17	12	10	2	25	13	497
VII 3	143	78	15	19	82	55	10	47	41	29	2	0	13	8	11	0	38	22	613
VIII 1	185	100	20	30	145	105	8	50	35	45	2	0	11	7	5	6	21	12	787
VIII 2	242	106	14	11	91	71	20	67	49	42	1	0	15	10	10	4	31	29	813
VIII 3	123	41	6	25	78	53	12	51	52	51	2	0	21	17	11	0	36	28	607
IX 1	339	29	5	9	168	38	9	48	21	50	2	0	4	6	3	2	16	10	759
IX 2	179	77	12	7	65	38	17	60	40	39	2	0	16	9	12	3	29	19	624
IX 3	152	69	5	10	58	51	11	61	39	31	1	0	8	5	8	0	34	20	563
X 1	217	43	7	10	92	47	10	52	23	33	2	0	6	8	4	1	17	11	583
X 2	184	71	8	14	67	39	8	44	41	28	1	0	17	13	9	3	26	18	591
X 3	173	69	5	9	80	52	13	63	49	23	1	0	9	7	5	0	37	27	622
Total	**2742**	**955**	**141**	**193**	**1249**	**678**	**173**	**696**	**506**	**510**	**21**	**0**	**164**	**124**	**107**	**28**	**380**	**257**	**8924**

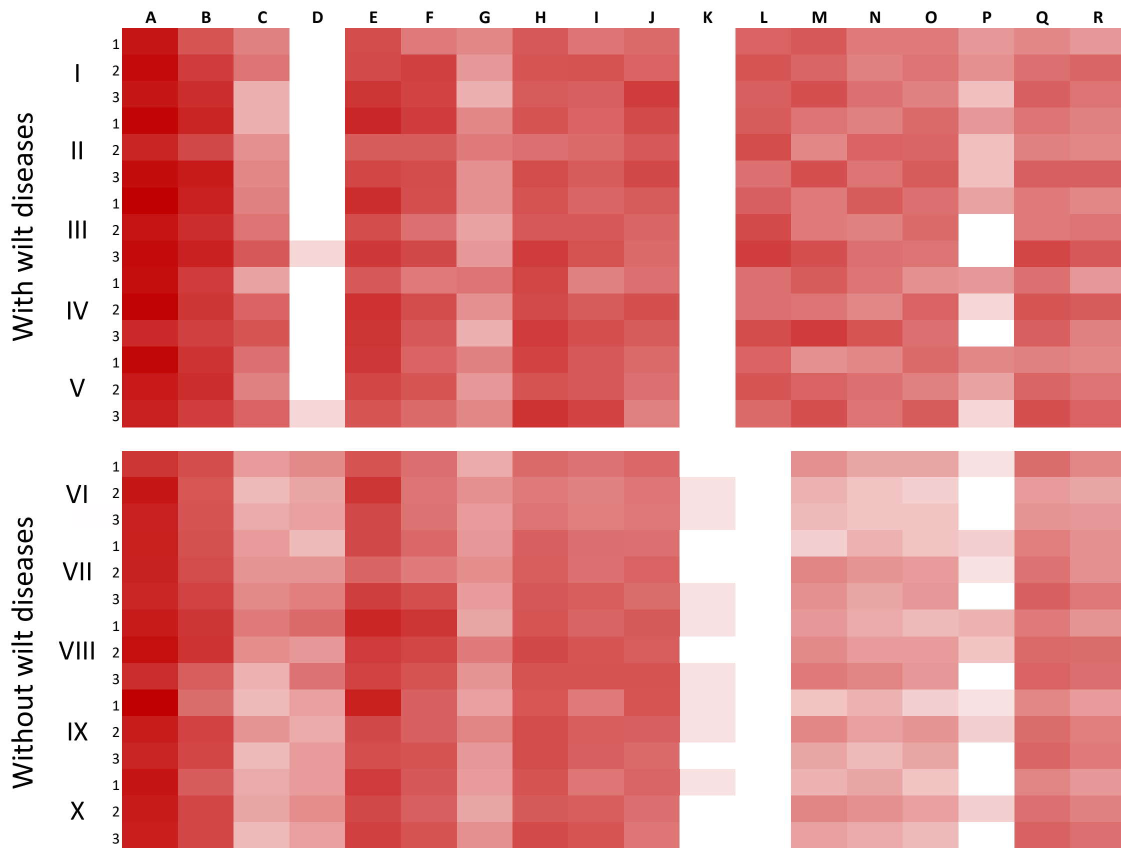

Figure 4.2. Color code chart of the different morphotypes (A to R) found in all pepper greenhouses (from I to X). The numbers 1, 2 and 3 correspond to the three samples collected for each greenhouse. The color gradient indicates the number of spores on a log10 scale found for each morphotype (white, 0-1; dark red, 50 or higher).

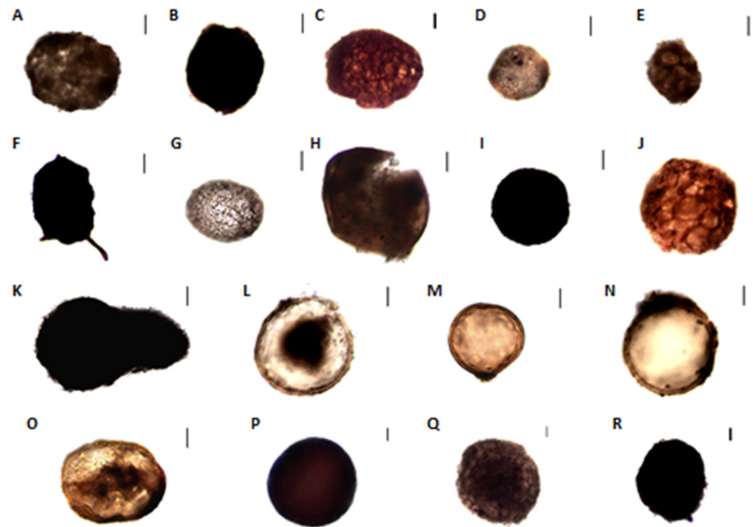

Figure 4.3. AMF spore morphotypes (A to R) isolated from pepper greenhouse soils at optical microscope. Scale bars: 20 µm.

The similarity between samples was performed by a non-metric multidimensional scale (NMDS) plot with Bray-Curtis coefficient using the Community Analysis Package 5.0 (CAP5) software (Henderson & Seaby, 2007). The coefficient of similarity (Bray-Curtis coefficient) (Bray & Curtis, 1957) is a coefficient of beta diversity that is calculated considering the differences in abundance of each species between sites. The NMDS analysis was performed using data from the number of spores for each morphotype. We observed that both types of greenhouses showed visible differences in abundance (Fig. 4.4); all samples from greenhouses without wilt diseases were completely separated from those samples with wilt diseases (Fig. 4.4).

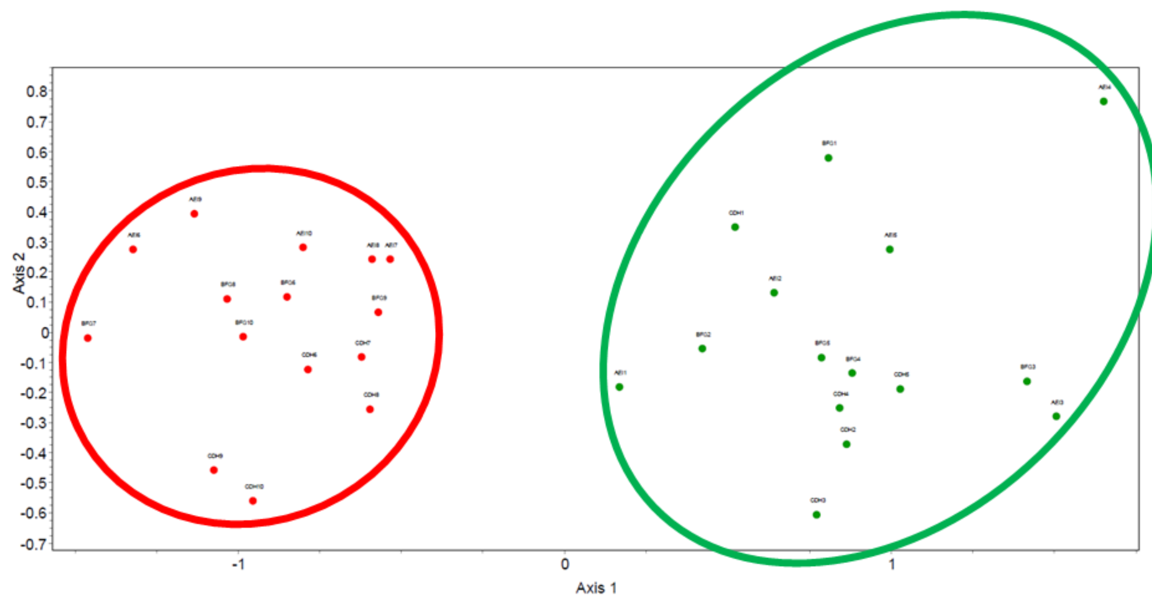

Figure 4.4. Non-metric multidimensional scale (NMDS) plot corresponding to the clustering analysis among samples for all greenhouses performed with Bray-Curtis coefficient (2D Stress = 0.0478968). The different set of colors represents different types of greenhouses (red: greenhouses presenting wilt diseases; green: greenhouses without wilt diseases).

Species Diversity and Richness 4.1.2. (SDR4) (Seaby & Henderson, 2007) was used to determine alpha diversity indexes in order to study the morphological species diversity within the same specific community, namely, Simpson diversity index (D), Shannon-Wiener diversity index (H') and Fisher's alpha indexes (Table 4.5). The number of morphotypes shared between samples was obtained by SDR4 software (S; Table 4.5). Alpha diversity indexes determine the diversity of a specific community considering both the number of species and the proportion in which each species is represented. Simpson's index (Simpson, 1949) measures the probability of any two randomly chosen individuals taken from an infinitely large community belong to the same species. This index is more focused on the abundance of species in the sample than the species richness itself. Shannon index (H') (Shannon, 1948) characterizes the species diversity in a community. This index computes the uncertainty associated with identifying species

in a community. Fisher's alpha index (Fisher *et al.*, 1943) accepts that species abundance follows a log series distribution. We observed significant differences between greenhouses with wilt diseases (I-V) and greenhouses without wilt diseases (VI-X) in the three alpha diversity indexes (Table 4.5), that is, the diversity is different between greenhouses with and without diseases. Greenhouses I-V displayed the highest diversity indexes in comparison with greenhouses VI-X (Table 4.5).

Table 4.5. Species richness and diversity parameters for AMF communities from the two types of soils studied: species richness (S), Simpson's index (D), Shannon index (H´) and Fisher's alpha. Statistically significant differences in ANOVA statistical analysis for each index were denoted by *, **, *** at $p \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Greenhouse	**S**	**D**	**H´**	**α Fisher**
I-V	17	10,75***	2,58***	2,323**
VI-X	17	6,751***	2,278***	2,096**

For comparing the morphotype diversity in each studied greenhouse, rarefaction curves were also determined. The rarefaction curves from all greenhouses reached a plateau, suggesting that the AMF community is well represented (Fig. 4.5). Moreover, the rarefaction curves suggest that greenhouses VI-X have the highest AMF diversity when compared with I-V greenhouses (Fig. 4.5).

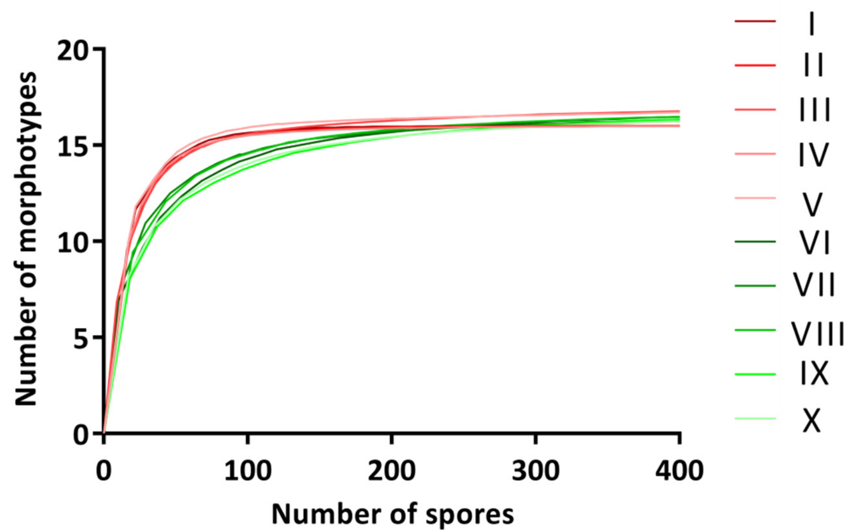

Figure 4.5. Rarefaction curves for the AMF community at 97% similarity. Representation of the rarefaction curves for the different study sites. Rarefaction curves computed in Species Diversity and Richness 4.1.2 (Seaby & Henderson, 2007) and plotted in GraphPad 6 (GraphPad Software).

Most of the identified AMF belong to Glomerales or Diversisporales orders

AMF molecular identification was performed through the DNA extracted from a single spore, which made it difficult to identify at the species or genus level. All the DNA sequences analyzed belong to Glomerales or Diversisporales orders. C, D, G, H and J belong to Diversisporales order, and E, F, K, L and R morphotypes belong to Glomerales order. We could not conclude if A, B, I, M, N, O, P and Q morphotypes belong to Diversisporales or Glomerales orders, but all of them are members of the Glomeromycetes class (Glomeromycota division).

Soil from diseased greenhouses produce disease symptoms in trap cultures (*P. lanceolata*, and *T. pratense*)

6 months after trap culture establishment, we observed that both *Plantago lancelata* L., and *Trifolium pratense* L. plants grown in I-V soils with vascular wilt diseases showed several disease symptoms such as chlorosis, wilting and dwarfism (Fig. 4.6) in comparison with plants grown in VI-X pepper soils, which barely showed symptoms (Fig. 4.6).

Figure 4.6. View of *T. pratense* and *P. lanceolata* plants grown in both types of greenhouses 6 months after trap culture establishment.

6 months after trap culture establishment, greenhouses VI-X had a significant higher percentage of germinated plants in comparison with greenhouses I-V (Fig. 4.7A). Moreover, plants from greenhouses VI-X had a significant greater height than plants from greenhouses I-V (Fig. 4.7B). We differenciated between plants with symptoms and plants without symptoms, and we observed that greenhouses I-V had a significant higher number of plants with symptoms in comparison with greenhouses VI-X, and the number of plants without symptoms was significantly lower in greenhouses I-V than in VI-X greenhouses (Fig. 4.8).

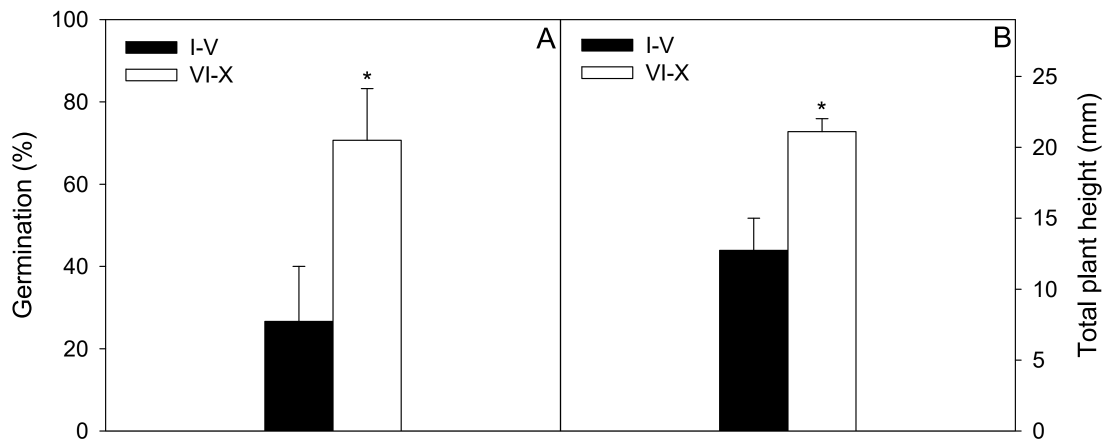

Figure 4.7. Germination and height of trap plants (*T. pratense* and *P. lanceolata*) 6 months after sowing in soil from both types of greenhouses. (A) Percentage of plant germination in I-V and VI-X greenhouses. (B) Total plant height in I-V and VI-X greenhouses. Asterisks indicate statistical differences ($p < 0.05$) in Student's t-Test.

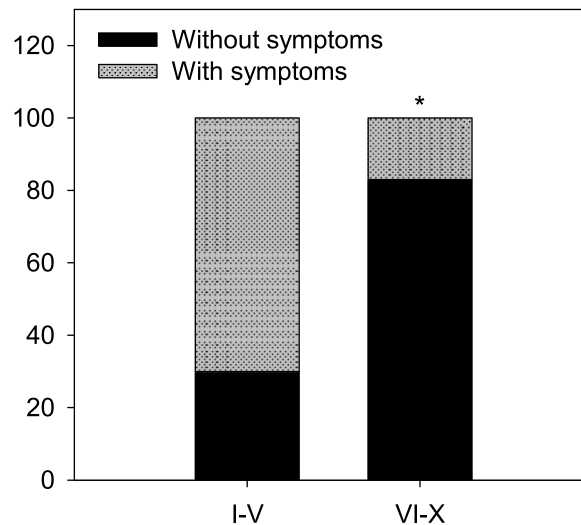

Figure 4.8. Percentage of trap plants with or without symptoms 6 months after sowing in soil from both types of greenhouses. Asterisk indicates statistical differences ($p < 0.05$) in Pearson's chi-squared test.

Similar abundance of mycorrhization between trap cultures growing in soils from disease-free and diseased greenhouses

AMF structures such as hyphae, spores and arbuscules were observed in roots stained with cotton blue (Fig. 4.9). However, we could not detect strong differences in arbuscular mycorrhization between plants in soil from both types of greenhouses.

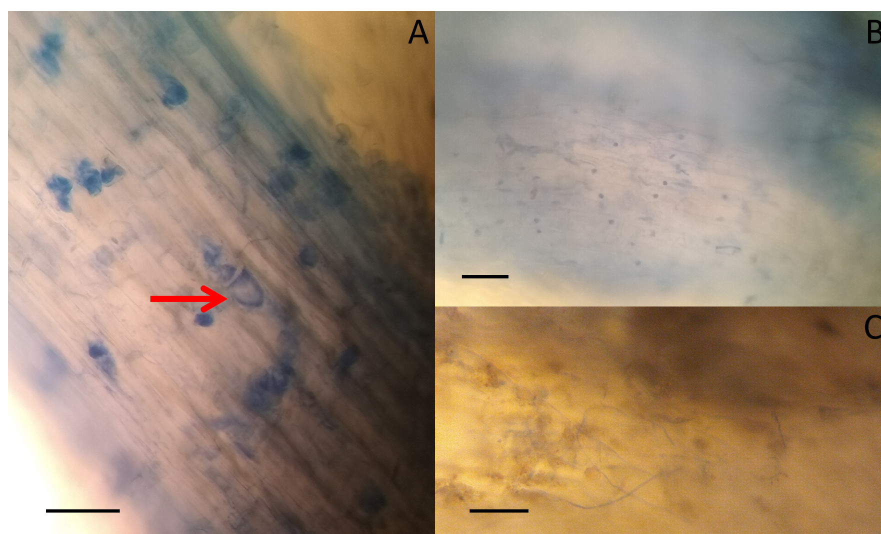

Figure 4.9. View of AMF colonization after 6 months of trap culture establishment. (A) Formation of arbuscules (arrow) in *Plantago lanceolata* root. (B) Spores atached to the root in *Trifolium pratense*. (C) Hyphae in *Plantago lanceolata* roots. Scale bar: 50 µm (A) and (B); 25 µm (C).

Soil from diseased greenhouses produce disease symptoms in pepper (*C. annuum*)

Disease severity (measured as percentage of wilted leaves) was significantly lower in VI-X soils (disease-free soils) than in soils from I-V greenhouses (diseased soils) 28 dat

(Fig. 4.10A; Fig. 4.11). Stem length and fresh weight were significantly higher in VI-X soils in comparison with I-V greenhouses 28 dat (Fig. 4.10B, C).

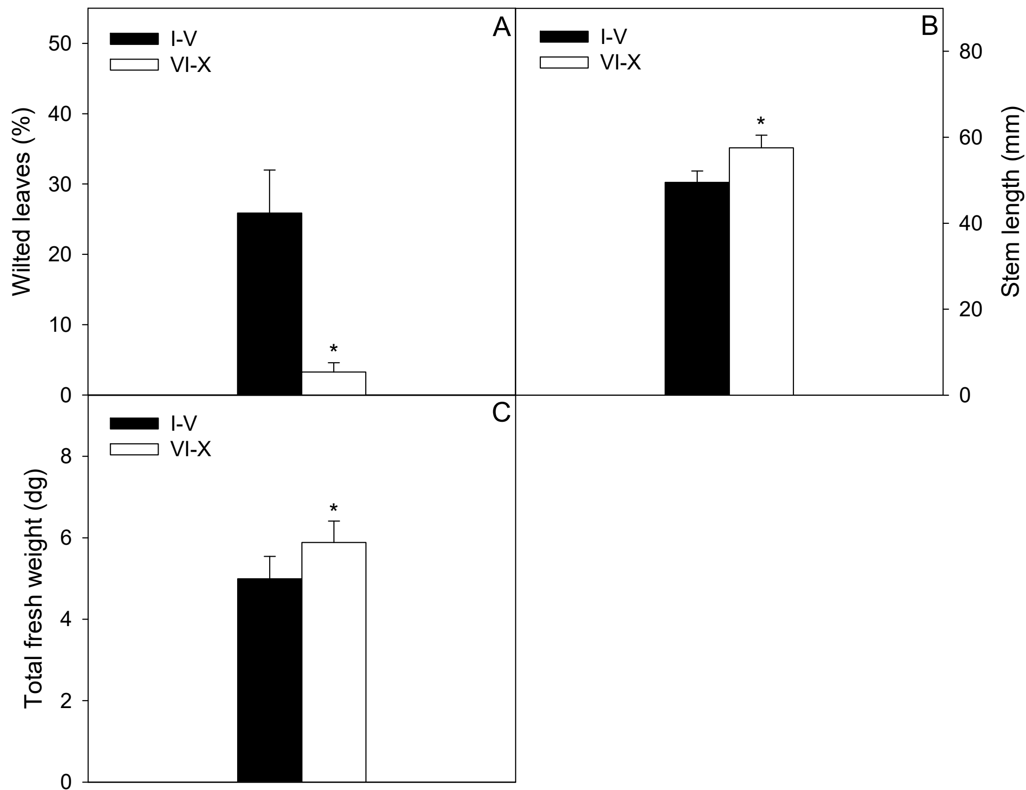

Figure 4.10. Disease symptoms and plant growth parameters of pepper grown in disease-free (VI-X) and diseased (I-V) soils 28 days after trasplantation (28 dat). (A) Percentage of wilted leaves (A), stem length (B) and total fresh weight (C) in pepper plants grown in both types of greenhouses. Asterisks indicate statistical differences between both types of greenhouses ($p < 0.05$) in a Mann-Whitney test. Two independent experiments were performed with 4 plants per treatment and per experiment (n=40).

Figure 4.11. Pepper plants grown in disease-free (VI-X; left) and diseased (I-V; right) soils 28 days after trasplantation (28 dat).

Verticillium dahliae and *Phytophthora capsici* are detected in roots of pepper plants growing in soil from diseased and disease-free greenhouses

Phytophthora capsici biomass in pepper roots was significantly higher in I-V soils in comparison with VI-X soils 28 dat (Fig. 4.12A). *Verticillium dahliae* biomass in pepper roots was higher in I-V soils in comparison with VI-X soils 28 dat; however, this difference was not statistically significant (Fig. 4.12B).

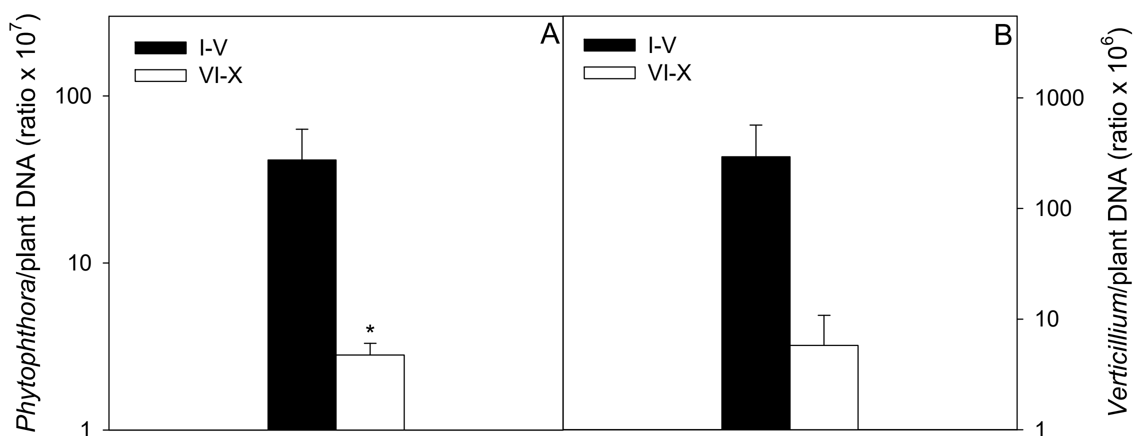

Figure 4.12. Pathogen colonization in roots of pepper plants grown in disease-free (VI-X) and diseased (I-V) soils 28 days after trasplantation (28 dat). (A) *P. capsici* colonization 28 dat expressed as the ratio between *P. capsici* DNA and *C. annuum* DNA. (B) *V. dahliae* colonization 28 dat expressed as the ratio between *V. dahliae* DNA and *C. annuum* DNA. Asterisks indicate statistical differences between both types of greenhouses ($p < 0.05$) in a Mann-Whitney test. Two independent experiments were performed with 5 greenhouses per group (n=10).

Several fungi were present in pepper grown in diseased or disease-free soils

AMF are difficult to culture, since they form multispecies symbiont communities in the same root. Moreover, the procedure to collect and separate different AMF species from the whole root is laborious and AMF spores may lose their viability. Therefore, we isolated other fungal and fungal-like species from pepper stems grown in soils of both types of greenhouses in the laboratory (Fig. 4.13). Fungal identification was permorfed by Jesús Collar Urquijo of Lafiga (Laboratorio Agrario e Fitopatolóxico de Galicia) in Mabegondo (Abegondo, A Coruña) based on macroscopic (e.g. color, rate of growth) and microscopic (e.g. hyphal morphology, asexual reproductive structures) features (Table 4.6).

According to morphological identification, we found different *Fusarium oxysporum* strains in both suppressive and conducive soils (Table 4.6). The saprophytic fungus *Chaetomium globosum* and the oomycete *Phytium* sp. were also present in both types of soils (Table 4.6). *Penicillium* sp. and *Cephalosporium* sp. were only found in suppressive soils, and *Cladosporium* sp. and *Alternaria* sp. were found in conducive soils (Table 4.6). Neither *P. capsici* nor *V. dahliae* were found in any of the analyzed soils, but quantification of pathogen biomass through real time qPCR in pepper roots grown in I-V and VI-X soils prove the presence of both phytopathogens.

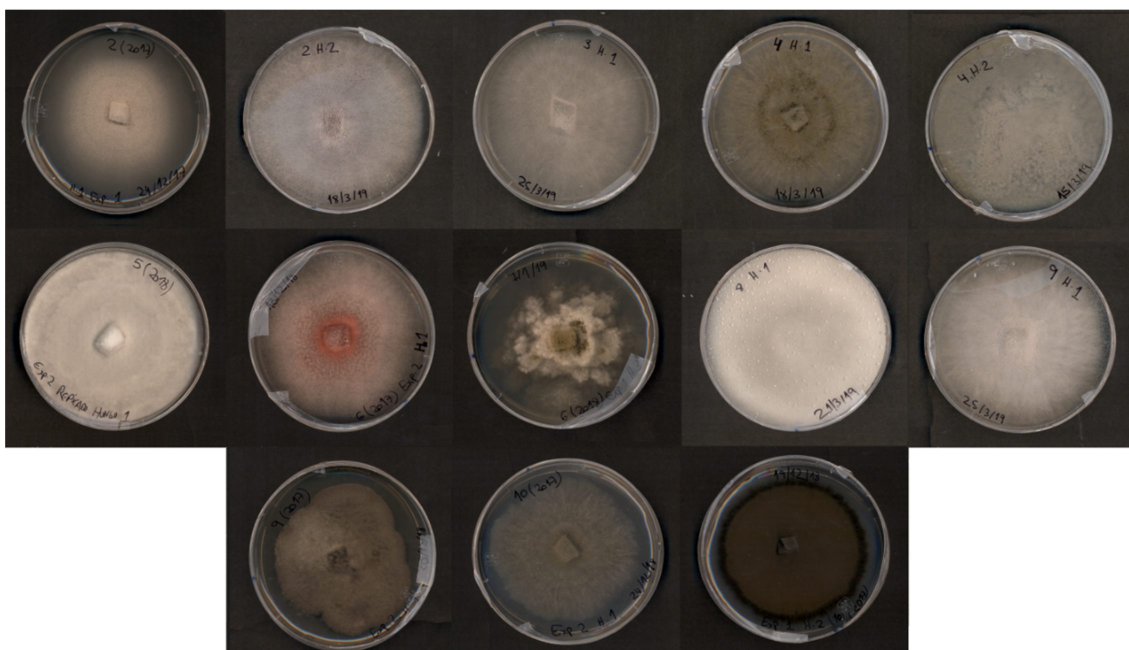

Figure 4.13. Several fungal and fungal-like isolates from pepper stems growing in both types of greenhouses 28 days after trasplantation (28 dat).

Table 4.6. Fungal and oomycete strains isolated from pepper stems in both suppressive and conducive soils.

Phylum	Genera	Species	Greenhouse
Ascomycota	*Fusarium*	*Fusarium oxysporum*	I
Ascomycota	*Chaetomium*	*Chaetomium globosum*	I
Oomycota	*Pythium*	*Pythium* sp.	III
Ascomycota	*Fusarium*	*Fusarium oxysporum*	IV
Ascomycota	*Alternaria*	*Alternaria* sp.	IV
Ascomycota	*Chaetomium*	*Chaetomium globosum*	V
Ascomycota	*Cladosporium*	*Cladosporium* sp.	V
Ascomycota	*Cephalosporium*	*Cephalosporium* sp.	VII
Ascomycota	*Fusarium*	*Fusarium oxysporum*	VII
Ascomycota	*Fusarium*	*Fusarium oxysporum*	VIII
Ascomycota	*Chaetomium*	*Chaetomium globosum*	IX
Ascomycota	*Penicillium*	*Penicillium* sp.	IX
Oomycota	*Pythium*	*Pythium* sp.	X

Discussion

Suppressive soils have acquired a great interest due to their potential for sustainable agriculture. Soils suppressive to diseases caused by many soil-borne fungi (e.g. *Fusarium* spp., *Thielaviopsis basicola*, *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, *Verticillium dahliae*), oomycetes (e.g. *Pythium* spp., *Phytophthora* spp.), bacteria (e.g. *Ralstonia solanacearum*, *Streptomyces scabies*) and nematodes (e.g. *Meloidogyne* spp.) have been described to occur worldwide (Gómez Expósito *et al.*, 2017).

Generally, disease suppressiveness is due to the concerted activities of multiple microbial genera working together at specific sites or operating at different stages of the infection process of the pathogen rather than to only one microbial species (Mahatma & Mahatma, 2015; Gómez Expósito *et al.*, 2017). It has been demonstrated that interactions with certain members of the rhizosphere community can significantly modulate the degree of suppressiveness of the soil (Weller *et al.*, 2002; Antoniou *et al.*, 2017; Durán *et al.*, 2017), which proves the importance of the root microbiome (and its activity) in disease suppressive soils. Furthermore, the microbial community in the rhizosphere is more abundant and more diverse than in the bulk soil, and also the first have greater biological activity (Garbeva *et al.*, 2004; Berg & Smalla, 2009; Mendes *et al.*, 2013; Durán *et al.*, 2017).

In this work, we found several Padron pepper greenhouse soils suppressive to *P. capsici* and *V. dahliae*. Moreover, analysis of AMF present in those soils revealed that these fungal microorganisms may be playing a role in the suppressiveness observed (specific suppression). As soil communities can differ between suppressive and conducive soils (Postma *et al.*, 2008; Penton *et al.*, 2014; Li *et al.*, 2015; Lee *et al.*, 2017; Inderbitzin *et al.*, 2018; Mitsuboshi *et al.*, 2018; Siegel-Hertz *et al.*, 2018), we analyzed AMF community in both suppressive and conducive soils and we found differences in AMF diversity and abundance between them. Generally, disease suppressive soils have higher microbial diversities in comparison with conducive soils (Cretoiu *et al.*, 2013; Chapelle *et al.*, 2015; Lee *et al.*, 2017), and abundance of several taxa can also correlate with soil suppressiveness (Cretoiu *et al.*, 2013; Penton *et al.*, 2014; Chapelle *et al.*, 2015; Orquera-Tornakian *et al.*, 2018; Siegel-Hertz *et al.*, 2018; Inderbitzin *et al.*, 2018; Mitsuboshi *et al.*, 2018). Surprisingly, in our case AMF diversity was significantly higher in conducive soils, as shown by significant higher values of alpha diversity

indexes in I-V soils; however, the AMF abundance was notably higher in suppressive soils, since the number of AMF spores in VI-X soils almost doubled the number of AMF spores in I-V soils. The significantly lower AMF diversity in our suppressive soils was also observed in the study of Lee *et al.* (2017), where bacterial community was less diverse in soil not showing the symptoms of the disease caused by *Ralstonia solanacearum* in the lower soil layer. The difference in AMF community between suppressive and conducive soils is probably due to changes in relative abundances, as in the study of Penton *et al.* (2014), where the abundance of fungal community differs between suppressive and conducive soils to *Rhizoctonia solani*, but the diversity of fungi was not different between both types of soils.

Interactions between the fungal pathogen, the plant and the rhizosphere microbiome are key elements in suppressive soils (Chapelle *et al.*, 2015). Hence, first we performed plant bioassays in a growth chamber using *Trifolium pratense* and *Plantago lanceolata* and the soils from Galician greenhouses described above, and second we also carried out the bioassays using Padron pepper plants to detect possible differences in symptoms and plant growth parameters, and we tried to identify potential pathogens acting in those soils. The percentage of germination and plant height in trap cultures were significantly higher in VI-X soils, but also the number of plants without symptoms, and pepper plants growing in VI-X greenhouses (suppressive soils) showed a significant lower percentage of wilted leaves in comparison with I-V greenhouses (conducive soils). This can be due to the less pathogen biomass observed in suppressive soils rather than the higher abundance of AMF. Antoniou *et al.* (2017) observed a significant lower *V. dahliae* and *F. oxysporum* disease severities in tomato plants grown in suppressive soils. We observed that stem length and fresh weight were significantly higher in pepper plants grown in suppressive soils; these results are in agreement with those obtained by

Antoniou *et al.* (2017), which observed a significant increase in plant height, total leaf surface, and fresh and dry weight in tomato plants grown in suppressive soils to *V. dahliae* and *F. oxysporum*. Inderbitzin *et al.* (2018) also observed that eggplant plants grown in Verticillium wilt-suppressive soils had also less disease severity and higher plant height.

In our work, we quantified *P. capsici* and *V. dahliae* colonization in pepper roots grown in suppressive (VI-X) or conducive (I-V) soils. We observed that *P. capsici* colonization was significantly higher in conducive soils 28 days after trasplantation to the greenhouse soils. *V. dahliae* biomass was also higher in pepper roots from conducive soils, but this difference was not statistically significant. However, quantification of both pathogens in soil would reveal if VI-X greenhouses are suppressive soils or just healthy soils in which both pathogens are absent or their inoculum is minimal to promote disease. In that case the soil would not be a suppressive soil but just a healthy soil. Lower fungal pathogen colonization in plants growing in suppressive soils was previously observed (Almario *et al.*, 2013; Antoniou *et al.*, 2017; Avilés & Borrero, 2017; Inderbitzin *et al.*, 2018). However, it would be necessary to quantify the inoculum of these pathogens in the soils from both types of greenhouses to confirm that VI-X soils are suppressive to vascular wilts. It is possible that VI-X soils simply had less pathogen inoculum and consequently the progress of the disease would be delayed.

Moreover, phytopathogens other than *V. dahliae* and *P. capsici* were present in stems of pepper plants grown in both suppressive and conducive soils such as *Pythium* sp. and *Fusarium* sp., which cause the disease known as damping off that affects seeds and new seedlings (Lamichhane *et al.*, 2017).

Regarding to the measured abiotic parameters, no noticeably differences were found in pH values among all the greenhouses. Usually, oomycete genera *Phytophthora* and *Pythium* are inhibited by a low pH (Jambhulkar *et al.*, 2015). In the study of Ann (1994), soils with a pH lower than 5.0 were more suppressive to *Phytophthora* spp. than those with a higher pH. None of the suppressive and conducive soils in our work had a pH lower that 5, and two of them (II and VII) had a pH higher than 7. In the study of Ochiai *et al.* (2008), pH played an important role in Verticillium wilt suppressive soils; soils with pH higher than 7 were more conducive to Verticillium wilt, and pH lower that 5.5 were more suppressive. In fact, we observed that greenhouses VI, VIII and IX are strongly acid. Generally, *Verticillium* spp. prefers alkaline conditions (Mahatma & Mahatma, 2015). However, there are other studies in which Verticillium wilt disease was favored by acidic soils (Jones *et al.*, 1971; Shao & Foy, 1982; Avilés & Borrero, 2017). Usually, fungi proliferate better in acid environments than bacteria (Postma *et al.*, 2008). Most of the soils analyzed in this work had an acid pH, but VI-X soils showed slightly more acid pH in comparison with I-V soils, so the highest abundance of AMF in these suppressive soils make sense. However, in the study of Cruz-Paredes *et al.*, (2019), AMF activities (e.g. mycorrhization, nutrient supply) were suppresed more frequently in low pH than in high pH soils. Also, the abundance of other fungal taxa different from AMF or bacterial taxa influence their activities (Cruz-Paredes *et al.* 2019). Therefore, the difference in AMF abundance between suppressive and conducive soils in our work may be due to a greater prevalence of specific fungi and/or bacteria in conducive soils that supress AMF activities and, therefore, decrease the abundance of these beneficial microorganisms, thus causing more disease in these soils.

On the other hand, none of the analyzed soils differed in nitrogen (N), carbon (C) or sulfur (S) contents. Only hydrogen (H) content was notably higher in I-V greenhouses,

and S content was noticeably higher in X greenhouse. In the study of Tenuta & Lazarovits (2004) a higher N content was associated with soil suppressiveness against Verticillium wilt. Higher C content was also associated with suppressiveness to *V. dahliae* (Ochiai *et al.*, 2008). In our work suppressiveness to *V. dahliae* and *P. capsici* does not appear to be related with mineral composition, and the biotic component appears to be the principal factor influencing soil suppressiveness. Mitsuboshi *et al.* (2018) reported the importance of both abiotic and biotic factors in suppressiveness depending on the plant-pathogen interaction and soil type. In the study of Durán *et al.* (2017), chemical properties and geographical origin of suppressive soils to *Gaeumannomyces graminis* var. *tritici* did not infuence soil suppressiveness. Other abiotic properties, such as aggregate size distribution, soil texture (loam, sandy loam, clay) or EC may play a role in suppressiveness (Penton *et al.*, 2014; Li *et al.*, 2018a). For example, in the study of Li *et al.* (2018b), both suppressive and conducive soils to Fusarium wilt had similar acidity and mineral composition, and this is in agreement with our results. In their study, however, the aggregate size distribution was different between suppressive and conducive soils; suppressive soils had a wider distribution of aggregates and higher contents of macro-aggregates. Moreover, Fusarium wilt suppressive soils had higher EC values in comparison with conducive soils and lower clay contents which can affect suppressiveness due to the limited pores which imply lower air flow and, therefore, lower oxygen content (Li *et al.*, 2018a). We also analyzed EC in pepper greenhouse soils, and we observed that IX and X suppressive soils had the highest EC values, but no noteworthy differences in EC were observed between conducive and suppressive soils. On the other hand, salinity can also increase Verticillium wilt disease severity in pepper (Pascual *et al.*, 2009), so the salts producing the high EC values in our conducive soils (II, III, V) could be an important factor in

Verticillium wilt development. The different EC values between soils from the same type of greenhouse in both suppressive and conducive soils could be due to greater amounts of pathogen inoculum, besides a higher salinity, in these specific soils. Moreover, soluble salts are not the only factor responsible for increasing the EC of a soil. The EC of soils varies depending on the soil particle size and texture, which are determined by the amount of moisture held by soil particles. For example, sands have lower conductivity than silts and clays. Therefore, the particle size in these pepper soils must be taken into account.

The suppressiveness of a soil may be affected by other factors. The crop itself, as well as weeds or cover crops, can enhance the suppressive level of the soil. The use of organic amendments (e.g. chitin, chitosan, composts, green manures), is known for its suppressive properties against different soil-borne pathogens, including *V. dahliae* and *P. capsici*. These organic amendments are based on the incorporation of large amounts of organic matter to reproduce the environment of naturally suppressive soils (Ochiai *et al.*, 2008; Larkin *et al.*, 2011; Ji *et al.*, 2012; Cretoiu *et al.*, 2013; Antoniou *et al.*, 2017; Avilés & Borrero, 2017; Inderbitzin *et al.*, 2018). Other agricultural practices such as crop rotation or tillage can also enhace microbial biomass and activity and, therefore, suppressivenes (Janvier *et al.*, 2007; Larkin *et al.*, 2011; Stirling *et al.*, 2012; Li *et al.*, 2015; Siegel-Hertz *et al.*, 2018). Nevertheless, crop rotation is less successful against pathogens with a wide range of hosts or with efficient survival forms as is the case for *V. dahliae*. Also, certain diseases are generally less severe in organic than in conventional farms due to monocropping and the increased use of pesticides in the latter (Shrestha *et al.*, 2014). All the analyzed soils in this work had similar previous treatments (e.g. Previcur®, sulfur treatment) and no specific cultural practices were used. Also, there were some conducive and suppressive soils with no crops at the time of sampling, and

other suppressive and conducive soils that had crops at the time of sampling, so suppressiveness appears to be unrelated with the crop itself. All these data constitute another evidence of the importance of soil microbiota in suppressivenes of pepper greenhouses to wilt diseases.

Biocontrol potential of AMF could be explained in terms of its ability to change root architecture, improved nutrient uptake, competition with the pathogen for infection site, activation of plant structural (e.g. lignification) and biochemical defenses (e.g. chitinases, peroxidases, superoxide dismutases, phenolic compounds, phytoalexins) (Zheng *et al.*, 2005; Garmendia *et al.*, 2006; Ozgonen & Erkilic, 2007; Sánchez-Bel *et al.*, 2016; Li *et al.*, 2018b). Li *et al.* (2015) found decreased abundances in several AMF belonging to Glomerales in soils with decreased suppressiveness, suggesting that the less mycorrhization of the rhizosphere affects soils suppressiveness. Penton *et al.* (2014) also found that mycorrhizal fungi were among the dominant members in the suppressive soils.

In addition to AMF, other fungal antagonists have been found in suppressive soils (e.g. *Trichoderma* spp., *Penicillium* spp., non-pathogenic *Fusarium* spp.) (Mahatma & Mahatma, 2015; Mitsuboshi *et al.*, 2018). Some of them are lignin and chitin degraders that also can degrade fungal melanin from microsclerotia walls and hyphal cell walls, (Inderbitzin *et al.*, 2018), and this can explain the reduced pathogen biomass observed in pepper roots from our suppressive soils. In the study Siegel-Hertz *et al.* (2018), the presence and abundance of *Penicillium* spp. in *Fusarium* suppressive soils was associated with reduced disease and reduced pathogen abundance. We also found these two fungal genera in the stems of pepper grown in our suppressive soils. As *Fusarium* also causes wilt in pepper, our soils could also be suppressive to Fusarium wilt. On the

other hand, the *Penicillium* strain found in pepper stems from the suppressive soil IX may be acting by inducing resistance against *P. capsici* and *V. dahliae* in that pepper soil, since other *Penicillium* strain has been demonstrated to protect pepper against *V. dahliae* (Chapter 1). Other several fungi were isolated from pepper stems grown in conducive soils (*Alternaria* sp.) which might have deleterious effects on Padron pepper. Moreover, some of the AMF that were more abundant in suppressive soils could facilitate Padron pepper protection, and some taxa that could be present in suppressive or conducive soils could have positive or negative effects respectively on pepper health. In the future, metagenomic approaches will help in our understanding about how fungal communities (specially AMF) present in pepper greenhouse soils influence their suppressiveness to *P. capsici* and *V. dahliae*.

The use of pepper greenhouse soils suppressive to vascular wilts with a specific AMF abundance and diversity could be combined with other well-known biocontrol agents such as PO212 to develop an effective strategy to manage vascular wilts in both greenhouses and open fields.

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General discussion

PO212 induced resistance against *V. dahliae* in the roots, reducing both symptoms and pathogen colonization. Therefore, we studied how PO212 affect the cell wall lignification, some biochemical defenses (*PR-1* gene, capsidiol biosynthesis gene, β-1,3-glucanase, chitinase and peroxidase activities) and the biosynthesis of the main hormones involved in defense. β-1,3-glucanases and chitinases are involved in fungal cell wall degradation and they play an important role intensifying the plant defense (Van Loon & Van Strien, 1999; Oliveira *et al.*, 2016).

48 hours after PO212-treatment, the plant showed a strengthen of cell wall (lignin deposition), increase of capsidiol biosynthesis gene and β-1,3-glucanase, chitinase and peroxidase activities, as well as an increase in the expression of ET, JA and SA biosynthesis genes. Similar results were obtained in PO212-elicited pepper cell cultures, except that lignin was not detected. Overall, the results point out that PO212 increases plant defenses. This fact would explain why PO212-treated plants had less pathogen biomass than control. However, these changes were not enough to avoid Verticillium wilt disease because pepper plants still showed symptoms. Then, we studied what happened in pepper roots after *V. dahliae* inoculation.

The first barrier that pathogens have to defeat is the cell wall. Cell wall plays two roles in a defensive process: a physical barrier and a source of signals (Bacete *et al.*, 2018). Plant cell wall is a critical point in defense because it is the first barrier that pathogens have to defeat. Moreover, alterations in cell wall, including lignin content, modify plant resistance (García *et al.*, 2018b). As we mentioned previously, the cell wall of pepper roots was reinforced with lignin accumulation mediated by peroxidases after PO212 treatment. After *Verticillium* inoculation, PO212-treated plants also showed higher lignin. These results suggested that the most active deposition of lignin started after

PO212 treatment. Normally, lignin deposition uses to be linked to pathogen attack, but in some cases the application of elicitors in absence of pathogen can have the same effect (García *et al.*, 2018b). Therefore, lignin would make more difficult the penetration of *V. dahliae* when the pathogen arrives to the root surface.

After recognition, the plant accumulated around the penetration point capsidiol and PR-1 proteins which have demonstrated to be important in defense against pathogens, including *V. dahliae* (Veloso & Díaz, 2012; Gamir *et al.*, 2017; García *et al.*, 2018a).

To study the lignification we measured the activity of the enzymes peroxidases, because they are involved in lignin polimerization (Almagro *et al.*, 2009), and soluble phenolics as a source of monomers to form lignin. The data pointed out the peroxidases were responsible of that lignification, particularly *CaPO1* and *CaPO2*, with consumption of phenolics. *CaPO1* is related with lignification (Chmielowska *et al.*, 2010) and *CaPO2* is related with H_2O_2 production (Choi *et al.*, 2007). PO212-treated plants showed high levels of H_2O_2. The data suggest that this H_2O_2 could be produced, at least in part, by *CaPO2*. This H_2O_2 could be used by *CaPO1*, among other peroxidases (e.g. *CaNPOD*), to increase the lignin content in cell wall. *CaNPOD* is also involved in defense responses to pathogens (Wang *et al.*, 2013).

Once *V. dahliae* arrives, it starts to degrade the cell wall to enter in the cell, and the plant detects the attack and responds by the generation of endogenous signals. We have measured the levels of hydrogen peroxide in the eight first hours both after induction and pathogen inoculation and we observed a significant increase 2 hours after PO212 treatmentand also a peak at 6 and 8 hours after *V. dahliae* inoculation. This peak was higher in PO212-treated plants. This could be the moment when plant recognizes the presence of *V. dahliae* and then, it activates defense responses. However, the functions

of H_2O_2 are not only acting as a signal but this ROS is also used by peroxidases to produce lignin or acts as toxic molecule for the pathogen (Lehmann *et al.*, 2015; Ali *et al.*, 2018).

As we mentioned previously, ROS are formed in the moment that plant sense the stress (Lehmann *et al.*, 2015; Ali *et al.*, 2018). 2 hours after inoculation with *V. dahliae*, pepper roots showed an accumulation of H_2O_2 creating an oxidative environment that can abolish the growth of the pathogen. Also in that moment, some changes are triggered inside the plant, and, as a consequence, PR-1 proteins, β-1,3-glucanases and chitinases started to be expressed and some hormones are synthesized.

Besides ROS, phytohormones are also important modulators of plant defense responses against pathogens, including salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) (Berens *et al.*, 2017). To study the role of hormones in pepper roots, we measured the expression of genes involved in hormone biosynthesis, we used tomato mutants impaired in those hormones, and we quantified the hormones as well. We got evidence from gene expression data that suggests that pepper started to synthesize the hormones SA, ET and JA as a response to PO212 both in pepper roots and in pepper cell cultures. These results suggested that the three hormones were modulating the response induced by PO212. However, in the case of SA, when it was measured in the roots, a decrease in its levels was observed as a response to PO212. That decrease happened at the same time when 4-HBA increased its levels in PO212-treated roots. In our laboratory, such a negative correlation between SA and 4-HBA was observed previously with other inducer (García, 2018). It is important to point out that SA is 2-hydroxybenzoic acid and 4-HBA is 4- hydroxybenzoic acid, therefore both can be synthesized from precursors derived from shikimate pathway and phenylpropanoid

pathway. Their relationship in plant defense deserves further research in the future. ON the other hand, we also got evidence about the involvement of ABA in the response of pepper to PO212. Overall, we concluded that SA, JA, ABA and ET are involved in PO212-induced resistance.

The combination of arbuscular mycorrhizal fungi (AMF) with other fungal strains has been shown to protect plants against different diseases (Møller *et al.*, 2009; Tanwar *et al.*, 2013; Li *et al.*, 2018). Therefore, the role of AMF in suppressiveness to vascular wilts caused by *V. dahliae* and *Phytophthora capsici* was studied in Padron pepper soils. The higher AMF abundance in suppressive soils suggests that these fungal microoganisms play an important role in soil protection against pathogens.

This study points out that PO212 protects pepper plants against *V. dahliae*, so it can be used as biofungicide. Combination of both PO212 and AMF could be an effective management strategy to control *V. dahliae* in pepper crops.

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Conclusions

Based on the results obtained in the previous chapters and discussed previously we can conclude the following points:

1. PO212 induces resistance in pepper against *Verticillium dahliae* when applied to the roots. The reduction of symptoms is correlated with a decreased pathogen biomass in pepper roots.

2. The resistance induced by PO212 in pepper plants is due to the deposition of lignin in the cell wall driven by peroxidases with comsumption of phenolic compounds, as well as to the expression of biochemical defenses in pepper roots as chitinase and β-1,3-glucanase activities, a capsidiol biosynthesis gene (*CaSC1*) and *PR* genes (*CaBPR1* and *CaBGLU*).

3. Elicitation of pepper cell cultures with PO212 conidia induces the expression of biochemical defenses as chitinase and β-1,3-glucanase activities, *PR*-genes (*CaBPR1*, *CaBGLU*, *CaCHI2* and *CaPO2*) and the gene *CaSC1*, involved in capsidiol biosynthesis. Moreover, elicitation with PO212 induces the early signaling molecule hydrogen peroxide and the expression of genes involved in ethylene, jasmonic acid and salicylic acid biosynthesis.

4. The resistance induced by PO212 in pepper roots is driven by the increase of several signals in roots: hydrogen peroxide, salicylic acid, 4-hydroxybenzoic acid, jasmonic acid, abscisic acid and ethylene. The defense response triggered by PO212 is very similar between pepper and tomato; salicylic acid, jasmonic acid and abscisic acid work in both plants, but the resistance induced by PO212 is independent of ethylene in tomato.

5. PO212 grows superficially around pepper roots, so competition for infection sites, resources and/or nutrientes would be other mechanism of action of this biocontrol agent against *V. dahliae*.

6. Supressiveness to *Verticillium dahliae* and *Phytophthora capsici* in soils from Padron pepper greenhouses is correlated with increased arbuscular mycorrhizal fungi abundance and decreased *V. dahliae* and *P. capsici* biomass in Padron pepper roots.

Annex I

Sinopsis

El pimiento es una planta herbácea anual perteneciente a la familia de las solanáceas. El origen geográfico del pimiento se localiza en Perú y Bolivia. Posteriormente el pimiento fue introducido en Europa tras el descubrimiento de América, y actualmente representa el quinto cultivo hortícola en cuanto a la superficie cultivada se refiere y el octavo en cuanto a producción total. Todas las formas del pimiento utilizadas por el hombre pertenecen al género *Capsicum*. La especie más cultivada es *Capsicum annuum* L., en la que están incluidos todos los cultivares gallegos de pimiento, algunos de los cuales han sido designados como Denominación de Origen Protegida o como Indicación Geográfica Protegida.

El cultivo de pimiento se puede ver afectado por diferentes enfermedades, entre ellas la verticilosis del pimiento causada por *Verticillium dahliae* Kleb. Este patógeno se halla presente en las explotaciones gallegas de pimiento, siendo la primera la causa de las mayores pérdidas en la producción de pimiento en Galicia. *V. dahliae* es un hongo asexual que coloniza los tejidos vasculares y se transmite por el suelo. Tiene un amplio rango de huéspedes a los que infecta, causando importantes pérdidas económicas, lo cual lo convierte en uno de los patógenos fúngicos más destructivos a nivel mundial. Actualmente no existe ningún fungicida eficaz contra *V. dahliae* aplicable durante la fase de cultivo, limitándose los métodos de control a la desinfección del suelo y otras medidas de carácter preventivo antes de la plantación. Hoy en día un programa de control integrado de enfermedades implica la combinación de pesticidas, agentes de control biológico (BCAs) y fitofortificantes. Los BCAs han sido regulados a nivel europeo por la Regulación Europea 1107/2009 y la Directiva Europea 2009/128/EC, donde el uso de pesticidas fue reducido para alcanzar un nivel sostenible mediante su combinación con los BCAs y otras técnicas de control. La explotación con fines

comerciales de ciertos BCAs ha adquirido gran importancia en la agricultura por su accesibilidad, propagación a gran escala, y capacidad para proporcionar una protección eficaz a largo plazo con un amplio espectro de acción y sin un impacto adverso sobre el medio ambiente o en la salud humana. El control biológico se refiere a cualquier acción directa o indirecta por parte del BCA que lleva a la reducción de la enfermedad. Por lo tanto, los BCAs actúan directamente sobre el patógeno (antibiosis, competición por el espacio o los nutrientes, parasitismo), o indirectamente sobre la planta huésped mediante la activación de las defensas vegetales (inducción de resistencia).

Las defensas vegetales ser constitutivas o inducidas, es decir, existe una defensa pasiva y activa respectivamente. A su vez, esta defensas pueden ser estructurales, como por ejemplo capas gruesas de cutícula, tricomas o depósitos de suberina, calosa o lignina en la pared celular vegetal, o puede ser químicas, basadas en la acumulación de substancias tóxicas en la célula vegetal como por ejemplo compuestos fenólicos, fitoalexinas o proteínas relacionadas con la patogénesis (proteínas PR). Cuando el patógeno penetra en la planta, las defensas vegetales inducidas se activan para prevenir el establecimiento del patógeno. Estas defensas son iniciadas gracias a receptores de la planta capaces de reconocer las moléculas del patógeno, conocidas como PAMPs (Patrones Moleculares Asociados a Patógenos) o MAMPs (Patrones Moleculares Asociados a Microorganismos). Las plantas también pueden reconocer la presencia del patógeno mediante moléculas derivadas del daño que causa el patógeno en la planta, denominadas DAMPs (Patrones Moleculares Asociados al Daño). Tras el reconocimiento de estos patrones moleculares por parte de la planta, se activa la PTI (Inmunidad Desencadenada por PAMPs), que representa la primera línea de defensa inducida por el patógeno. La PTI puede ser suprimida mediante la secrección por parte del patógeno de moléculas denominadas efectores, pasando la planta a un estado conocido como ETS

(Susceptibilidad Desencadenada por Efectores), en el que la planta es susceptible al patógeno. Los efectores, no obstante, pueden ser también reconocidos por la planta, iniciándose así el mecanismo de defensa conocido como ETI (Inmunidad Desencadenada por Efectores).

Tanto la PTI como la ETI pueden activar las defensas en los tejidos vegetales sistémicos que no han sido atacados por el patógeno, un fenómeno conocido como SAR (Resistencia Sistémica Adquirida). Las plantas también pueden adquirir este estado de resistencia mediante la exposición a organismos beneficiosos (bacterias y hongos promotores del crecimiento vegetal, PGPR y PGPF respectivamente), un fenómeno conocido como ISR (Resistencia Sistémica Inducida). La SAR se caracteriza por proporcionar una resistencia de larga duración junto con la expresión de proteínas PR. Varias señales son necesarias para el establecimiento de la SAR, pero el ácido salicílico (SA) es la más representativa. Este tipo de resistencia es efectiva contra un amplio rango de patógenos, pero suele ser más efectiva contra patógenos biotrofos y hemibiotrofos. Por otra parte, la ISR suele ser más efectiva contra patógenos necrotrofos. Las señales necesarias para el establecimiento de la ISR dependen del ácido jasmónico (JA) y del etileno (ET), pero la acumulación de ambas hormonas y la activación de los genes regulados por las mismas no tiene lugar hasta que la planta es atacada por el patógeno. Por otra parte, la Resistencia Inducida por Micorrizas (MIR), desencadenada por hongos micorrícicos, puede considerarse una variación de la ISR, ya que ambos tipos de resistencia inducida se activan ante la exposición de la planta a microorganismos beneficiosos y las respuestas de defensa producidas en ambas son similares.

En general, SA, JA y ET son las principales hormonas que regulan las defensas vegetales, pero otras como el ácido abscísico (ABA), citoquininas (CT), brasinoesteroides (BR) y auxinas (IAA) también participan en la modulación de la respuesta de defensa.

Tanto la SAR como la ISR constituyen respuestas de defensa inmediatas al estrés, pero también pueden desencadenar un estado de *priming*. El *priming* es un estado de "alerta" en el que la planta responde de forma más intensa y rápida a un segundo ataque patógeno, por lo que se considera un estado de memoria que podría participar en la adaptación de las plantas a condiciones de estrés.

Este trabajo de investigación plantea el estudio del BCA *Penicillium rubens* cepa 212 (PO212) como método de control de la verticilosis en pimiento.

Se evaluó el efecto de PO212 contra *V. dahliae* mediante inmersión de las raíces de pimiento en una solución de conidios del BCA. Las plantas tratadas con PO212 mostraron una reducción en los síntomas causados por este hongo. Además, las raíces de pimiento tratadas con PO212 mostraron una menor biomasa de *V. dahliae*, lo cual confirma que PO212 es capaz de inducir resistencia en pimiento contra este patógeno.

Posteriormente se estudiaron las defensas inducidas (defensa activa) por PO212, tanto estructurales como bioquímicas, en las raíces de pimiento. Con respecto a las defensas estructurales, se estudió la lignificación a través de la actividad peroxidasa y dos genes que codifican para estas enzimas, así como el contenido en compuestos fenólicos y la lignina. Las raíces de pimiento inducidas con PO212 mostraron un aumento en lignina con la participación de las peroxidasas y a expensas del consumo de compuestos fenólicos. Las defensas bioquímicas se estudiaron a través de la expression de los genes *CaSC1*, *CaBPR1*, *CaBGLU*, y las actividades β-1,3-glucanasa y quitinasa, implicadas

en la degradación de la pared celular de los patógenos. Se observeó que PO212 induce la expresión en las raíces de los tres genes y las dos actividades enzimáticas.

También se estudió el papel de las especies reactivas de oxígeno (ROS) y las principales hormonas implicadas en la defensa vegetal (SA, JA y ET) en la resistencia inducida por PO212. Se determinaron los niveles de peróxido de hidrógeno (H_2O_2) en las raíces de pimiento durante las primeras 8 horas tras la inoculación, y se observó que PO212 induce la acumulación de este compuesto. Se midió también la expresión de genes implicados en la biosíntesis de hormonas, ya que en pimiento no hay genes marcadores que respondan únicamente a una sola hormona. Los genes seleccionados fueron los siguientes: *CaPAL1* y *CaICS* para el SA, *CaAOS* para el JA y *CaACO* y *CaACS3* para el ET. También se llevaron a cabo ensayos con mutantes de tomate insensibles o que no sintetizan estas hormonas (*never ripe* para el ET, *defenseless1* para el JA y *nahG* para el SA) y también para el ácido abscísico (*sitiens*), y se cuantificaron los niveles hormonales en las raíces de pimiento mediante espectrometría de masas. Se observó que las tres hormonas (SA, JA y ET), además del ácido abscísico (ABA), están implicadas en la regulación de la resistencia inducida por PO212 en pimiento.

Con el fin de conocer posibles mechanismos de acción del BCA, se estudió la interacción directa entre pimiento y PO212 mediante microscopía confocal. Se observó que PO212 crece superficialmente alrededor de las raíces de pimiento. Además, el BCA es capaz de colonizar superficialmente las raíces de pimiento en las primeras 24 horas tras su aplicación.

PO212 también se aplicó como elicitor a suspensiones celulares de pimiento de Padrón para observar las defensas inducidas en un sistema de cultivo *in vitro*. Tras la aplicación de una suspension de conidios de PO212 en los cultivos celulares de pimiento, se

midieron las actividades β-1,3-glucanasa, chitinasa y peroxidasa así como la cantidad de compuestos fenólicos tanto en el medio extracelular como en la fracción celular. También se analizó la expresión de genes implicados en la producción de proteínas PR y la biosíntesis de hormonas, la producción de lignina y el papel del H_2O_2 en las suspensiones celulares de pimiento elicitadas con PO212. Se observó que las respuestas de defensa elicitadas por PO212 en las suspensiones celulares de pimiento y las respuestas inducidas *in planta* son similares, pero difieren en la cantidad de compuestos fenólicos y en la producción de lignina.

Finalmente se realizó un estudio sobre el papel de los hongos micorrícicos arbusculares (AMF) en la supresión de *V. dahliae* y *Phytophthora capsici* en suelos de invernaderos de pimiento de Padrón. Se observó que los AMF influyen positivamente en la supresión de la enfermedad causada por ambos patógenos en los suelos de pimiento. Las raíces de las plantas de pimiento crecidas en los suelos supresivos mostraron una menor biomasa de ambos patógenos en comparación con las plantas de pimiento crecidas en los suelos conductivos. Además, se observó que los factores abióticos (pH, conductividad eléctrica) y las prácticas agrícolas (aplicación de fungicidas, sulfatación) no influyen de forma notable en la supresividad de los suelos de pimiento.

En resumen, PO212 induce resistencia en pimiento contra *V. dahliae*, induce el refuerzo de la pared celular mediante el depósito de lignina mediado por las enzimas peroxidasas, así como varias defensas bioquímicas moduladas por el H_2O_2, SA, ET y JA.

Basándonos en los resultados obtenidos en los capítulos anteriores, podemos concluir los siguientes puntos:

1. PO212 induce resistencia en pimiento contra *Verticillium dahliae* cuando es aplicado en las raíces. La reducción de síntomas se correlaciona con un descenso de biomasa del patógeno en las raíces de pimiento.

2. La resistencia inducida por PO212 en plantas de pimiento es debida a la deposición de lignina en la pared celular llevada a cabo por las peroxidasas con un consumo de compuestos fenólicos, así como la expresión de defensas bioquímicas en las raíces de pimiento como las actividades quitinasa y β-1,3-glucanasa, un gen implicado en la biosíntesis de capsidiol (*CaSC1*), y genes *PR* (*CaBPR1* y *CaBGLU*).

3. La elicitación de cultivos celulares de pimiento con conidios de PO212 induce la expresión de defensas bioquímicas como las actividades quitinasa y glucanasa, genes *PR* (*CaBPR1*, *CaBGLU*, *CaCHI2* y *CaPO2*), y el gen implicado en la biosíntesis de capsidiol *CaSC1*. Además, la elicitación con PO212 induce la molécula de señalización temprana peróxido de hidrógeno y la expresión de genes relacionados con las vías de biosíntesis del etileno, el ácido jasmónico y el ácido salicílico.

4. La resistencia inducida por PO212 en pimiento es llevada a cabo por el incremento de varias señales en las raíces: peróxido de hidrógeno, ácido salicílico, ácido 4-hidroxibenzoico, ácido jasmónico, ácido abscísico y etileno. La respuesta de defensa desencadenada por PO212 es muy similar entre pimiento y tomate; el ácido salicílico, ácido jasmónico y ácido abscísico funcionan en ambas plantas, pero la resistencia inducida por PO212 es independiente del etileno en tomate.

5. PO212 crece superficialmente alrededor de las raíces de pimiento, por lo que la competición por los sitios de infección, recursos y/o nutrientes puede ser otro mecanismo de acción de este agente de biocontrol contra *V. dahliae*.

6. La supresividad a *Verticillium dahliae* y *Phytophthora capsici* en invernaderos de pimiento de Padrón se correlaciona con un incremento en la abundacia de hongos micorrícicos arbusculares y un descenso de la biomasa de *V. dahliae* y *P. capsici* en las raíces de pimiento de Padrón.