Analysis of capsinoids in Galician pepper cultivars and characterization of their properties for plant protection

Tania García Lamas

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Supervisors: Dr. José Díaz Varela, Dr. Javier Veloso Freire

Tutor: Dr. José Díaz Varela

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UNIVERSIDADE DA CORUÑA

José Díaz Varela, Profesor Titular de Universidad del área de Fisiología Vegetal en el Departamento de Biología de la Universidad de A Coruña, y **Javier Veloso Freire**, Contratado postdoctoral del área de Fisiología Vegetal en el Departamento de Biología de la Universidad de A Coruña,

INFORMAN:

Que la presente tesis doctoral titulada "Analysis of capsinoids in Galician pepper cultivars and characterization of their properties for plant protection" presentada por la licenciada **Tania García Lamas** 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 Grado de Doctor en Biología,

Y para que así conste a los efectos correspondientes, firmamos el presente informe en A Coruña a 5 de Octubre de 2018,

Fdo. Dr José Díaz Varela

Fdo. Dr Javier Veloso Freire

Facultade de Ciencias, Universidade da Coruña
Campus da Zapateira s/n E-15071 A Coruña, España-Spain
Tel.: 981167000 ext. 2139, Fax: 981167065

Esta tesis está dedicada a la memoria de Manuel Lamas Bugallo (1955-2007), cuya tenacidad y superación han sido toda una lección de vida. Gracias por enseñarme que un "no" puede convertirse en un "tal vez" con solo intentarlo.

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Abstract

The content in capsaicinoids and capsinoids was analysed in several Galician pepper cultivars. No capsinoids were detected, but capsaicin and dihydrocapsaicin were measured in cultivars Padrón and Mougán. The antimicrobial activity of a capsinoid (vanillyl nonanoate) and its precursors were tested; the capsinoid inhibited *Phytophthora capsici* spore germination. On the other hand, vanillyl nonanoate protects pepper plants against *Botrytis cinerea* and *Phytophthora capsici* through induced resistance. Vanillyl nonanoate induces resistance systemically in both pepper and Arabidopsis plants. The observed induced resistance involves cell wall reinforcement with an increase in lignin as well as other defences as PR proteins in both plant species. Signalling of the vanillyl nanoate systemic response is mediated by hydrogen peroxide, salicylic acid, ethylene and jasmonates in the case of pepper, and salicylic acid and jasmonates in the case of Arabidopsis. Changes in the levels of 4-hydroxybenzoic acid were also detected in pepper and a role for this compound in resistance is discussed.

Resumen

Se analizó el contenido en capsicinoides y capsinoides en varios cultivares de pimiento de Galicia. No se detectaron capsinoides, pero se midieron capsicina y dihidrocapsicina en los cultivares Padrón y Mougán. Se ensayó la actividad antimicrobiana de un capsinoide (el vanillil nonanoato) y de sus precursores; el capsinoide inhibió la germinación de esporas de *Phytophthora capsici*. Por otro lado, se demostró que el vanillil nonanoato tiene la capacidad de conferir protección local en pimiento frente a *Botrytis cinerea* y *Phytophthora capsici*, obteniendo evidencias de que su modo de acción es la resistencia inducida. También se demostró la sistemicidad de la resistencia inducida por este compuesto, tanto en plantas de pimiento como en Arabidopsis. La resistencia inducida observada implica el reforzamiento de la pared celular con lignina y el incremento de varias defensas de tipo bioquímico como las proteínas PR en ambas especies. La señalización de la respuesta sistémica al vanillil nonanoato está mediada por peróxido de hidrógeno, ácido salicílico, etileno y jasmonatos en el caso de pimiento y ácido salicílico y jasmonatos en el caso de Arabidopsis. Asimismo, se detectaron cambios en los niveles de ácido 4-hidroxibenzoico en pimiento y se ha discutido su papel en la resistencia.

Resumo

Analizouse o contido en capsicinoides e capsinoides en varios cultivares de pemento de Galicia. Non se detectaron capsinoides, pero se mediron capsicina e dihidrocapsicina nos cultivares Padrón e Mougán. Ensaiouse a actividade antimicrobiana dun capsinoide (o vanillil nonanoato) e dos seus precursores; o capsinoide inhibiu a xerminación de esporas de *Phytophthora capsici*. Por outra banda, demostrouse que o vanillil nonanoato ten a capacidade de conferir protección local en pemento fronte a *Botrytis cinerea* e *Phytophthora capsici*, obtendo evidencias de que o seu modo de acción é a resistencia inducida. Tamén se demostrou a sistemicidade da resistencia inducida por este composto, tanto en plantas de pemento como en Arabidopsis. A resistencia inducida observada implica o reforzamento da parede celular con lignina e o incremento de varias defensas de tipo bioquímico como as proteínas PR en ambas especies. A sinalización da resposta sistémica ao vanillil nonanoato está mediada por peróxido de hidróxeno, ácido salicílico, etileno e xasmonatos no caso do pemento e ácido salicílico e xasmonatos no caso de Arabidopsis. Así mesmo, detectáronse cambios nos niveles de ácido 4-hidroxibenzoico en pemento e se discutiu o seu papel na resistencia.

Preface

One challenge for current agriculture is to feed an increasing world population that already has reached 7.6 billion of people. One of the problems to solve in agriculture is the impact of plant diseases in crop yield. This problem has existed since the beginning of agriculture and causes important losses in crop production. In order to manage plant diseases, farmers use different strategies, but pesticide application is the most used so far. However, isolates resistant to fungicides usually arise in the field and, as a consequence, their effectiveness decreases. Moreover, the current trend in the law is to forbid the use of the pesticides that are the most damaging to the environment. New strategies are needed to supplement the ban of such pesticides. In the last years, biological control and the use of plant resistance inducers have been developed in order to reduce the use of pesticides and its environmental impact.

In this thesis we studied the effect of vanillyl nonanoate, a synthetic capsinoid, as an inducer of resistance in plants of Padrón pepper against several pathogens. In addition, we determined if some capsinoids were present in the pepper fruits of several Galician ecotypes.

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Some work from this thesis has already been published in a book chapter and several scientific journals as well as showed in scientific meetings.

The publications are:

- Díaz, J., García, T. & Veloso, J. (2012). Enfermidades do pemento causadas por fungos e oomicetos. Xornadas Técnicas sobre do

pemento do Couto - 3ª Xornadas Técnicas sobre do pemento do Couto. Deputación da Coruña. ISBN: 978-84-9812-194-0.

- Díaz, J., Carballeira, C., Veloso, J. & García, T. (2013). Induction of resistance to *Botrytis cinerea* by ethylene is evolutionary conserved but ethylene inhibits vanillylnonanamide induced resistance. IOBC/WPRS Bulletin. 88: 113 - 117
- Veloso, J., García, T., Bernal, A. & Díaz, J. (2014). New bricks on the wall of induced resistance: salicylic acid receptors and transgenerational priming. European Journal of Plant Pathology, 138: 685-693.
- García, T., Veloso, J. & Díaz, J. (2018). Properties of vanillyl nonanoate for protection of pepper plants against *Phytophthora capsici* and *Botrytis cinerea*. European Journal of Plant Pathology, 150: 1091-1101.
- García, T., Veloso, J. & Díaz, J. (2018) Vanillyl nonanoate induces systemic resistance and lignification in pepper plants. Journal of Plant Physiology. https://doi.org/10.1016/j.jplph.2018.10.002.

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

- García, T., Veloso, J., Díaz, J. (2012). El capsinoide vanillilnonanoato protege al pimiento frente a *Phytophthora capsici* y *Botrytis cinerea*. XVI Congreso de la Sociedad Española de Fitopatología. Málaga (Spain). Poster.
- García, T., van Kan, J., Veloso, J., Díaz, J. (2014). Vanillylnonanoate induces systemic resistance in pepper and *Arabidopsis thaliana* against *Botrytis cinerea*. XVII Congreso de la Sociedad Española de Fitopatología. Lleida (Spain). Poster.

- García, T., Veloso, J., Díaz, J. (2014). El vanillilnonanoato induce resistencia sistémica en pimiento frente a *Phytophtora capsici*. XVII Congreso de la Sociedad Española de Fitopatología. Lleida (Spain). Oral communication.
- García, T., Lois, M., Veloso, J., Díaz, J. (2015). Induced resistance in pepper against *Phytophthora capsici* by vanillyl nonanoate, a Fo47 crude elicitor and a Bacillus-based product. XXI Reunión de la Sociedad Española de Fisología Vegetal y XIV Congreso Hispano-luso de Fisiología Vegetal. Toledo (Spain). Poster.
- García, T., Lois, M., Veloso, J., Díaz, J. (2016). Induced resistance against *Phytophthora capsici* in pepper by Vanillyl nonanoate, a Fo47 elicitor and a Bacillus product. XVIII Congreso de la Sociedad Española de Fitopatología. Palencia (Spain). Poster.
- García, T., Veloso, J., Díaz, J. (2016). Vanillyl nonanoate induces physiological changes in pepper that cause a systemic resistance in pepper against *Botrytis cinerea*. XVIII Congreso de la Sociedad Española de Fitopatología. Palencia (Spain). Oral communication.
- García, T., Pastor, V., van Kan, J., Flors, V., Carrillo-Barral, N., Veloso, J., Díaz, J. (2018) Vanillylnonanoate induced resistance in Arabidopsis against *Botrytis cinerea*: a story of lignification and hormones. XIX Congreso de la Sociedad Española de Fitopatología. Toledo (Spain). Oral communication.

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Abreviations

4-HBA	4-Hydroxybenzoic Acid
ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-Carboxylic Acid
ACL	Acyl Carrier Protein
ACO	ACC Oxidase
ACS	ACC Synthase
ACT	Actin
AOS	Allene Oxide Synthase
ASM	Acibenzolar-Smethyl
AT3	Acyltransferase 3
AUDPC	Area Under Disease Progress Curve
BABA	β-Amino-Butiric Acid
BCAT	Branched-Chain Amino acid Transferase
BSMT1	Benzoic acid/ Salicylic acid carboxyl MethylTransferase 1
BTH	Benzothiadiazole
COI1	CORONATINE INSENSITIVE1
CS	Capsaicin Synthase
CTR	CONSTITUTIVE TRIPLE RESPONSE
DAB	3,3’-Diaminobenzidine
DAMPs	Damage-Associated Molecular Patterns
DMSO	Dimethyl Sulfoxide
DTT	Dithioethreitol
EIN	ETHYLENE INSENSITIVE
ERS	ETHYLENE RESPONSE SENSOR
ET	Ethylene

ABREVIATIONS

ETI	Effector-Triggered Immunity
ETR	ETHYLENE RESPONSE
ETS	Effector-Triggered Susceptibility
FMO1	FLAVIN-DEPENDEN-NOOXYGENASE1
HPLC	High Performance Liquid Chromatograph
HR	Hypersensitive Response
IAA	Indole Acetic Acid
ICS	Isochorismate Synthase
INA	2,6-dichloroisonicotinic acid
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JA-ILE	(+)-7-iso-jasmonoyl-L-Isoleucine
JAZ	JASMONATE ZIM
KAS	Ketoacyl-ACP Synthase
MAMPs	Microbe-Associated Molecular Patterns
MCP	1-Methylcyclopropene
MeJA	Methyl Jasmonate
MeSA	Methyl Salycilate
NB-LRR	Nucleotide-Binding Leucin-Rich Repeat
NNA	Nonanoic Acid
NO	Nitric Oxide
NPR1	NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1
OPDA	cis-12-Oxo-Phytodienoic Acid
PAL	Phenylalanine Ammonia-Lyase
PAMPs	Pathogen-Associated Molecular Patterns
pAMT	putative Aminotransferase
PCD	Programmed Cell Death

PDA	Photo Diode Array
PDA	Potato Dextrose Agar
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
PGIP	Polygalacturonase Inhibiting Protein
PGPF	Plant Growth-Promoting Fungi
PGPR	Plant Growth-Promoting Rhizobacteria
PIP	Pipecolic Acid
PR	Pathogenesis-Related
PRRs	Pattern Recognition Receptors
PTI	PAMP-Triggered Immunity
R	Resistance
RLK	Receptor-Like Kinases
RLP	Receptor-Like Proteins
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SA	Salicylic Acid
SAG	Glucosylated Salicilic Acid
SAR	Systemic Acquired Resistance
SC	Sesquiterpene Cyclase
VNT	Vanillyl Nonanoate
VOH	Vanillyl Alcohol

GENERAL INTRODUCTION

Part of this introduction has been published in:

Veloso, J., García, T., Bernal, A. & Díaz, J. (2014). New bricks on the wall of induced resistance: salicylic acid receptors and transgenerational priming. European Journal of Plant Pathology, 138: 685-693.

Díaz, J., García, T., & Veloso, J. (2012). Enfermidades do pemento causadas por fungos e oomicetos. Xornadas Técnicas sobre do pemento do Couto - 3ª Xornadas Técnicas sobre do pemento do Couto. Deputación da Coruña. ISBN: 978-84-9812-194-0.

I.-*Capsicum annuum* L.

The genus Capsicum is one of the most economically important crops from the Solanaceae family, commonly known as peppers (Qin *et al.*, 2014). Pepper is native of tropical America and West Indies and was one of the first plants to be domesticated and cultivated by natives in 6000 B.C. (Qin *et al.*, 2014; Wang & Bosland, 2006). After Columbus voyages the pepper cultivation spread to the rest of the world, being nowadays one of the most used condiments in human diet (Wang & Bosland, 2006; Moscone *et al.*, 2007). This spreading was due to pepper grows in different climatic regions and its high versatility in food and medicine (Qin *et al.*, 2014). In 2016, the pepper world yield was 3.9 million tonnes of dry fruit and 34.5 million tonnes of green fruit (FAOSTAT, 2018).

Pepper fruit has all the necessary nutritional components as sugars, lipids, proteins and minerals as well as colouring and flavouring substances (Estrada *et al.*, 2000a). Due to these properties, pepper was used as a food additive in pre-columbian America. In addition, pepper fruit has a high content of vitamin A and C (Estrada *et al.*, 2000a).

Currently 32 species are known to belong to the genus Capsicum but only 5 of them have been domesticated and cultivated (Qin *et al.*, 2014). They are *Capsicum baccatum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Capsicum pubescens* and *Capsicum annuum* L. (Bosland, 1996). Several Galician ecotypes of pepper were included into the Protected Designation of Origin and the Protected Geographical Indication for agricultural products provided by the EU Regulation No 1151/2012. These ecotypes were Padrón ecotype designated as Herbón PDO (Protected Designation of Origin) and Couto, Mougán, Oinmbra and Arnoia ecotypes got the recognition of PGI (Protected Geographical Indication) (Casal, 2010; Taboada *et al.*, 2010). All the Galician ecotypes belong to the species *Capsicum annuum.*

C. annuum is an annual herbaceous plant (Figure I.A) with a central root from which lateral roots branch (Figure I.B) (Nuez *et al.*, 1996). The pepper has glabrous appearance, it stands erect and the stems have limited growth. Branching follows a single basic model characterized by the formation of sympodial units. The leaves are whole and attached to the stem by a long petiole. Moreover, they have a lanceolate

shape with entire or very slightly sinuate edge at the base (Figure I.C) (Nuez *et al.*, 1996).

The flowers are hermaphrodite and appear usually alone in each node. Generally they have a milky white corolla and a twisted down petiole (Figure I.1.D, I.1.E) (Nuez *et al*., 1996; Estrada *et al*., 2000b). The fruit is a hollow berry with the shape of a capsule and smooth and shiny surface (Figure I.F). There are 2 or 4 incomplete septa inside the fruit, that end in placental tissue. In this region are located the seeds, which are crushed, typically 4 to 5 mm in diameter and white yellowish (Figure I.G) (Nuez *et al.*, 1996).

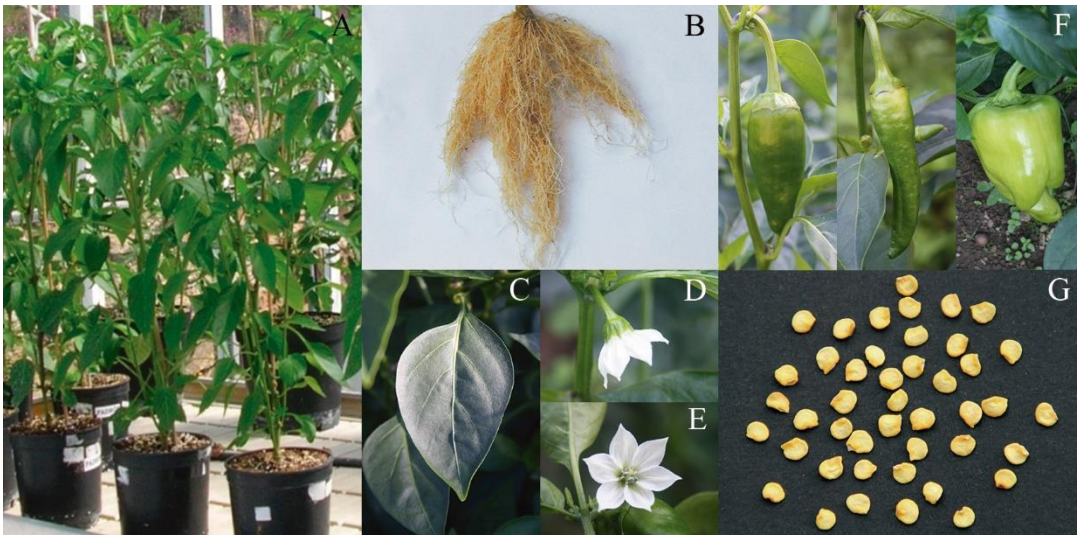

Figure I. *Capsicum annum*. A: general aspect of the plant; B: pepper root; C: shape of pepper leaf; D-E: flower appearance; F: different shape of the pepper fruit; g: pepper seeds.

Some of the pathogens that attack *C. annuum* are *Phytophthora capsici* Leon., causing Phytophthora root rot and Phytophthora blight, and *Verticillium dahliae* Kleb., causing verticillium wilt. Another pepper pathogen is *Botrytis cinerea*, although the yield loss caused by this pathogen is minor compared with the pathogens mentioned above. However, *B. cinerea* is interesting because it is considered a model necrotroph (Díaz *et al.*, 2012).

II. *Phytophthora capsici* Leon.

Phytophthora capsici is a hemibiotrophic pathogen that belongs to the class Oomycetes, order Peronosporales and family Peronosporaceae. It is a highly dynamic

and destructive pathogen (Lamour *et al.*, 2012). It can cause rot in roots, stems and fruits of a wide range of solanaceous plants as pepper, eggplant and tomato, cucurbits as melon, cucumber, pumpkin, watermelon and courgette, and legumes as snap bean and lima bean (Erwin & Ribeiro, 1996; Hausbeck & Lamour, 2004). It was first discovered in 1922 in New Mexico as the causal agent of wilting pepper. Since then *P. capsici* has been identified in different crops around the world (Hausbeck & Lamour, 2004).

The mycelium of *P. capsici* presents a large number of branched tubular structures called hyphae. The mycelium is hyaline in the suitable cultured medium or in infected tissue. Its growth begins in the apical portion of each hypha (Erwin & Ribeiro, 1996).

P. capsici can reproduce asexually by sporangia (Figure II). These structures can be semipapillate or papillated. In some cases this structure can have two or three apices. The shape of the sporangia varies with cultural conditions. Sporangia have a conical base with a long pedicel with variable length (between 35 and 138 µm) and are deciduous. These structures grow on sporangiophores which are irregularly branched and sympodically shaped when the pathogen grows in water and light conditions (Erwin & Ribeiro, 1996). After rain or irrigation, mature sporangia germinate and quickly release 20-40 biflagellated and mobile zoospores (Hausbeck & Lamour, 2004; Lamour *et al.*, 2012). These zoospores show negative geotropism and swim chemotactically towards the plant. Upon plant surface, zoospores lose their flagella, become encysted, adhere to the surface and produce a germ tube. The germ tube penetrates the cuticle helped by enzymes and then colonizes the host tissue (Lamour *et al.*, 2012).

On the other hand *P. capsici* can also reproduce sexually by oospores (Figure II). They are mainly plerotic and their average diameter ranges from 23.7 to 34.9 µm depending on the isolate (Erwin & Ribeiro, 1996). More than a half of the Phythophthora species are homothallic which means one isolate is enough to complete sexual cycle. However, the rest of the species, including *P. capsici*, are heterothallic, that is, they show two compatible mating types called A1 and A2. Thus oospores are formed when A1 and A2 interact (Lamour *et al.*, 2012). At the beginning of sexual phase each parental produces hormones that are responsible for the differentiation in male (antheridium) and female (oogonium) gametangia. The last one can be spherical or subspherical with colour from hyaline to brown and variable size (Erwin & Ribeiro,

1996) (Figure II). When the antheridium and the oogonium interact, the first one surrounds the female gametangia and then this one penetrates the male gametangia. After that, the oogonium receives one or more haploid nuclei from the antheridium. Then the oogonium increases its size and causes the formation of a single oospore (Erwin & Ribeiro, 1996). Oospores serve as inoculum for next season. These oospores can germinate directly forming a germ tube or indirectly forming sporangia (Ristaino & Johnston, 1999).

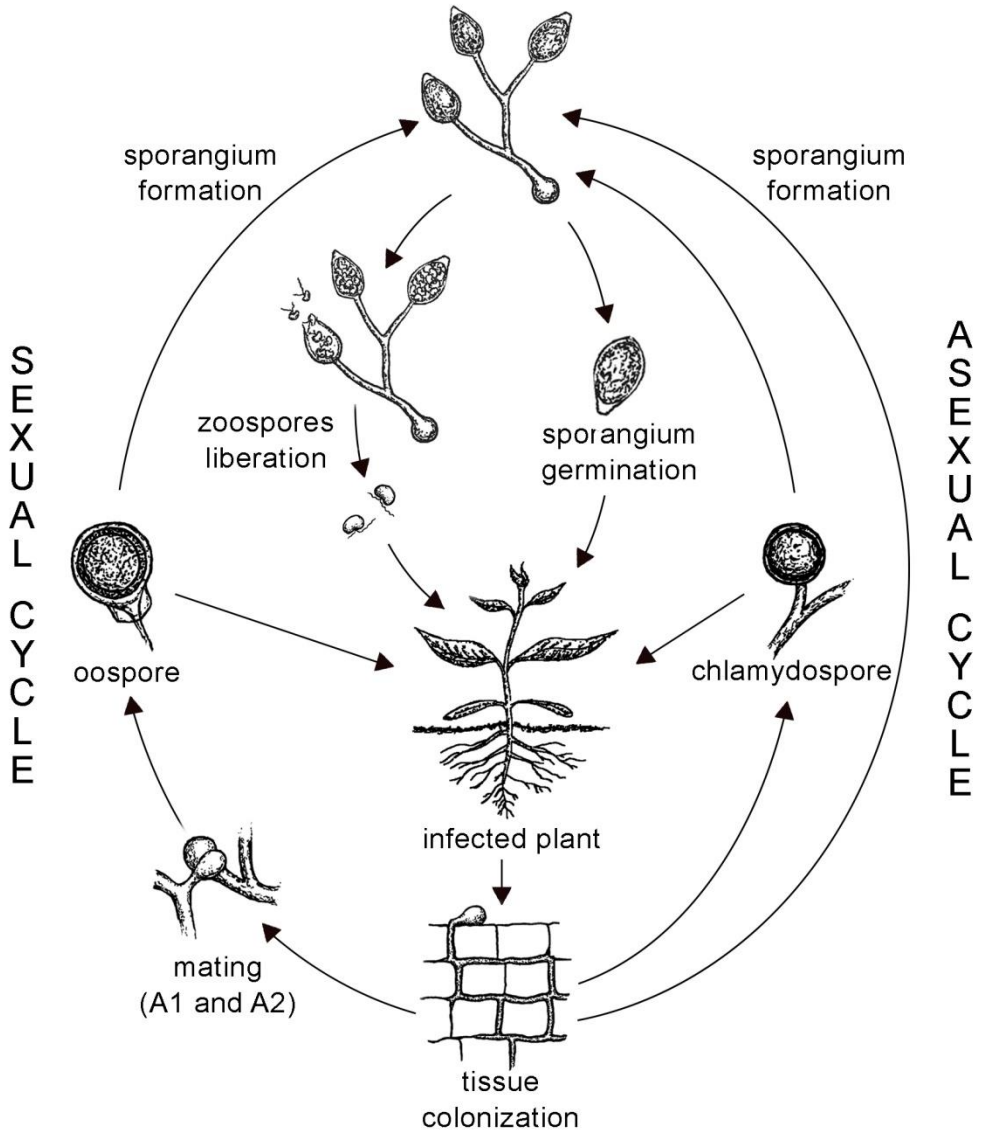

Figure II. *Phytophthora capsici* life cycle.

Finally *P. capsici* can also reproduce by resistant asexual spores or chlamydospores (Figure II). However, their formation is not observed in all hosts and only occurs in adverse conditions (Erwin & Ribeiro, 1996; Nuez *et al.*, 1996).

P. capsici can infect any part of the plant at any stage of development. The most characteristic symptoms are crown rot (Figure III.A and III.C) and wilting (Figure III.B and III.D). The symptoms in the crown are a depressed and black annular zone which first affects the cortical tissues and subsequently the vascular tissues. This lesion develops both upstream and downstream from the starting point and finally chokes the plant. This phenomenon occurs quickly and the leaves look hanging and still green (Nuez *et al.*, 1996). *P. capsici* can infect other plant organs although it is less common. Infection of other organs is usually produced by splashing water or high humidity air containing zoospores (Nuez *et al.*, 1996).

Figure III. Symptoms caused by *P. capsici* in pepper. A and C: Phytophthora rot. Blue arrow points the disease area B and C: Phythophthora blight. The first row of the figure shows the symptoms observed in the laboratory. The second row show the symptoms observed in the field.

III. *Botrytis cinerea* Pers.: Fr.

Botrytis cinerea Pers.: Fr. [teleomorph *Botrytinia fuckeliana* (de Bary) Whetzel], (Dean *et al.*, 2012) is a very destructive and necrotrophic polyphagous pathogen. It is the causal agent of grey mold. This ascomycete belongs to the class Leotiomycetes, order Helotiales, family Sclerotiniaceae and genus Botryotinia. It produces huge losses in over 1000 species of plants (Veloso & van Kan, 2018), although the most affected crops are vegetables such as lettuce, broccoli or cabbage, and crops of small fruits such as grapes, strawberries or raspberries. Moreover, it causes rot in flowers, fruits, leaves, buds and underground storage organs (Williamson *et al.*, 2007). *B. cinerea* has a worldwide distribution (Agrios, 2005).

B. cinerea produces abundant mycelium which first has thin and hyaline hyphae and then turns grey and septated. Conidiophores are developed on hyphae. They are septate, erect, long, branched pseudo-dicotomically and light brown. Hyaline or grey cell groups, oval or globular cells are developed in the apical region of conidiophores and are called conidia or conidiospores (Michailides & Elmer, 2000; Agrios, 2005; Pande *et al.*, 2006).

During the winter *B. cinerea* remains in soil as resistant structures called sclerotia. In temperate regions these sclerotia begin to grow in early spring and produce conidiophores and multinucleate conidia which serve as primary inoculum. On the other hand, the fungus can also survive as mycelium infecting dead plants or seeds. The pathogen also forms abundant microconidia that function as spermatids (Figure IV) (Agrios, 2005; Williamson *et al.*, 2007).

Sclerotia can be fertilized by uninucleated microconidia. This process forms the apothecia or teleomorphic phase known as *Botryotinia fuckeliana* (de Bary) Whetzel. Two sexual mating types are required for the apothecia formation. These two mating types are called MAT 1-1 and 1-2 isolates or MAT-1/2 pseudohomotalic isolates. After sexual mating of two compatible MAT types, asci are formed in the apothecia and each ascus contains 8 sexual spores, called ascospores. The ascospores are the infective structures after sexual reproduction and will be dispersed by wind mainly in the spring and in periods with high rainfall (Pande *et al.*, 2006; Williamson *et al.*, 2007).

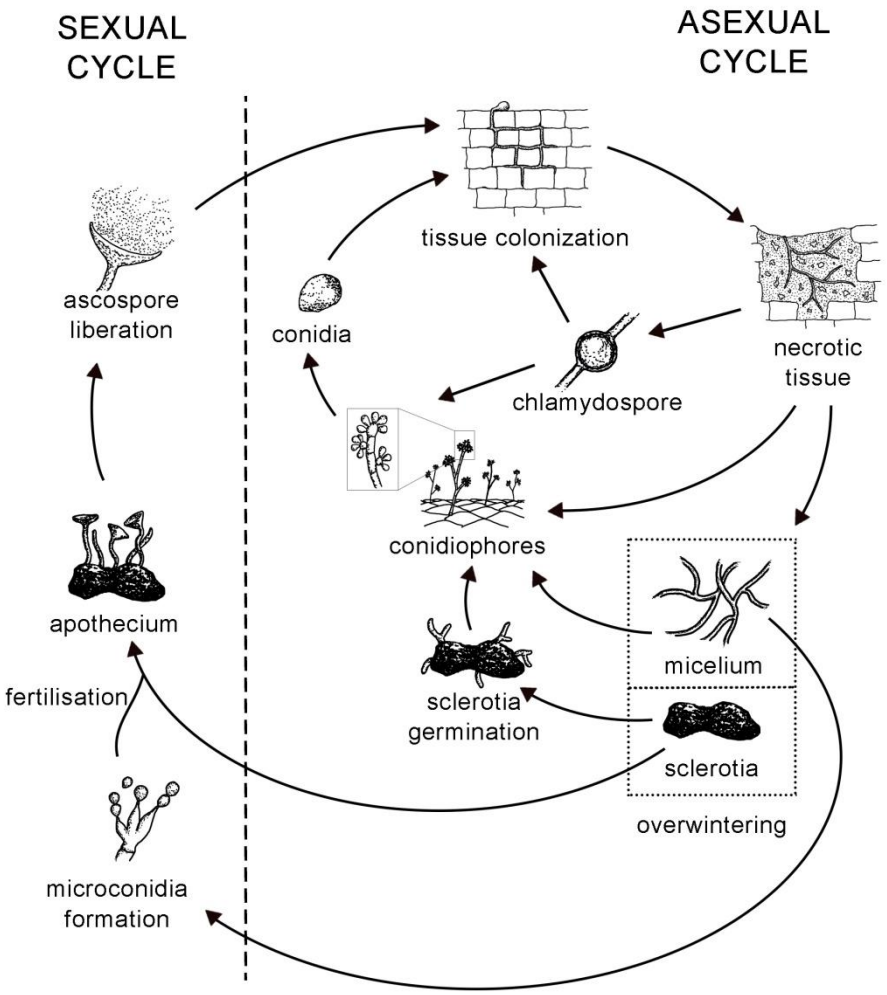

Figure IV. *Botrytis cinerea* life cycle.

B. cinerea forms chlamydospores under adverse conditions, such as drought, lack of nutrients or oxygen, bacterial attack or pH changes. The chlamydospores serve as infection and survival structures. They germinate and produce mycelium that serves as secondary inoculum or forms macroconidia (Pande *et al.*, 2006).

B. cinerea is responsible for a wide range of symptoms that depends on the tissue and the organ (Figure V). For example, the typical symptom in leaves and red berries is the rot accompanied by the collapse and flooding of parenchymal tissues followed by a rapid onset of masses of conidia. The symptoms in petals range from small spots to large-scale rots. In stems, lesions appear near to the neck or wounds caused by pruning and later they spread. This infection does not affect the axillary buds due to the existence of the periderm but delays the development of shoots the following year (Pande *et al.*, 2006; Williamson *et al.*, 2007).

Figure V. Symptoms caused by *B. cinerea* in pepper.

IV. *Verticillium dahliae* Kleb.

Verticillium dahliae Kleb. is a vascular pathogen that causes Verticillium wilts. It is classified taxonomically into the phylum Ascomycota, class Sordariomycetes, order Phyllachorales and genus Verticillium. It causes crop loses of billions of dollars in annual plants worldwide but especially in temperate and subtropical regions (Fradin & Thomma, 2006; Klosterman *et al.*, 2009). *V. dahliae* attacks over 200 species of dicotyledonous. However, some isolates show host specificity (Fradin & Thomma, 2006).

This pathogen is characterized by a hyaline mycelium. The mycelium shows septate and branched hyphae that favours colonization and the formation of conidiophores. Conidiophores are organized in mucilaginous groups that contain elongated conidia-forming cells and are arranged in whorls (Pegg & Brady, 2002).

V. dahliae lives in the soil and attacks the plant roots. This pathogen causes a monocyclic disease (Fradin & Thomma, 2006). Generally speaking there are three phases in the cycle: dormancy, parasitic phase and saprophytic phase. In the first stage the resistant structures germinate (Figure VI). These structures are called microsclerotia; they are dense and dark mycelium aggregates. They are present in the soil or associated with dead plants (Fradin & Thomma, 2006; Klosterman *et al.*, 2009). When microsclerotia germinate, hyphae grow towards plants roots. The distance that hyphae can cover is limited and it is directed by a gradient of nutrients. Microsclerotia

germination can occur several times. There is no evidence that special conditions are necessary for plant root colonization (Fradin & Thomma, 2006; Klosterman *et al.*, 2009).

Once hyphae infect the roots the parasitic phase begins. In this phase *V. dahliae* needs to go through the endoderm to reach the xylem (Figure VI) (Fradin & Thomma, 2006; Klosterman *et al.*, 2009). Once there, conidia are produced and transported acropetally by the sap due to transpiration. Conidia may be retained in small cavities or in the end walls of vessels. These places are called capture or trapping sites. It is here where the conidia germinate and penetrate the adjacent vascular elements and continue the plant colonization. In this way a new infection cycle begins (Fradin & Thomma, 2006; Klosterman *et al.*, 2009).

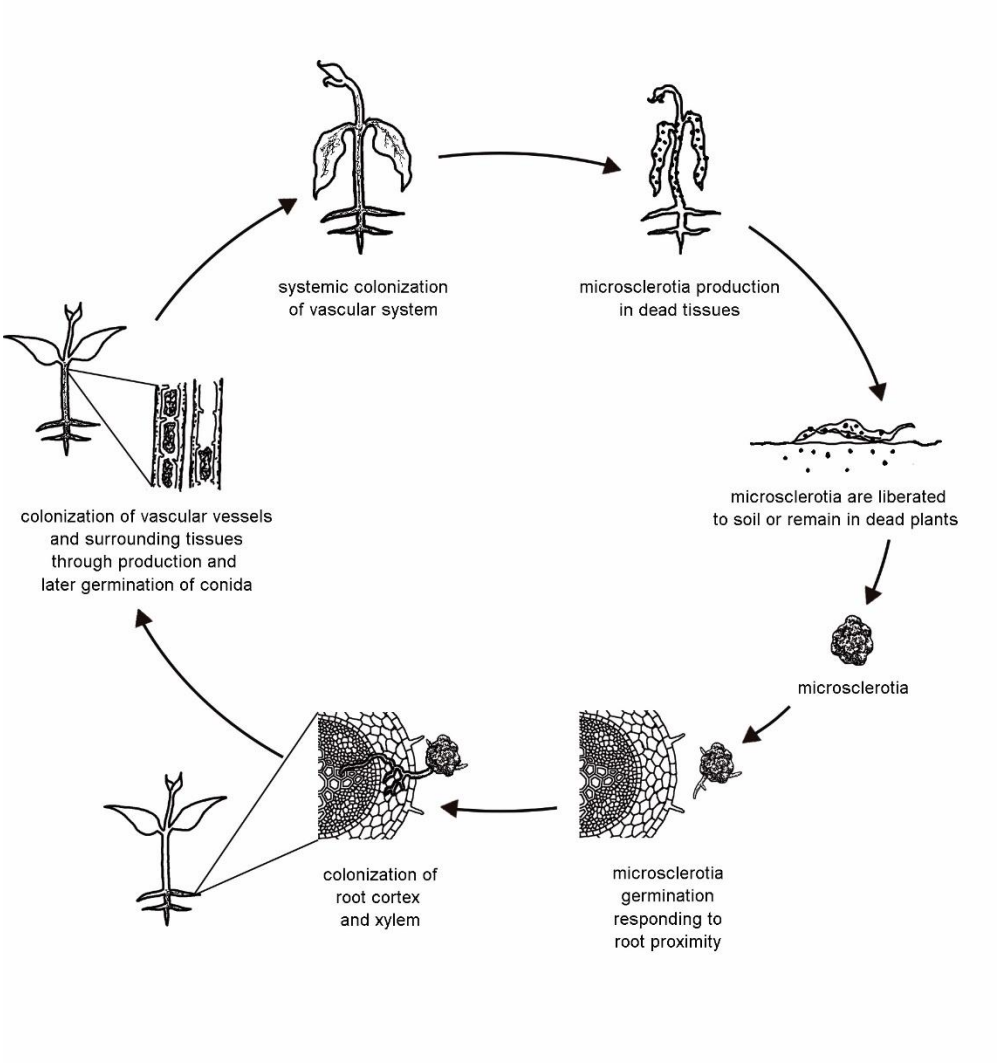

Figure VI. *Verticillium dahliae* life cycle.

The pathogen enters in saprophytic phase during tissue necrosis or plant senescence. During this phase the fungus also colonizes shoots and roots. In addition the fungus produces many microsclerotia that will be released to the soil during decomposition of the plant (Figure VI) (Fradin & Thomma, 2006).

V. dahliae infects a wide range of species including vegetables, fruit trees, flowering plants, oil plants, fibre crops and perennial trees. It causes a wide variety of different symptoms, namely rot, total or partial loss of turgor, brown spots, leaf abscission, chlorosis, necrosis and dwarfism (Figure VII) (Fradin & Thomma, 2006).

Figure VII: Symptoms caused by *V. dahliae* in pepper observed in the laboratory (A) and in the field (B).

V. Plant defensive response to pathogen challenge

Unlike animals, plants are sessile organisms, then, they cannot escape and have to cope with adverse situations. These can come from abiotic or biotic factors. The first group include all factors related with the environment such as light, temperature and amount of nutrients among others. On the other hand, biotic stress includes the damage caused by other organisms as fungi, virus and insects among others. Those attacks can devastate whole fields but first they have to overcome plant defences. These mechanisms are no easy to defeat thus not all of pathogens are able to penetrate and to colonize the host. They must find an adequate host. When the pathogen finds a host the outcome of the interaction can fail (incompatible interaction) or be successful (compatible interaction). The first case occurs when the plant is a non-host or the host is

resistant to that pathogen. The compatible interaction occurs when the host is susceptible and, therefore, disease occurs (Muthamilarasan & Prasad, 2013).

Plants have different defensive mechanisms that can be classified as constitutive and inducible barriers. Constitutive barriers can be chemical or physical. Some examples are cuticle, cell wall and phytoanticipins (Burketova *et al.*, 2015). These defences are considered the first level that the pathogen has to overtake. Most of the pathogens are not able to avoid this basic resistance and this is the so-called incompatible interaction (Muthamilarasan & Prasad, 2013).

Inducible defences need to be activated through the recognition of the microbes. The majority of pathogens have molecules named Pathogen- or Microbe-Associated Molecular Patterns (PAMPs or MAMPs). PAMPs are highly conserved within a specific microorganism class and are essential for their survival and their fitness (Thomma *et al.*, 2011). Normally these molecules belong to components of pathogen cell wall as flagellin from bacteria or chitin or glucan from fungi (Burketova *et al.*, 2015). PAMPs also include other components such as oligogalacturonides, ergosterol, xylanase and Pep-13 (Muthamilarasan & Prasad, 2013). In addition the plant cell also can perceive host-molecules that are produced by the pathogen during the penetration. They are known as DAMPs (Damage-Associated Molecular Patterns) (Burketova *et al.*, 2015). Both PAMPs and DAMPs are recognized by receptors called PRRs (Pattern Recognition Receptors). They are considered ancient from the evolutionary point of view. These types of receptors are located at the plasma-membrane level (Thomma *et al.*, 2011) and they belong to two types: receptor-like kinases (RLK) and receptor-like proteins (RLP). RLKs are formed by putative extracellular ligand-binding domain, a single transmembrane domain and intracellular serine/threonine kinase domain. RLPs have an extracellular domain and a membrane-spanning domain but they need an intracellular adaptor molecule to transmit the signal (Muthamilarasan & Prasad, 2013). PRRs recognize a specific epitope from PAMPs or DAMPS but this epitope can vary depending on plant species (Boller & He, 2009; Thomma *et al.*, 2011).

Once PRR receptors recognize PAMPs/DAMPs, they trigger a signalling cascade that finally activates the PAMP-Triggered Immunity (PTI) (Figure VIII). PTI is the first active barrier and includes changes in ion fluxes, increment of cytoplasmic Ca^{2+}, production of early signals such as ROS (reactive oxygen species) and NO (nitric

oxide) and activation of MAP and CDP kinases (Mitogen-Activated and Calcium-Dependent Protein Kinases). These signals cause the stomatal closure, PR protein accumulation, phytoalexin biosynthesis and cell wall strengthening through lignin and callose deposition. In addition ROS also have a direct role due to their antimicrobial capacity (Chisholm *et al.*, 2006; Burketova *et al.*, 2015; Hammond-Kosack & Jones, 2015). All this process is mainly mediated by phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Burketova *et al.*, 2015). Recently studies have shown that hormones such as abscisic acid, brassinoesteroids, giberellins, cytokinins and auxins also play a role in plant defence against biotic stress (Bigeard *et al.*, 2015).

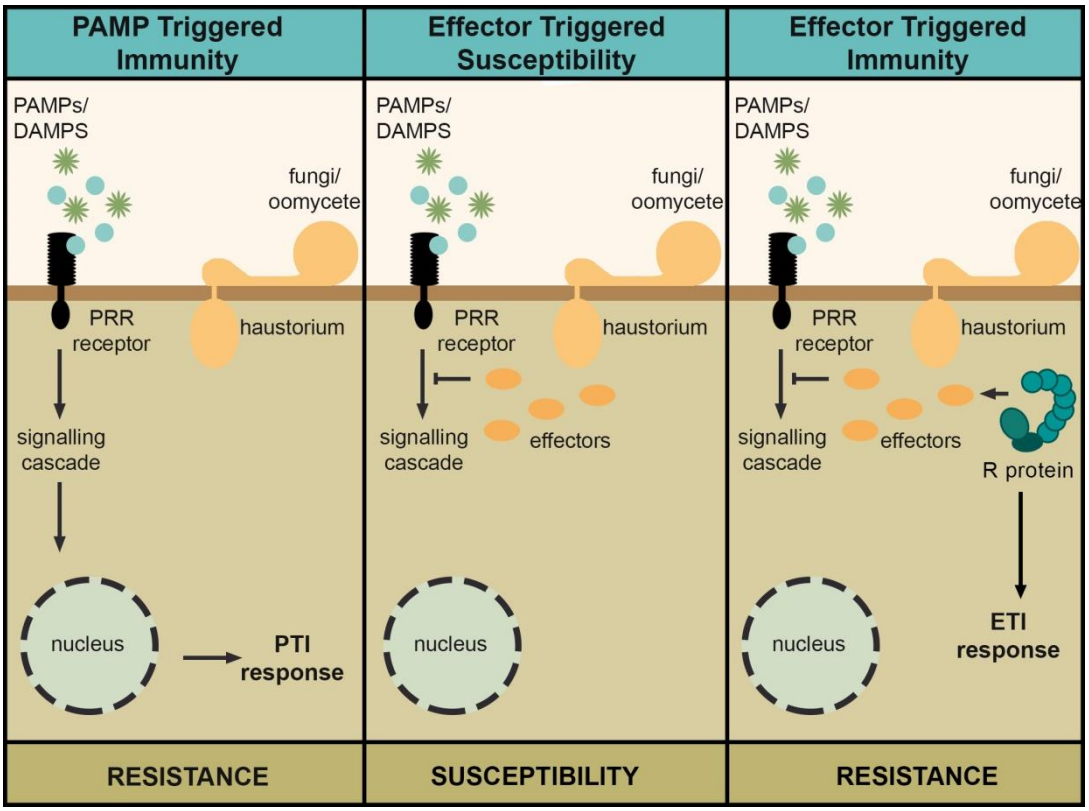

Figure VIII. Model of ways how plants detect pathogens and how they avoid their recognition by the plants. On the left, plants recognize PAMPs and DAMPs by PRR receptor. Therefore PAMP triggered immunity is activated and plant is able to resist the pathogen attack. On the centre, pathogen evolves to avoid the recognition. To do that, pathogen secrets effectors and then PTI response is suppressed. In this case, plant is in a susceptible state. On the right, plant also evolves and recognizes effectors by R proteins. Therefore effector triggered immunity is activated and the plant back to resistant state. This figure was adapted from Chisholm *et al.*, (2006).

PTI has caused a selective pressure on pathogen selecting those which are able to overcome PTI. To suppress the PTI the pathogens produces molecules called effectors that cause Effector-Triggered Susceptibility (ETS) (Hammond-Kosack & Jones, 2015). Effectors were defined by Hogenhout *et al.*, (2009) as "all pathogen proteins and small molecules that alter host-cell structure and function". Therefore, effectors interact with different PTI components blocking the PTI activation or altering the plant physiology to facilitate the pathogen infection (Win *et al.*, 2012). The effectors can be liberated in the apoplast acting in the interface between the host cell and the pathogen or secreted into the host cell (Hogenhout *et al.*, 2009).

The pathogens produce new effectors to counteract the plant defense and the plant produces new R proteins to recognize these new effectors. This arms race between pathogen and plant leads to step-wise co-evolution between pathogen and plant for effector and R gene production. (Hammond-Kosack & Jones, 2015). These R genes codify proteins located mainly in the cell cytoplasm and usually they are receptors of the nucleotide-binding leucin-rich repeat (NB-LRR) protein family. They have a leucin-repeat rich domain, a varying N-terminal effector domain and a central NB domain which control the signalling activity (Muthamilarasan & Prasad, 2013). Moreover, some R genes codify for extracellular LRR (eLRR) proteins which comprises RLP receptors, RLK receptors and PGIP (polygalacturonase inhibiting protein) receptors (Muthamilarasan & Prasad, 2013).

The recognition of effectors by R proteins can be achieved by direct interaction or helped by accessory-proteins. After that, the plant activates the Effector-Triggered Immunity (ETI) or R gene-mediated defence which is considered a second level of plant defence. Defence mechanisms activated during ETI are stronger and faster than PTI. Moreover, ETI is associated with hypersensitive response (HR), a kind of programmed cell death that helps to stop the infection (Jones & Dangl, 2006; Hammond-Kosack & Jones, 2015).

Formerly ETI was called gene-for-gene model. This model postulates that the plant is resistant when it carries a dominant resistant (R) gene complementary to the dominant recognized effector (Ree) (Hammond-Kosack & Jones, 2015).

In a situation in which pathogens are unable to avoid plant recognition it is necessary for them to evolve. This evolution can be caused by mutation in PAMPs or in

effectors. Thus plants also have to evolve to detect the pathogen. This cycle is repeated time after time and it supposes a coevolution between plant and pathogen (Hammond-Kosack & Jones, 2015). A representation of this evolution is shown in Figure XIX.

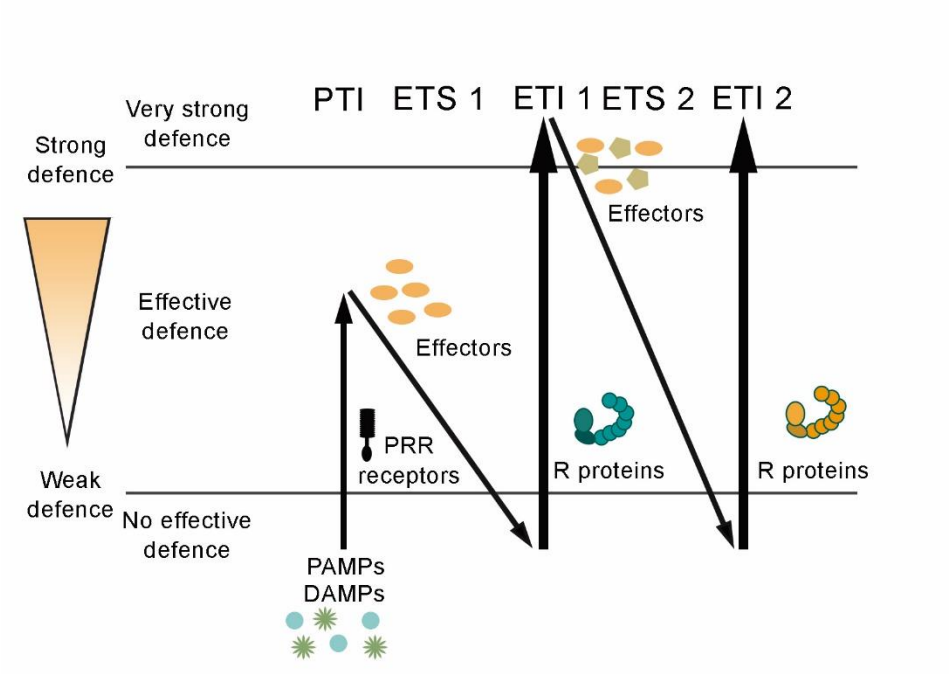

Figure XIX: Zig-zag model. This scheme shows how the defence state in plant is during process of PAMP triggered immunity (PTI), effector-triggered susceptibility (ETS) and effector-triggered immunity (ETI). This model also shows plants and pathogens have to evolve to survive. In a first stage plants recognize PAMPs and DAMPs (blue circles and green asterisks) by PRR receptors and trigger PTI. Some pathogens evolve and secrets effectors (orange ellipses) which block PTI and the plant defence decreases. This process is called ETS 1. Then some plants possess among their alleles genes that codify for R proteins which recognize effectors. Interaction between R proteins and effectors trigger ETI 1 and defence state is increased. In this situation pathogens that deliver new effectors (brown pentagon) could provoke a new ETS 2. Therefore plants capable of detecting those effectors could activate ETI 2. This figure was adapted from Jones & Dangl (2006).

Sometimes PTI and ETI can activate the defences in systemic tissues which have not been attacked by any pathogen. This fact promotes the resistance in the whole plant. This type of plant resistance is normally known as Systemic Acquired Resistance (SAR). Plants have another type of systemic resistance but, in this case, pathogen challenge does not promote it. This kind of systemic resistance is called Induced Systemic Resistance (ISR) and is triggered by beneficial microorganisms (Gourbal *et al.*, 2018).

VI. Systemic acquired resistance (SAR)

SAR is developed during an incompatible interaction in which a plant is able to stop the pathogen attack. During this interaction necrotic tissue is formed as a consequence of programmed cell death or symptom progress. In early stages several signals are formed, among them, the production of ROS is one of the most important. These signals trigger SA accumulation in both local and distal tissues. This accumulation induces plant defence in non-infected tissues and therefore SAR is established (Durrant & Dong, 2004; De Vleesschauwer *et al.*, 2009; Spoel & Dong, 2012; Choi & Hwang, 2015). The systemic response is completely established in several days and it can protect the plant for weeks and even months (Conrath, 2006). The degree of protection depends on the initial stimuli. It was observed that the number of inducing inoculations can increase the plant resistance level (Hammerschmidt & van Loon, 2009). One characteristic of SAR development is the formation of necrotic tissue, but in some systems it is no necessary (Hammerschmidt & van Loon, 2009) although SA accumulation is essential (Pieterse *et al.*, 2009).

SAR activation in tobacco and Arabidopsis involves SAR gene expression which includes pathogenesis-related (PR) genes (Conrath, 2006). So far 17 families of PR genes have been defined according to their sequence (Liu & Ekramoddoullah, 2006). Some of these proteins have been identified as acidic β-1,3-glucanases and chitinases, which are enzymes able to degrade the fungal cell wall (Conrath, 2006). In general PR proteins are characterized by their antifungal properties, although some of them show antibacterial, insecticide, nematicide and antiviral activity (Reddy, 2013). In addition to the synthesis of PR proteins, SAR defences also include lignification, deposition of callose in pathogen penetration points and HR (Baysal *et al.*, 2005).

SAR is effective against a broad-spectrum of pathogens including virus, bacteria, fungi and oomycetes (Durrant & Dong, 2004). However, in Arabidopsis, SAR is more effective against biotrophs and hemibiotrophs (Hammerschmidt & van Loon, 2009).

VI.I. SAR signals

SA is the most important signal for SAR activation (Manohar *et al.*, 2015). In fact, the exogenous application of SA can mimic the activation of SAR in the absence of pathogen attack (Hayat *et al.*, 2012).

SA can be biosynthesized by two different pathways: the isochorismate pathway and the phenylalanine ammonia-lyase pathway. Both pathways use chorismate, the final product from shikimate pathway, as precursor (Gao *et al.*, 2015). The involvement of each pathway in SA biosynthesis varies depending on the plant species. For example, in Arabidopsis SA production seems to originate from isochorismate pathway during pathogen attack. However, in tobacco SA synthesis derives from phenylalanine ammonia-lyase pathway (Ogawa *et al.*, 2006; Gao *et al.*, 2015).

During a pathogen infection SA accumulates first at the site of infection and later on at the distal tissues and organs (Jung *et al.*, 2009). Initially it was suggested that SA itself translocates from local infection to non-infected-distal tissues. However, Vernooij *et al.* (1994) proved long time ago that SA is not translocated from the original site of infection to the distal tissues. They used transgenic tobacco expressing a salicylate hydroxylase (NahG), which degrades SA. These plants were unable to produce SAR. Using grafting experiments between wild-type and *NahG* plants, they observed that SAR was restored (Vernooij *et al.*, 1994). In addition, the experiments of Vernooij *et al.* (1994) also showed that de novo biosynthesis of SA is required for SAR mounting in the distant tissues.

Several studies have showed the participation of several amino acid pathway products. Among them Návarová *et al.* (2012) discovered that accumulation of pipecolic acid (Pip), a non-protein amino acid, increase in both local inoculated and systemic non-inoculated leaves. Furthermore, they observed that this accumulation precedes SA accumulation in distal leaves. Moreover, experiments carried out in *ald1* mutant, Pip-deficient plants, showed that SAR was completely blocked (Návarová *et al.*, 2012). Thus, these results suggest that Pip is an important signal to SAR establishment. Subsequent studies confirmed this idea and showed that Pip controls SAR activation via FLAVIN-DEPENDENT-MONOOXYGENASE1 (FMO1) (Bernsdorff *et al.*, 2016). Bernsdorff *et al.* (2016) also observed an additive effect between Pip and SA signals.

Other compound identified as a putative long distant signal for SAR was methyl salycilate (MeSA) (Park *et al.*, 2007). Grafting experiments in Arabidopsis showed that MeSA formation is required at the site of infection and MeSA conversion in SA is required at the systemic level to generate SAR (Park *et al.*, 2007). However, other authors observed that Arabidopsis mutants lacking methyltransferase activity (necessary to produce MeSA) have the ability to accumulate SA in distant tissues and to mount SAR (Attaran *et al.*, 2009). This data suggests that the role of MeSA is influenced by additional factors. Arabidopsis *dir1* mutants are deficient in systemic immunity but are still able to activate local immune responses (Liu *et al.*, 2011). This mutation impairs the plant to mount the systemic response. *dir1* mutants present higher expression of the major MeSA synthesising enzyme in Arabidopsis (BSMT1, benzoic acid/ salicylic acid carboxyl methyltransferase 1) and also higher levels of MeSA but, in contrast, the levels of SA were lower (Liu *et al.*, 2011). These data suggest that DIR1 acts to repress the production of MeSA from SA by inhibiting BSMT1. DIR1 has also been involved in the generation of putative lipidic signals since DIR1 has a predicted function of lipid transfer protein (Truman *et al.*, 2007). In addition, it was probed that DIR1 is able to move using plasmodesmata from local to distal leaves during SAR (Champigny *et al.*, 2013; Cameron *et al.*, 2016). DIR1 cooperatively functions with other putative long distant signals, as azelaic acid and glycerol-3-phosphate, both requiring a functional DIR1 to induce systemic responses (Chanda *et al.*, 2011). The diversity in candidates observed for the long distal signal suggests an integration of different stimuli perceived at the site of infection that transfer information to the distal tissues about the most adequate response to mount. The distant tissue responds to the long distance signal by increasing the SA level. Therefore, SA signalling is required both locally and systemically.

VI.II. SAR regulation: role of NPR1, NPR3 and NPR4

NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1), an ankyrin repeat protein, plays a key role in plant defence regulation in both local and systemic level (Hammond-Kosack & Jones, 2015). NPR1 is positively regulated by SA and activates all the genes influenced by SA (Shah & Zeier, 2013).

The regulation of NPR1 by SA was described by Mou *et al.* (2003). They discovered that the inducers of SAR regulate the NPR1 activity through redox changes. If the plant is not attacked by a pathogen, NPR1 remains in cytosol in an oligomeric oxidized form. This form is maintained through intermolecular disulfide bridges (Hammond-Kosack & Jones, 2015). When the plant recognizes the pathogen attack, it starts a sequence of events that involves the production of SA which is accompanied by an increase in ROS (Fobert & Despres, 2005). These increases in SA and ROS are followed by a defence-associated programmed cell death (PCD). Both SA and ROS levels increase also in the tissues surrounding HR lesions and in the uninfected distal tissues (Zurbriggen *et al.*, 2010). This oxidative burst alters the redox status in cytoplasm leading to the reduction of the disulfide bridges of NPR1. This fact produces the dissociation of the NPR1 complex into reduced NPR1 monomers that migrate to the nucleus where they activate the transcription of defence genes (Hammond-Kosack & Jones, 2015).

In spite of NPR1 being a key regulator in SAR, Fu *et al.* (2012) proposed two NPR1 paralogues, NPR3 and NPR4, as receptors of SA. They have also demonstrated that NPR3 and NPR4 are adaptor proteins for the CUL3 E3 ligase that target NPR1 for specific polyubiquitination and subsequent degradation by the 26S proteasome (Fu *et al.*, 2012). In addition these paralogues present different affinity to SA: NPR3 possess low affinity and NPR4 high affinity (Fu *et al.*, 2012). Thus Fu *et al.* (2012) suggested that NPR3 and NPR4 respond to different concentrations of SA and in this way SA controls the role of NPR3 and NPR4. According to their results, Fu *et al.* (2012) proposed the following model: NPR1 is degraded in the nucleus of cells of healthy plants with very low or no levels of SA since NPR4 will target it for degradation preventing unnecessary defence gene activation. After a pathogen attack SA levels highly increase in the site of inoculation; these levels of SA are sufficiently high to activate the low affinity NPR3 triggering its linkage to NPR1 leading to NPR1 degradation and triggering of HR. Therefore both, high and very low (or any) levels of SA, bring about NPR1 turnover. However, in the tissue surrounding HR or systemic tissue, low levels of SA disrupt NPR4-NPR1 interaction but are not sufficiently high as to lead to NPR3-NPR1 formation resulting in NPR1 stabilization (Figure X). Only in this later case NPR1 is available to induce defence gene expression.

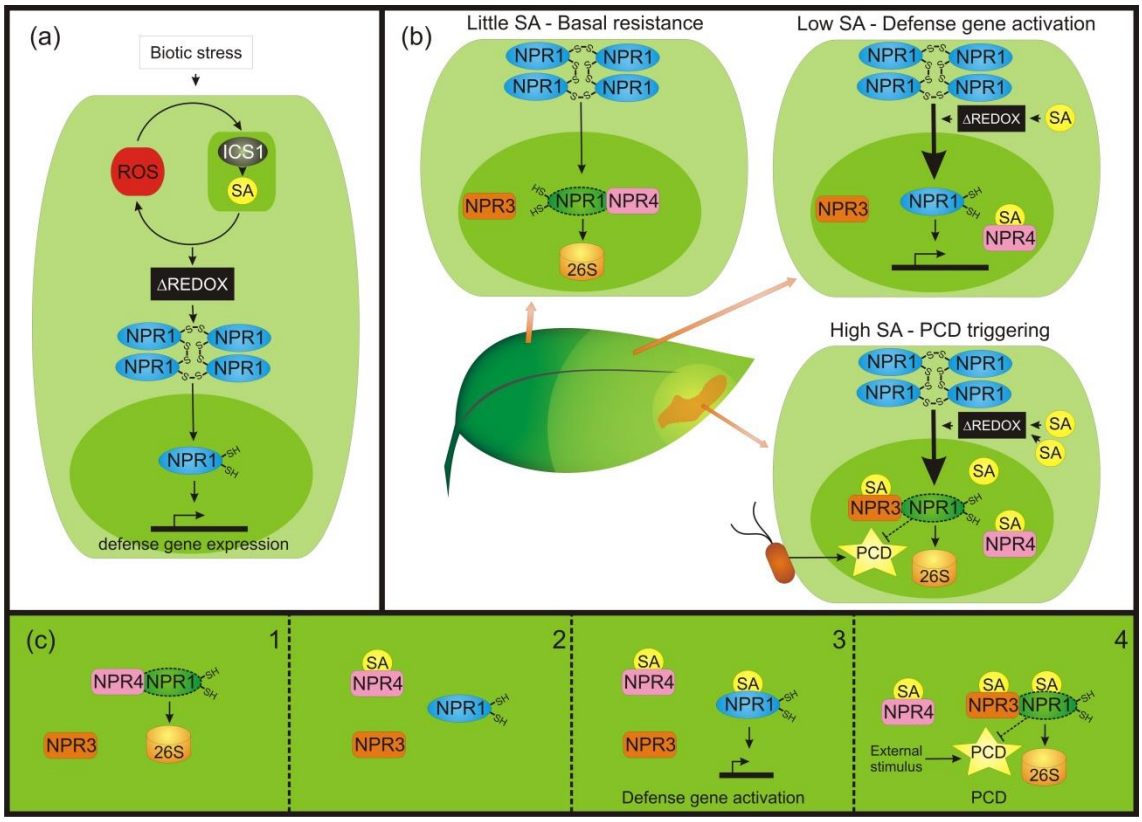

Figure X. Models of SA perception. A: Model proposed by Mou *et al.* (2003). SA regulates the activation of the acquired response by the generation of reactive oxygen species modifying the oxidative status of NPR1. B: Model proposed by Fu *et al.* (2012). Two SA receptors, NPR3 and NPR4 regulate NPR1 activation by degradation equilibrium. C: Model integrating NPR3, NPR4 and NPR1 as SA receptors. 1, 2, 3 and 4 represent increasing SA concentration. 1, in the lowest levels of SA, NPR4 binds to NPR1 and triggers its degradation. 2, NPR4 has the highest affinity to SA, when NPR4 is bound to SA, NPR1 is released from the NPR4 triggered degradation. 3, NPR1 has an intermediate affinity to SA (Wu *et al.*, 2012). NPR1 activation by SA binding leads to defence gene activation. 4, NPR3 has the lowest affinity to SA, when NPR3 is bound to SA, NPR1 is degraded by NPR3 triggered ubiquitination. NPR1 degradation releases NPR1-repression of the programed cell death (PCD). NPR1 in blue means intact protein, NPR1 in green and dotted contour means that the protein has been degraded by ubiquitin-mediated proteolysis. (Reproduced from Veloso *et al.*, 2014a).

However, at the same time that Fu *et al.* (2012) described NPR3 and NPR4, Wu *et al.*, (2012) observed that NPR1 is able to bind directly with SA. Later on Manohar *et al.* (2015) confirmed that NPR1 can bind SA. This adds to the model a direct step where SA binds to NPR1 that regulates the breakdown of the NPR1 complex and the NPR1 defence gene activation.

Besides NPR1 and its paralogues, SA is capable to bind to other proteins as catalase or carbonic anhydrase. Recently, Manohar *et al.* (2015) show nine new proteins

capable of binding to SA with diverse functions, e.g. assisting oxidation, reduction, and/or isomerization of disulphide bonds or involved in photorespiration (Manohar *et al.*, 2015).

VII. Induced Systemic Resistance (ISR)

ISR is induced by beneficial microorganisms. Among them the most studied are plant growth-promoting rhizobacteria (PGPR) (Pieterse *et al.*, 2000), such as *Pseudomonas*, and plant growth-promoting fungi (PGPF), such as *Trichoderma* sp. and *Piriformospora indica* (Shoresh *et al.*, 2010).

An important issue in ISR is the establishment of the interaction between the plant and the beneficial microbe. The cross-talk between the host plant and the microorganism is the key for the establishment of the defence response in the plant. The way in which PGPR or PGPF associates with the host plant is specific for each microorganism. For example, *Bacillus subtillis* produces a biofilm and *Trichoderma* sp. forms an appressorium-like structure in the root hairs (Pieterse *et al.*, 2014). Besides to guarantee the successful and prolonged mutualistic connection, PGPR and PGPF have to evade their recognition by the plant avoiding the plant defensive machinery being activated against them. They use the same strategies than pathogens use to infect the plants such as the use of effectors, as SP7 that suppress ethylene -mediated defensive responses, or activate an antagonistic signalling pathway to block the defence response (Pieterse *et al.*, 2014).

Once mutualistic interaction is established successfully, a signal is generated in the roots and moves, via phloem, through the rest of the plant tissues increasing the host defence responses against pathogenic organisms (van Loon & Bakker, 2005; Pieterse *et al.*, 2014). These defences are controlled by the hormones JA and ET (van Loon & Bakker, 2005; Choudhary *et al.*, 2007). However, the accumulation of these hormones and subsequent gene activation does not occur until a pathogen attacks the plant (Pieterse *et al.*, 2000). It has also been observed that some rhizobacteria as *Paenibacillus alvei* K165 induces defences regulated by SA, while others such as endophytic actinomycetes are capable of inducing both pathways, SA and JA / ET (van Wees *et al.*, 2008).

ISR has been observed in bean, carnation, cucumber, radish, snuff, tomato and *Arabidopsis thaliana*. It is effective against different types of pathogens, although it is more effective against necrotrophs (Pieterse *et al.*, 2000).

However, the relationship between PGPR or PGPF and plants is not so simple. It was observed that the iron availability can modify the susceptibility or the resistance of a plant against a pathogen. This connection was discovered in 1996 by Leeman and co-authors (Verbon *et al.*, 2017). In fact, the pathways of ISR and iron homeostasis share the component MYB72 which is induced by several beneficial microbes and also it is involved in the biosynthesis and secretion of iron-mobilizing phenolic compounds under iron-limiting conditions (Verbon *et al.*, 2017). Moreover, MYB72 activates BGLU42 (β-GLUCOSIDASE 42), involved in fluorescent phenolic compounds production, and, when this gene is mutated, the plant is unable to trigger the ISR (Verbon *et al.*, 2017).

VIII. Priming and inheritance of this state by the plant progeny

Priming is described as a sensitization state in which plants respond faster and stronger to very weak stimuli during a stress (biotic or abiotic) (Conrath *et al*., 2015). In fact, priming strengthens plant basal resistance and this state has a very low cost for the plant (Pastor *et al*., 2013; Veloso *et al*., 2014a).

This state can be established by plant exposure to a pathogen, an elicitor, an herbivore, a chemical compound or beneficial microorganisms (bacteria or fungi) (Gamir *et al*., 2014; Conrath *et al*., 2015). Also the defensive responses SAR and ISR can trigger this state. This sensitization state can go on for a long time since the moment of establishment. Thus priming can be considered a kind of memory and could participate in plant adaptation to stress conditions (Pastor *et al*., 2013).

Three stages can be distinguished in the establishment of priming. The first stage is called the pre-challenge priming stage and occurs before the pathogen interacts with the plant. This phase involves the generation of signals and components involved in the triggering of the plant responses to challenge, e.g. changes in phosphorylation and/or accumulation of transcription factors. The second stage occurs upon pathogen attack and it involves the fast and strong activation of defences. Finally the last stage involves

the long lasting resistance state which includes changes in DNA methylation patterns that produces transgenerational resistance (Gamir *et al*., 2014).

When plants are exposed to pathogen challenge conditions for long periods of time, the primed state can be transmitted to the progeny and they respond to biotic stress in a similar way as their parents (Figure XI). Luna *et al.* (2012) inoculated parental plants several times in 3 weeks with *P. syringae* DC3000 with other non-inoculated parental plants used as controls. The progenies of both groups were inoculated with the oomycete pathogen *Hyaloperonospora arabidopsidis* and the authors observed that pathogen colonization was reduced in the progeny of infected parental plants, and that PR1 gene expression was enhanced. A similar experiment was carried out by Slaughter *et al*. (2012). They infiltrated Arabidopsis plants with *Pseudomonas syringae* pv t*omato* carrying the avirulence gene avrRpt2 and compared them to another group of plants that were mock inoculated. These two groups were considered parental plants. Of these plants they obtain a first progeny named ColM (from mock plants) and ColP (from Pseudomonas challenged plants). Then, they challenged the progeny lines with virulent *P. syringae* and observed less symptoms in ColP in comparison to ColM, as well as higher priming of PR1 gene expression. Both Luna *et al.* (2012) and Slaughter *et al*. (2012) found the same results: the offspring of plants subjected to pathogen stress presented reduced symptoms because of priming inherited from their parents (Figure XI).

The transmission of induced resistance to progeny is not exclusive of the interaction Arabidopsis-bacteria or - oomycete, but also works in other plant-pathogen interactions, for example, *Nicotiana tabacum*-tobacco mosaic virus (TMV) when parental plants were pre-treated with an elicitor (Kathiria *et al.*, 2010). In the monocotyledonous plant *Hordeum vulgare*, the progeny of plants pre-treated with the SA-mimic elicitor acibenzolar-Smethyl (ASM, synonymous with benzothiadiazole, BTH) or saccharin was more resistant to the fungus *Rhynchosporium commune*, and the relative growth rate of the offspring was unaltered (Walters & Paterson, 2012).

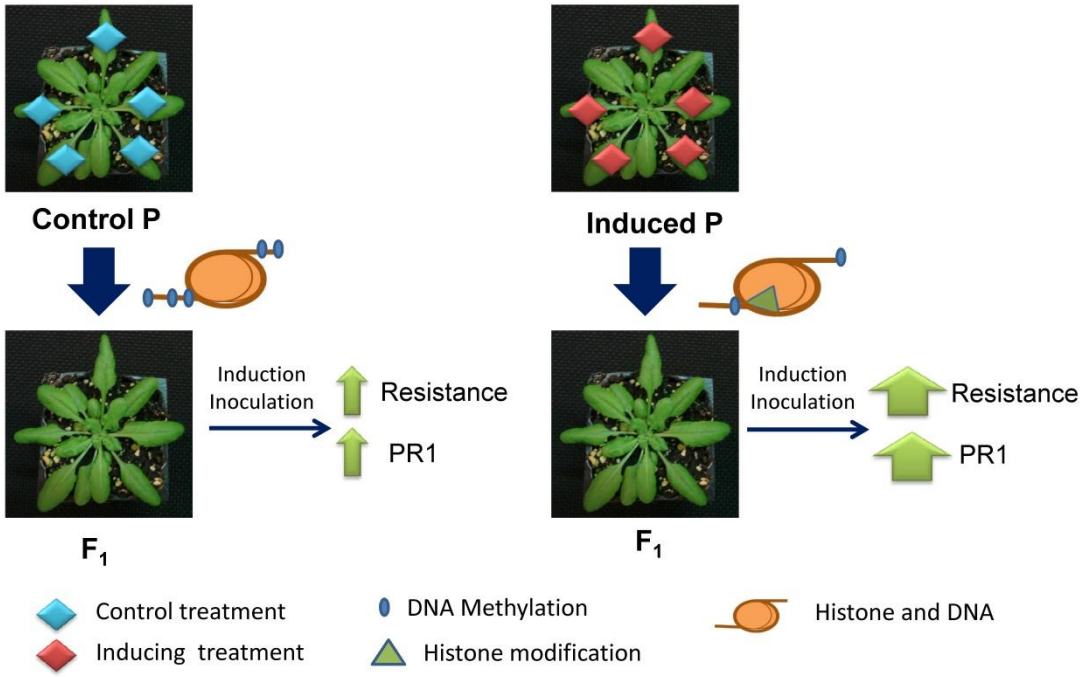

Figure XI. Model for transgenerational priming according to findings by Luna *et al*. (2012) and Slaughter *et al*. (2012). The progeny of primed plants (right) shows a higher response to pathogens and chemical inducers than the progeny of control plants (left), in terms of a further increased induced resistance and expression of defence mechanisms as PR1. Such transmission of priming across generations is caused by histone modifications and a reduction in DNA methylation. (Reproduced from Veloso *et al*., 2014a).

Another important finding of the experiments of Luna *et al*. (2012) and Slaughter *et al*. (2012) is related to the durability of the transgenerational priming. In order to test this, one half of the plants that presented transgenerational priming was exposed to elicitor or pathogen treatment and the other half of the plants was grown under stress-free conditions. Luna *et al*. (2012) found that induced resistance and defence gene priming persisted after one stress-free generation. However, Slaughter *et al*. (2012) observed that the second generation progeny whose parents were not subjected to a new priming treatment lost induced resistance to *H. arabidopsidis* and also showed a slower and lower induction of PR1 gene expression. Possibly such different results are caused by the difference in priming treatment in the parental generation: several repeated treatments in the case of Luna *et al*. (2012) and one time treatment in Slaughter *et al*. (2012). These two papers also focused on the way this priming memory is transmitted from one generation to the next. One of the possible ways is the accumulation of compounds such as elicitors or hormones in the seed, thus the progeny would be primed (Pastor *et al*., 2013). On the other hand, plant hormones as

jasmonates and salicylates were not more abundant in progeny from primed parental plants (Luna *et al*., 2012).

Therefore, there must be some other mechanism involved in the transfer of the priming. Youngson & Whitelaw (2008) discuss soft inheritance or non-Mendelian inheritance, that is, the transmission of nongenetic information from parental plants to the offspring through epigenetic mechanisms. Such changes can be reversible when the biotic stress is removed (Boyko & Kovalchuk, 2011), since the plant-pathogen interaction causes fast changes at the DNA level (Gómez-Díaz *et al*., 2012). The epigenetic mechanisms involved in the transgenerational priming could be modifications of histones and changes in DNA methylation (Pastor *et al*., 2013). Transgeneratinal priming is being studied as a tool for pest management (Ramírez-Carrasco *et al*., 2017)

IX. Phytosanitary products to control plant disease and pests

Since the beginning of agriculture, pests and diseases have diminished crops yield. Thus farmers have had to find ways to control and maintain the quality of their production (Pal & Gardener, 2006; Oerke *et al*., 2012). Different strategies have been developed for this purpose, but the most used is the application of fungicides, bactericides, insecticides and herbicides (Edevra, 2004).

Since the late XIX century and early XX centuries, several chemical management strategies have been used, such as those based on sulfur, lime and copper sulfate as antifungal agents against downy mildew and powdery mildew. These initial treatments led to the establishment of an industry responsible for developing many fungicides with different modes of action against pathogens and effects on the plant (Russell, 2005; Deising *et al*., 2008). However, the social alarm related to residues from these products in fruits and vegetables began to rise. As a consequence, in the 1930s, these highly toxic compounds were replaced for pesticides based on synthetic organic compounds (Pretty, 2005).

The use of fertilizers and pesticides bond to the use of high producing varieties and the general use of irrigation techniques have meant that the crop yield has been tripled in a period of 50 years (1961-2010). This phenomenon is known as Green

Revolution (Zeng *et al.*, 2014). This moment meant the widespread use of plant protection products that opened a new market and needed the establishment of a legal framework regulating the use of these products. Currently in Spain, commercialization and use of plant protection products is regulated by Royal Decree 1311/2012, of September 14th, which updates the Law 43/2002, of November 20th, plant health with the provisions of Regulation (EC) No. 1107/2009 of the European Parliament and Council of October 21th 2009. The aim of this Regulation is to ensure a high level of protection of human and animal health and the environment while safeguarding the agriculture community competitiveness.

Article 2 of Regulation (EC) No. 1107/2009 of the European Parliament and the Council of 21th October 2009 stipulates that plant protection products are those products that contain or are composed by active substance, protective or synergists compounds, and set aside for one of the following uses:

a) "protecting plants or plant products against all harmful organisms or preventing the action of such organisms, unless the main purpose of these products is considered to be for reasons of hygiene rather than for the protection of plants or plant products"
b) "influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient"
c) "preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives"
d) "destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants"
e) "checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants"

This regulation defines as well the requirements that plant protection products should have to be approved, registered and sold. These are:

1. Dossier: refers to all the information necessary for an estimate of the fate and distribution of active substance in the environment and its impact on non-target species.
2. Efficacy

3. Relevance of metabolites: help to establish the toxicological, ecotoxicological or environmental relevance of metabolites.
4. Composition of the active substance, safener or synergist
5. Methods of analysis
6. Impact on human health
7. Fate and behaviour in the environment: refers to the persistence, bioaccumulation and toxicity of the active substance.
8. Ecotoxicology
9. Residue definition
10. Waste and behaviour concerning groundwater

Nevertheless Royal Decree 1311/2012, of September 14th, advise to reduce the use of phytosanitary products by means of using alternative techniques such as the use of appropriate agricultural techniques and/or use of resistant cultivars, among others. Regarding the use of phytosanitary products, it should be used those products that contain low-risk active substances and do it at the lowest recommended dose. Moreover it is also recommended to complement the phytosanitary products effect using basic substances or the natural plant defences using alternative substances as biostimulants or pheromones.

In spite of the legal framework behind the use of phytosanitary products and the numerous studies done to understand the mode of action of the product itself and its residues, there are still products that have a negative impact in environment as well as human and animal health (Edevra, 2004). It has been observed that when a pesticide is applied to crops, most of the product is either taken by animals and plants or eventually degraded. However, other compounds are vaporized, then the rainfall rinses them and they are deposited in the soil. Thus some pesticides persist as organochlorines in the soil which have become a serious problem for the environment (Pretty, 2005).

In addition to the enviromental problem, phytosanitary products also produce the appearance of resistant strains of the pathogens. As a consecuence a lot of products have been removed from the market (Deising *et al.*, 2008). This is because the resistance to fungicides or other phytosanitary products is inherited, causing a reduction in organism sensitivity to the compound and thus becoming less efficient (Ma & Michailides, 2005).

These problems create the need to find new strategies to control pests and diseases. In the last years new strategies, as biological control or the use of elicitors, are being introduced into the classical pest management. These strategies are based on their capacity to make use of the natural plant defence system to reduce the use of classical phytosanitary products.

X. Resistance inducing agents

Plant resistance can be activated by a variety of inducers which can be of biological or chemical origin. Biological inducers include all those molecules that form during a plant-pathogen interaction. Some examples are lipopolysaccharides from bacteria and fungi cell wall, e.g. chitin and glucan, or some siderophores and enzymes from bacterial exudates. Also in this group are include substances belonging to plant secondary metabolism (see point below). Biological inducers may refer as well to the whole organism that produces them. Currently there are many organisms that are used alive in agriculture as inducers of resistance because they produce this kind of substances and are not detrimental for the plant. These kinds of beneficial microorganisms are called biocontrol agents. Examples of biocontrol agents are well characterized rhizobacteria as *P. fluorescens*, or some fungi such as *Trichoderma* sp. (Reddy, 2013).

On the other hand chemical inducers include synthetic products that induce plant defence. The first chemical inducers emerged after the identification of SA as an essential signal in the SAR establishment by mimicking this hormone. The first synthesized compounds able to induce SAR were 2,6-dichloroisonicotinic acid (INA) and its methyl ester (Goellner & Conrath, 2008). However, the INA is phytotoxic in some cultures leading to the development of the second SA mimicking compound, BTH (Goellner & Conrath, 2008). Both compounds are capable of activating SAR by the same pathway as the SA (Goellner & Conrath, 2008). This type of chemical inducers is effective against fungi, bacteria and viruses, e.g. *Peronospora tabacina*, *Cercospora nicotianae*, *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pv. *tabaci* and TMV (Edevra, 2004).

The application of chemical inducers can directly activate plant defences but can also prime the plants. When an inducer drives priming, defences are not activated until

the plant comes into contact with the pathogen. When the pathogen is detected by a primed plant, the activation of defences is faster and stronger than if the plant is in a non-primed state. The phenomenon of priming does not exclude direct induction of defences (Walters *et al*., 2013).

The use of chemical inducers for controlling crop diseases meets all requirements for safe application both in greenhouses and in the field. That means that they are not directly toxic for pathogens or plants or animals, have no negative effects on growth, development or production, have a broad spectrum of action, are used in low concentrations, produce lasting protection and reduce economic costs for farmers (Edevra, 2004).

XI. Secondary metabolism involved in plant defence

Plants produce a wide range of antimicrobial compounds either as part of the basal resistance or as a consequence of induced resistance (SAR, ISR). Several criteria were established to classify these compounds, e.g. the core structure of the compound, common precursors and mechanisms of action, but the most common is based on their biosynthesis and accumulation. This classification establishes two groups: phytoanticipins and phytoalexins (Piasecka *et al*., 2015). Phytoalexins are antimicrobial compounds with low molecular weight and they are synthetized and accumulated during pathogen challenge. The term of phytoalexins was coined by Müller and Börger in 1940 (Jeandet *et al*., 2014). They observed as the infection of potato with one strain of *Phytophthora infestans* was able to block the next infection with the same pathogen but different strain. They thought that the accumulation of a substance generated during the infection was responsible for the subsequent unsuccessful infection (Jeandet *et al*., 2014). In this group are included camalexins, phenylalanine-derived phytoalexins and terpenoids (Piasecka *et al*., 2015).

For a long time it had been known that plants possessed compounds involved in plant defences that were present before pathogen challenge. Initially scientists included these compounds as phytoalexins, but in 1994 VanEtten *et al*. proposed the term phytoanticipins and it was defined as “low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents” (VanEtten *et al*., 1994). Within this

group we can find saponins, glucosinolates, cyanogenic glucosides and benzoxazinone glucosides (Piasecka *et al*., 2015).

As it was mentioned above some components from secondary metabolism can be used as inducers. There are some studies that apply plant extracts to treat the plant and check if they protect the plants against pathogens or pests. Some of them can be artificially synthesized such as anthraquinones (Song *et al*., 2013). In the case of pepper, the expectations are on its fruit because it is a clear example of how the chemical composition protects the seeds from pathogens. A feature of the fruits of some varieties of pepper is the presence of pungency. Tewksbury *et al*. (2008) said that the pungency is an adaptive response to selective pressure exerted by pathogens. The compounds responsible for the pungency can be used to control diseases caused by pathogens in pepper.

XII. Capsaicinoids and their analogues

One characteristic of pepper fruits is the presence of pungency. This property is a consequence of the accumulation of compounds known as capsaicinoids. These secondary metabolites are located in the placenta of pepper fruit from some cultivars and some species of pepper (*Capsicum* spp.). This property is inherited as a dominant trait (Díaz *et al*., 2004). Several studies show that dominant allele *Pun1* and enzyme pAMT (putative aminotransferase), involved in vanillin amination, are essential for synthesis and accumulation of capsaicinoids. Therefore both components determine the presence or absence of the pungency trait (Díaz *et al*., 2004; Jang *et al*., 2015; Ogawa *et al*., 2015).

In nature the presence of pungency in pepper fruits avoid that mammals like rodents eat them because seeds are destroyed during the digestive process, but birds neither cause such a detrimental effect on seeds nor are deterred by capsaicinoids (Tewksbury & Nabhan, 2001; Levey *et al.*, 2006). Mammals are able to sense capsaicinoids and similar compounds, named vanilloids, therefore they are a good deterrent (Diaz *et al.*, 2004). The hot sensation is caused by a direct effect of capsaicinoids on pain receptors located in mouth and throat (Díaz *et al.*, 2004).

Several studies have shown that some cultivars possess non-pungent compounds similar to capsaicinoids. These metabolites are capsinoids and capsiconinoids. Capsinoids were first identified in *C. annuum* var. CH-19 Sweet (Sutoh *et al.*, 2006; Lourdes-Reyes-Escogido *et al.*, 2011) which produces huge amounts of capsinoids. Nevertheless there are trace quantities of capsinoids in the most pungent cultivars. Capsiconinoids were firstly detected in *Capsicum baccatum* var. *praetermissum* (Tanaka *et al.*, 2009). Several studies have shown that both capsaicinoids and their non-pungent analogues possess the same pharmacological properties, but non-pungent analogues cause no irritation (Lourdes-Reyes-Escogido *et al.*, 2011).

XII.I. Molecular structure and biosynthesis

Since the beginning of the XIX century capsaicinoids and their analogues have been studied extensively. In their molecular structure can be distinguished two different parts: an aromatic ring and an aliphatic chain (Figure XII). Both structures are bind by an amide bond in capsaicinoids and by an ester bond in capsaicinoids and capsiconinoids (Mazourek *et al.*, 2009; Aza-González *et al.*, 2011; Lourdes-Reyes-Escogido *et al.*, 2011).

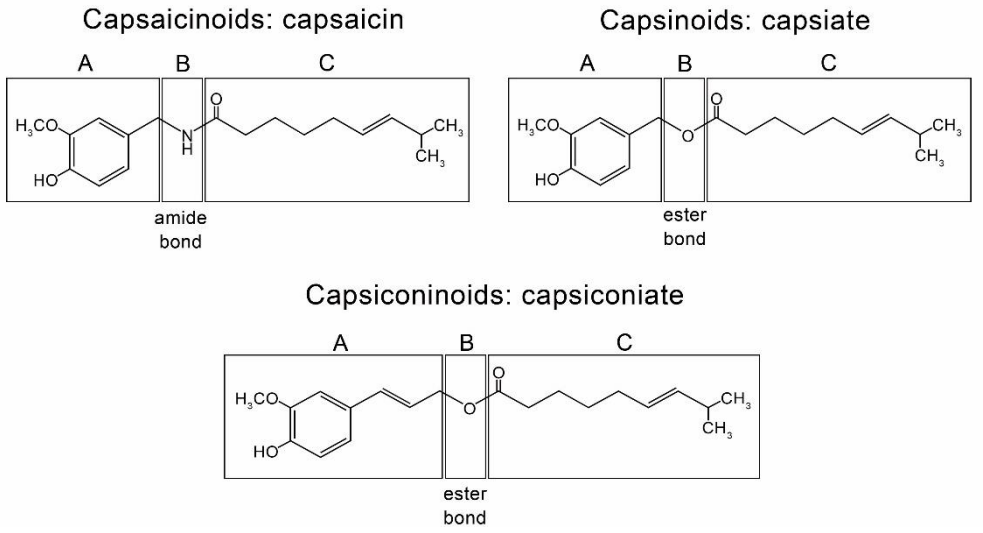

Figure XII. Regions of chemical structure of the capsaicinoids and their analogues (capsinoids and capsiconinoids). This figure was adapted from Lourdes-Reyes-Escogido *et al*. (2011).

The precursor of the phenolic portion varies depending on the compound: vanillylamine in capsaicinoids, vanillyl alcohol in capsinoids and coniferyl alcohol in capsiconinoids (Figure XII). However, all of them are derived from phenylalanine

following phenylpropanoid pathway (Vázquez-Flota *et al*., 2007; Lourdes-Reyes-Escogido *et al*., 2011). Phenylalanine is transformed after several changes in ferulic acid that, in turns, is transformed in coniferyl alcohol and vanillin following different pathways. In the next step the vanillin is aminated by pAMT to yield vanillylamine (Figure XIII) (Vázquez-Flota *et al.*, 2007; Kobata *et al.*, 2011). This process is very fast because the vanillin is very reactive (Prasad *et al.*, 2006). However, in some cases the vanillin is transformed in vanillyl alcohol (VOH) because pAMT has an insertion of a T-nucleotide that results in a new stop codon and therefore a truncated-non-functional enzime. This mutation was only observed in pepper cultivar CH-19 Sweet (Sutoh *et al.*, 2006; Lang *et al.*, 2009). In other non-pungent cultivars, as Himo, the insertion is located in other position (Tanaka *et al.*, 2010).

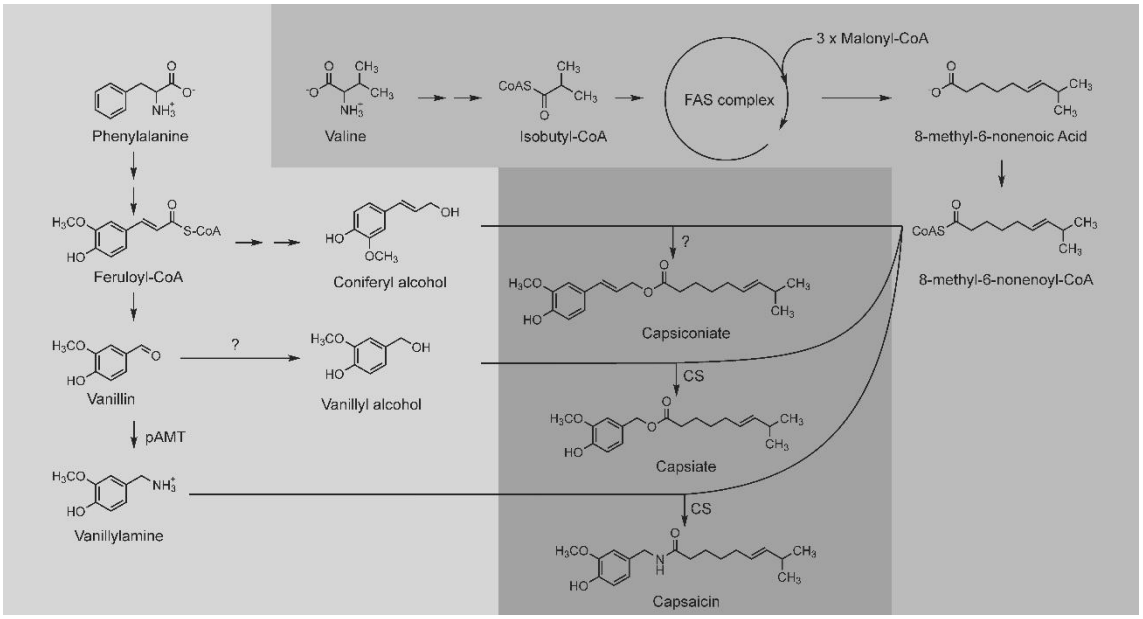

Figure XIII. Biosynthesis pathway of capsaicinoids and non-pungent analogues. pAMT- putative aminotransferase; CS: capsaicin synthase. (García *et al*., 2018)

The aliphatic chain of capsaicinoids is formed from the amino acids valine, leucine and isoleucine (Appendino, 2007; Mazourek *et al.*, 2009). The length of the chain, between 9 and 11 carbon atoms, and the number of insaturations determine the different types of capsaicinoids (Figure XIV) (Lourdes-Reyes-Escogido *et al.*, 2011). The most abundant capsaicinoids are capsaicin and dihydrocapsaicin which represent around 90% of the total (Aza-González *et al.*, 2011). Moreover modifications in the aliphatic chain length and the presence of double bonds have influence in pungency degree (Mazourek *et al.*, 2009).

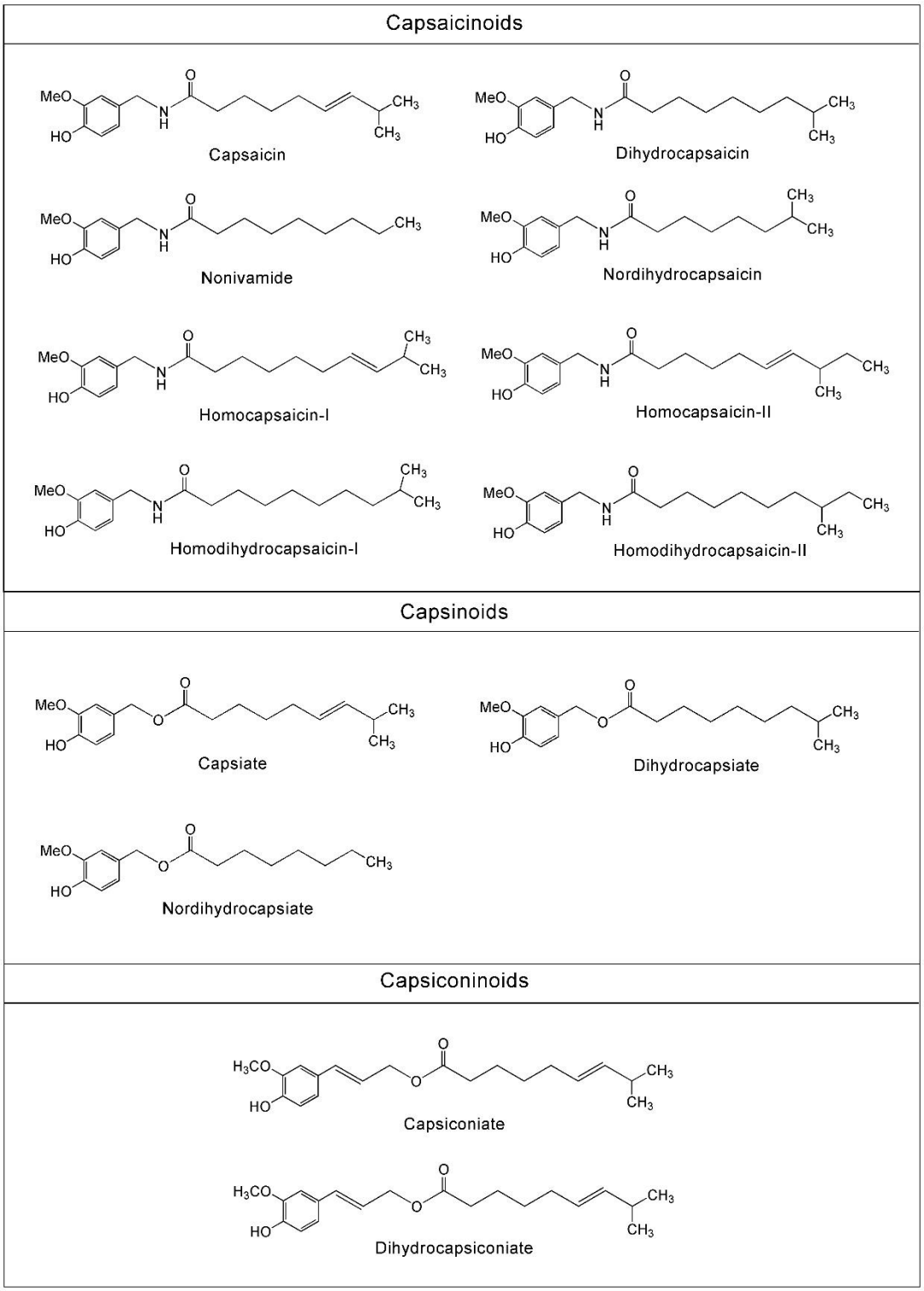

Figure XIV. Chemical structure of different types of capsaicinoids, capsinoids and capsiconinoids. This figure was adapted from Mazourek *et al*. (2009).

Finally the aromatic portion and aliphatic chain are condensed in one molecule by capsaicin synthase (CS). This enzyme specifically works on the aliphatic chain and needs Mg^{2+}, ATP and coenzyme A (CoA) (Lourdes-Reyes-Escogido *et al.*, 2011). CS is

poorly known because it has not been purified and characterized. However, the studies carried out by Stewart *et al.* (2005; 2007) showed that this enzyme is coded by *AT3* (acyltranferase 3) gene, namely Pungent gene 1 (*Pun1*). In fact, experiments carried out by Arce-Rodríguez & Ochoa-Alejo (2015) showed a correlation between expression of this gene and capsaicin accumulation being the concentration of this metabolite a negative regulator of *AT3* (Kim *et al*., 2009; Arce-Rodríguez & Ochoa-Alejo, 2015). Moreover, the silencing of *AT3* also affected the gene expression of *pAmt*, *BCAT* (branched-chain amino acid transferase), *Kas* (ketoacyl-ACP synthase) and *Acl* (acyl carrier protein) (Arce-Rodríguez & Ochoa-Alejo, 2015).

Capsaicinoids are synthesized in the endoplasmatic reticulum and, then, they are trasported in vesicles across the cytoplasm to be fused with plasmalemma. Cell cuticle separates from cell wall and form a blister that accumulates the capsaicinoids (Díaz *et al.*, 2004; Stewart *et al.*, 2007). At the end of ripening blisters can be observed on placental surface (Aza-González *et al.*, 2011). These structures only appear in pepper fruits with pungent genotype (Broderick & Cooke, 2009).

Normally the capsaicinoid accumulation starts ca. 14 days after blooming. Their levels remain low till 28 days after flowering and then rise till the end of ripening (Estrada *et al.*, 2000b).

XII.II. Properties and uses of capsaicinoids

For a long time it has been known that capsaicinoids have an antimicrobial activity (Billing & Sherman, 1998; Ceylan & Fung, 2004). In fact pungent pepper fruit show less infected seed with *Fusarium* than non-pungent fruits (Tewksbury *et al.*, 2008). Moreover it has been shown that capsaicinoids possess antimicrobial activity against 50 species of bacteria and *Candida albicans* (Cichewicz & Thorpe, 1996). Also it was observed that capsaicinoids affect insect although there are specialist insects, such as *Helicoverpa assulta* (tobacco budworm) that are able to feed on tobacco and pepper (Ahn *et al*., 2011). However, the growth of generalist insects decreased and the larvae mortality increased when they were feeding on a diet supplementary with capsaicin. Moreover, the injection of capsaicin in those insects produced abdomen paralysis and even self-cannibalism (Ahn *et al*., 2011).

As it was mentioned above, capsaicinoids and their analogues have two motifs that can influence the antimicrobial activity of these compounds (Veloso *et al.*, 2014b). Several studies have shown that the aromatic portion is able to alter the cell membrane and to provoke ion leakage resulting in loss of homeostasis and alteration of cellular respiration (Fitzgerald *et al.*, 2004). On the other hand the aliphatic chain is an analogue of alkamides, which are aliphatic amides, composed of unsaturated fatty acids. These compounds also have antimicrobial properties and their mode of action has been described as an interference of the enzymatic activity responsible for the synthesis of unsaturated fatty acids, which is a vital function of many microorganisms (Veloso *et al.*, 2014b).

Besides the antimicrobial activity of the two precursors, it has also been observed that the capsaicinoids are able to strongly inhibit the growth of *Bacillus subtilis* and to delay it in *Escherichia coli* and *Pseudomonas solanacearum* (Molina-Torres *et al*., 1999). In yeast, it has been observed that the application of capsaicin induces the response to osmotic stress and the synthesis of phosphatidylinositol and phosphatidylcholine, which are cell membrane components. This suggests that treatment with capsaicin causes osmotic stress and damage in the structure and fluidity of the cell membrane (Kurita *et al*., 2002). In fact Aranda *et al*. (1995) found that capsaicin molecule was intercalated between the acyl chains of the membrane phospholipids and thus disturbed its behaviour.

In addition to this antimicrobial activity, capsaicinoids are also able to induce systemic resistance in Arabidopsis against *Pseudomonas syringae* and *Pectobacterium caratovorum* (Song *et al*., 2013), and in pepper against *V. daliae* and *B. cinerea* (Veloso *et al.*, 2014b). The reduction in symptoms is accompanied by the induction of defences related to SA and JA in Arabidopsis and synthesis of capsidiol, glucanases and chitinases in *C. annuum* (Song *et al.*, 2013; Veloso *et al.*, 2014b).

Despite its great benefits, toxicological studies showed that capsaicinoids have low selectivity and high toxicity, which limits its application both at the clinical (Arora *et al*., 2011) and agriculture level. For this reason capsinoids have caused great interest as substitutes for capsaicinoids. Capsinoids are non-pungent and therefore are less toxic, but have the other characteristics of the capsaicinoids (Luo *et al.*, 2011). Thus they would be good candidates as substitutes of capsaicinoids.

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OBJECTIVES

There are few studies on the effect of capsinoids in plants. Since the tests performed with animal cells treated with these compounds show a similar effect than capsaicinoids it is also expected a similar effect in plants. Nevertheless, plant and animal cells respond differently to stimuli. Therefore, the present thesis addresses the study of the non-pungent capsinoid, vanillyl nonanoate (VNT) to determine its protective effect and the mechanism of action in pepper and Arabidopsis plants. Also this thesis quantifies the content in capsinoids in several pepper ecotypes from Galicia. The objectives of this work were the following:

- To quantify the content in capsinoids and capsicinoids in fruits of several Galician pepper ecotypes (Chapter 1).
- To determine the antimicrobial activity of vanillyl nonanoate and its two precursors, vanillyl alcohol and nonanoic acid (Chapter 2).
- To determine the protection obtained by VNT at the place of application (local level) (Chapter 2).
- To determine the VNT-induced systemic resistance against *Phytophthora capsici*, *Botrytis cinerea* and *Verticillium dahliae* in Padrón pepper (Chapter 3).
- To characterize the VNT-induced systemic resistance in pepper by quantifying the cell wall lignification, the pathogenesis-related protein 1 (PR1), the phytoalexin capsidiol, the β-1,3-glucanase and chitinase activities (Chapter 3).
- To elucidate the signals involved in the resistance induced by vanillyl nonanoate in Padrón pepper against *Phytophthora capsici* and *Botrytis cinerea* (Chapter 4).
- To determine the VNT-induced systemic resistance against *Botrytis cinerea* in *Arabidopsis thaliana* (Chapter 5).
- To characterize the VNT-induced systemic resistance in *Arabidopsis thaliana* by quantifying the cell wall lignification, biochemical defences and different signalling hormones, as wel as testing knock-out mutatns (Chapter 5).

CHAPTER 1.

Quantification of the capsinoid and capsicinoid content in fruits of several Galician pepper ecotypes.

1.1. Introduction

Pepper plants were introduced in Spain in 1493 after the first Columbus voyage to America. After that, pepper seeds were spread throughout Spain and later to the rest of Europe (Casal, 2010).

In Galicia the introduction of pepper is dated in the 17th century when Franciscan monks took the seeds to Galicia. After that, farmers from different locations carried out a selective process by plant breeding until they obtained the present ecotypes (Taboada *et al*., 2010). Nowadays, there are mainly five Galician ecotypes which can be classified in short fruit and in mid or long fruit. Ecotypes Padrón and Couto are included in the first group and ecotypes Arnoia, Branco Rosal and Oímbra belong to the second group. In addition to the most produced ecotypes there are other varieties like Mougán, Piñeira, Punxín, Vilanova and Couto Grande (Taboada *et al*., 2010).

Galician varieties are known among consumers due to their organoleptic properties. These varieties have been characterized by their nutritional components, being the content in capsaicinoids one method to classify the cultivars. According to the content in capsaicinoids, there are pungent and non-pungent cultivars. However, the content in capsinoids, non-pungent analogues of capsaicinoids, has not been measured for Galician ecotypes. Capsinoids have shown the same pharmacological properties as capsaicinoids except pungency (Lourdes-Reyes-Escogido *et al*., 2011). Therefore, the knowledge of capsinoid content could be and additional proof of the nutritional value of Galician pepper cultivars. For this reason the aim of this chapter is to quantify the content of capsinoids in the diferents Galician ecotypes. Following, there is a description of the cultivars used to perform the measurement of capsinoids.

1.1.1. Ecotype Padrón

Padrón is the most known among Galician ecotypes and is recognized as Protected Designation of Origin “Pemento de Herbón” (Commission Regulation (EU) No 700/2010, 2010; Taboada *et al*., 2010). The main production areas are Padrón, Dodro and Rois in the province of A Coruña and Pontecesures and Valga in the province of Pontevedra (Figure 1.1.A) (Council Regulation (EC) No 510/2006, 2009).

The fruit of this ecotype is short (3.5 to 6 cm of length) and 1.5 to 2 cm of maximum diameter. Its longitudinal section is shaped as a cone or a truncated cone, apex sunken between clearly defined 3 or 4 lobes and fruit wall is thin (approximately 1.5 mm) (Figure 1.1.B). The fruit is hanging and its size and shape are variable. In addition the fruit of these ecotypes has moderate aroma intensity, sweet flavour and sometimes mildly spiciness due to the presence of capsaicin (Council Regulation (EC) No 510/2006, 2009).

Normally, the fruit is consumed in immature stage (Taboada *et al*., 2010).

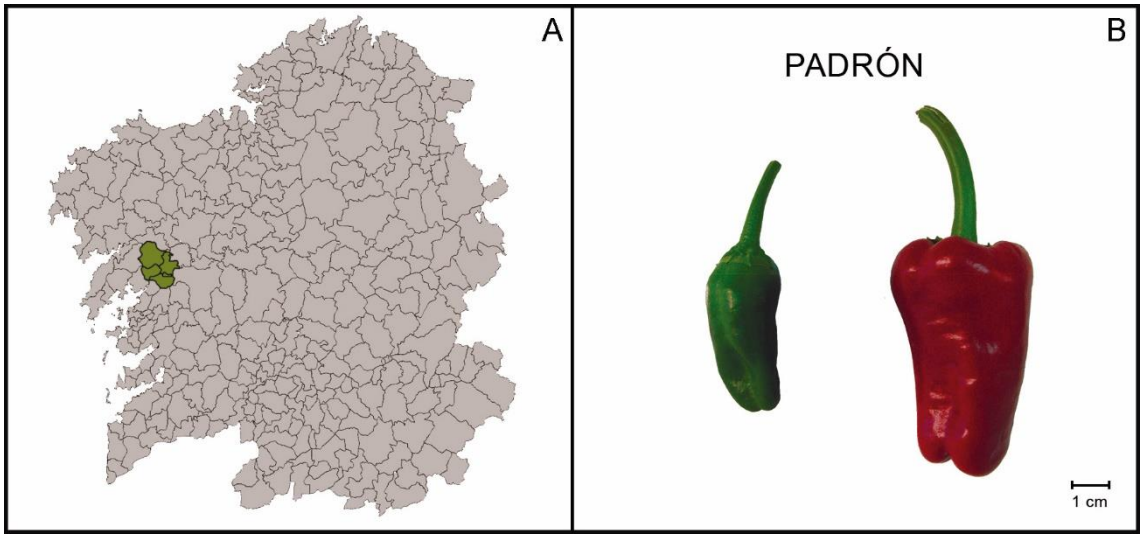

Figure 1.1. Ecotype Padrón. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.1.2. Ecotype Couto

This ecotype has got the recognition of Protected Geographical Indication "Pemento do Couto". The name "O Couto" corresponds with the location of the monastery that began the breeding and cultivation of this ecotype (Commission Regulation (EU) No 147/2010, 2010).

The producing area is located in the region of Ferrol that includes municipalities of Ferrol, Narón, Valdoviño, Cedeira, Moeche, As Somozas, San Sadurniño, Neda, Fene, Mugardos and Ares (Figure 1.2.A) (Council Regulation (EC) No 510/2006, 2009).

The aspect of this ecotype is very similar to ecotype Padrón. The longitudinal section is trapezoid truncated conical and the cross-section is round. The fruit wall is thin (approximately 1 to 1.5 mm). Normally, the fruit length is 4 to 8 cm and the fruit width is 2 cm. The fruit stalk is shorter than the fruit itself, stiff and straight or slightly curved that makes the fruit to grow erect (Figure 1.2.B) (Council Regulation (EC) No 510/2006, 2009). Besides, this ecotype has fine-textured juicy flesh, sweet flavour, slightly herbaceous with no pungency due to the absence of capsaicin, with a moderately strong aroma and few seeds (Council Regulation (EC) No 510/2006, 2009).

The fruit is consumed in immature stage (Taboada *et al*., 2010).

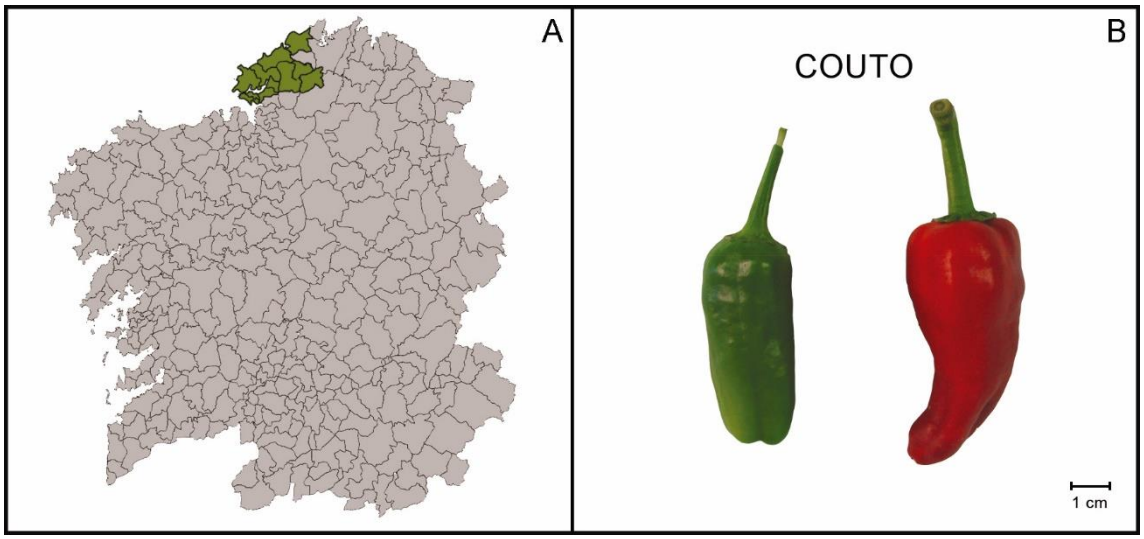

Figure 1.2. Ecotype Couto. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.1.3. Ecotype Arnoia

This ecotype has got the recognition of Protected Geographical Indication "Pemento de Arnoia" (Commission Regulation (EU) No 444/2010, 2010).

The producing area is located in the region of O Ribeiro which include the municipality of A Arnoia and the parish of Meréns in the Municipality of Cortegada (Figure 1.3.A) (Council Regulation (EC) No 510/2006, 2009).

The fruit of this variety presents mid-long size having 7.5 cm to 11 cm of length and 5 cm to 7 cm of width. Its shape is conical or bell-shaped, with three or four lobes and four grooves and a varying number of well-defined longitudinal septa. The apex is

cleft or rounded. The skin is light green in colour; smooth and bright (Figure 1.3.B). The longitudinal section is trapezoidal and fruit wall is 2.6 to 7.7 mm (Council Regulation (EC) No 510/2006, 2009).

Arnoia pepper fruit possesses an intense smell, sweet flavour and very low pungency (Council Regulation (EC) No 510/2006, 2009).

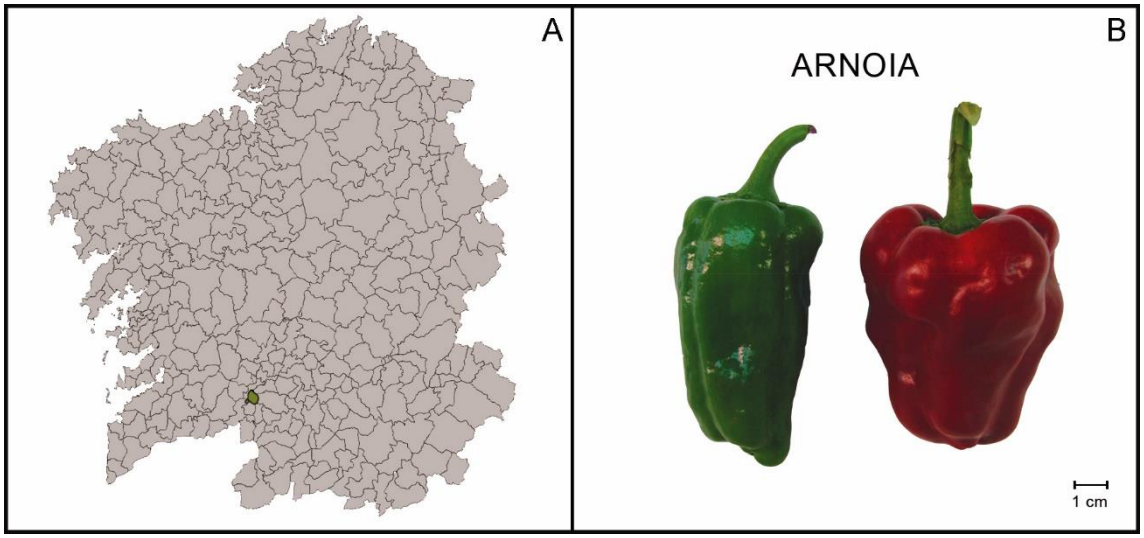

Figure 1.3. Ecotype Arnoia. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.1.4. Ecotype Branco Rosal

The production area is located in region of Baixo Miño (Figure 1.4.A) (Taboada *et al*., 2010).

The fruit has long size, pointed, and with light green peel in immature stage which became orange in mature stage (Figure 1.4.B). The longitudinal section is triangular and fruit wall is intermediate (Taboada *et al*., 2010).

1.1.5. Ecotype Oimbra

This ecotype has got the recognition of Protected Geographical Indication "Pemento de Oimbra" (Commission Regulation (EU) No 429/2010, 2010).

The producing area encompasses the district of Verín in the province of Ourense which include the municipalities of Oímbra, Verín, Castrelo do Val, Monterrei,

Cualedro, Laza, Riós and Vilardevós (Figure 1.5.A) (Council Regulation (EC) No 510/2006).

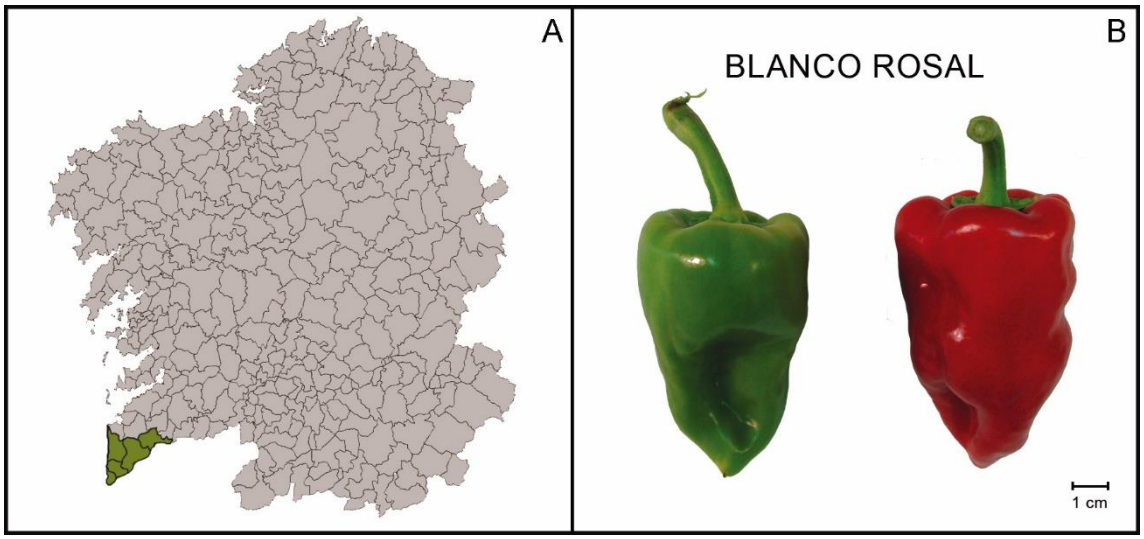

Figure 1.4. Ecotype Blanco Rosal. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

The fruit of this variety has big size with 10 cm to 20 cm of length and from 6 cm to 8 cm in width at the base. Its shape is regular, elongate, with a single lobe and three or four grooves with no marked vein. The apex is pointed or round. The peel is smooth, shiny and light green to almost yellow in immature stage becoming red in ripe stage (Figure 1.5.B). The fruit wall is 6 mm to 8 mm thick (Council Regulation (EC) No 510/2006).

This ecotype does not possess capsaicin in ideal growth conditions (Taboada *et al.*, 2010).

1.1.6. Ecotype Mougán

This ecotype has got the recognition of Protected Geographical Indication "Pemento de Mougán" (Commission Implementing Regulation (EU) No 1199/2014, 2014).

The producing area encompasses the whole municipality of Guntín in province of Lugo (Figure 1.6.A) (Council Regulation (EC) No 510/2006, 2014).

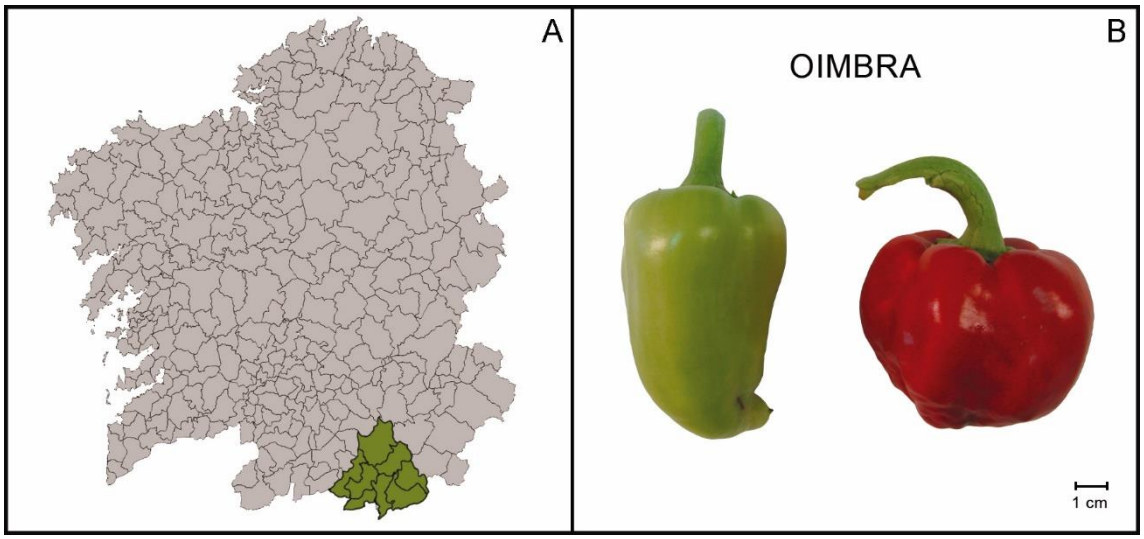

Figure 1.5. Ecotype Oimbra. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

The fruit of this variety has short size with 3 to 6.5 cm long and 2.5 to 4 cm wide. The longitudinal section is square and the cross-section slightly grooved with three or four ridges at the tip. The stalk is always shorter than the fruit, measuring 2 to 5 cm, and it is rigid and curved. The skin is dark, glossy green turning into dark red after ripening (Figure 1.6.B). Fruit wall is very thin (1.5 mm) (Taboada *et al*., 2010; Council Regulation (EC) No 510/2006, 2014).

Fruits are pungent because of their high amount of capsaicin (Taboada *et al*., 2010).

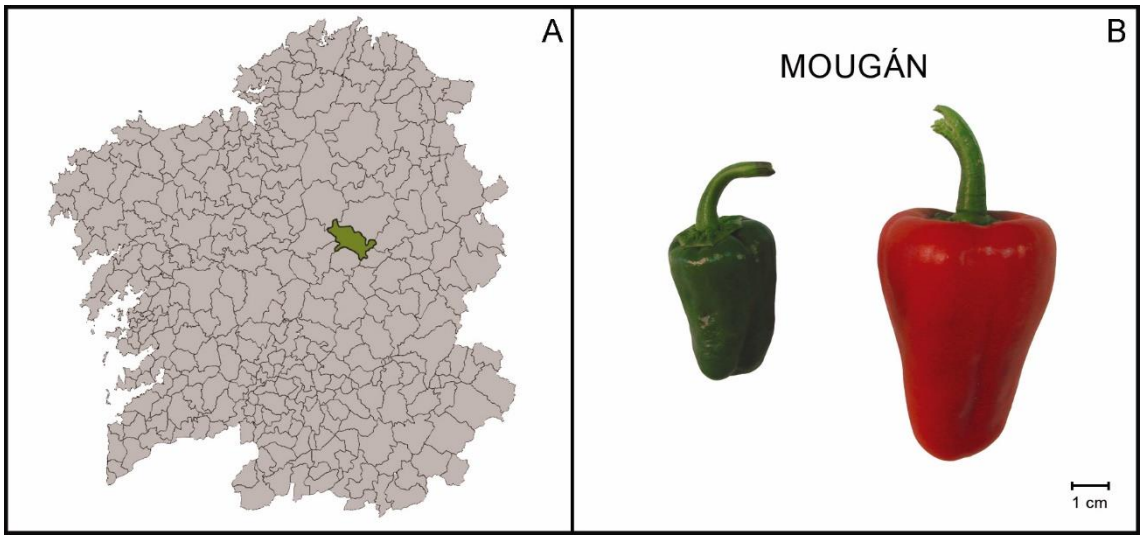

Figure 1.6. Ecotype Mougán. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.1.7. Ecotype Piñeira

The production of fruits from this ecotype is focused in the area of Piñeira (Figure 1.7.A) (Taboada *et al*., 2010).

Their fruits are big, squared and ended in 4 lobes. The skin is dark green in immature stage and red in ripe stage (Figure 1.7.B). Fruit wall is very thick (Taboada *et al*., 2010).

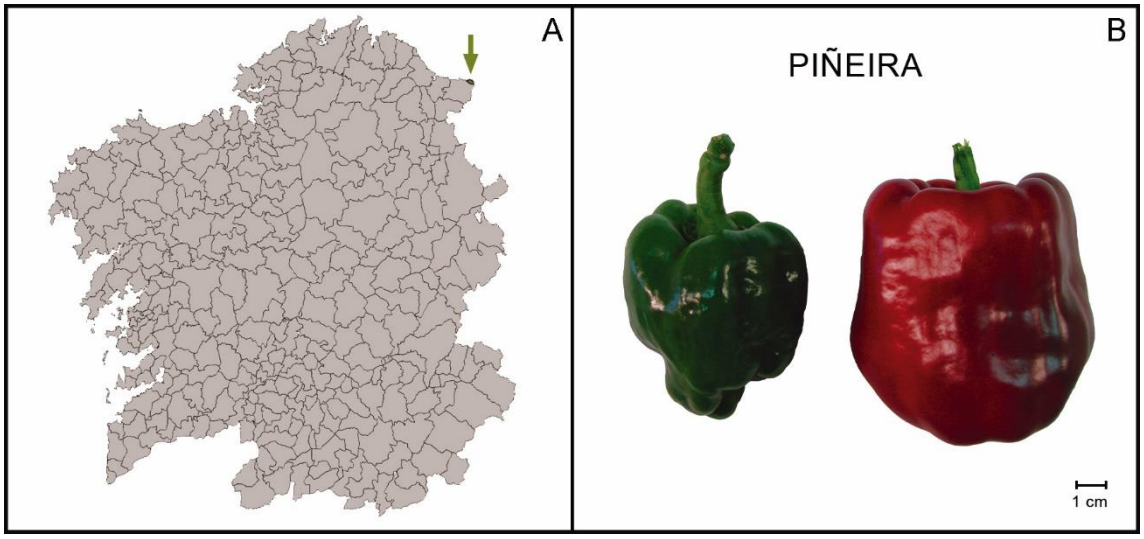

Figure 1.7. Ecotype Piñeira. A: main production area in Galicia. Green arrow points out the location in the map; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.1.8. Ecotype Punxín

The production area of this ecotype is mainly the region of Ribeiro (Figure 1.8.A) (Taboada *et al*., 2010).

The fruit size of this ecotype is medium. Its shape is squared and pointed. The skin is bright green in immature stage and red at the end of its development (Figure 1.8.B). Fruit wall is intermediate (Taboada *et al*., 2010).

1.1.9. Ecotype Vilanova

The production area of this ecotype is in the region of Vilanova de Arousa (Figure 1.9.A) (Taboada *et al*., 2010).

The fruit size is big and the shape is squared with 4 lobes. The skin is dark green in immature stage and dark red at the end of its development (Figure 1.9.B). Fruit wall is thick (Taboada *et al*., 2010).

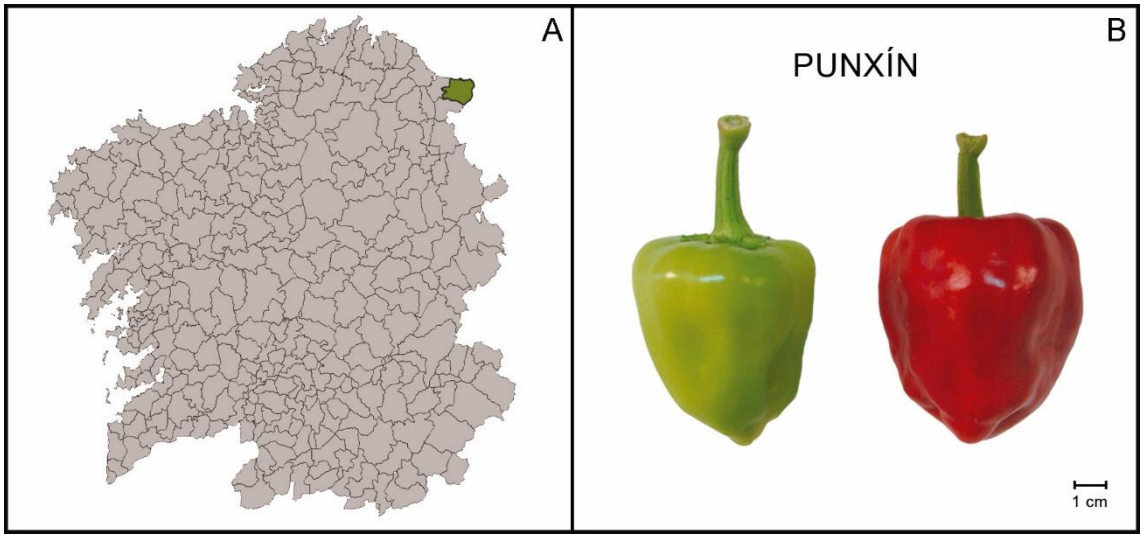

Figure 1.8. Ecotype Punxín. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

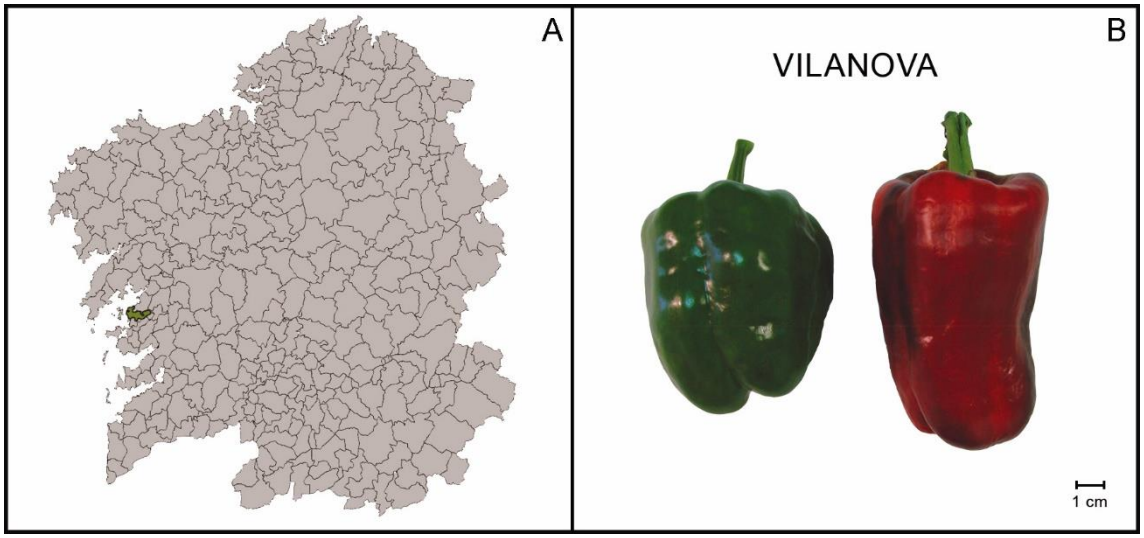

Figure 1.9. Ecotype Vilanova. A: main production area in Galicia; B: Aspect of the fruits in immature state (left) and ripe state (right).

1.1.10. Ecotype Couto Grande

The production area of this ecotype is mainly in region of Ferrol (Figure 1.10.A) (Taboada *et al*., 2010).

The fruit size of this ecotype is big and its shape is square. The peel is green in immature stage and red in ripe stage (Figure 24B). Fruit wall is thick (Taboada *et al*., 2010).

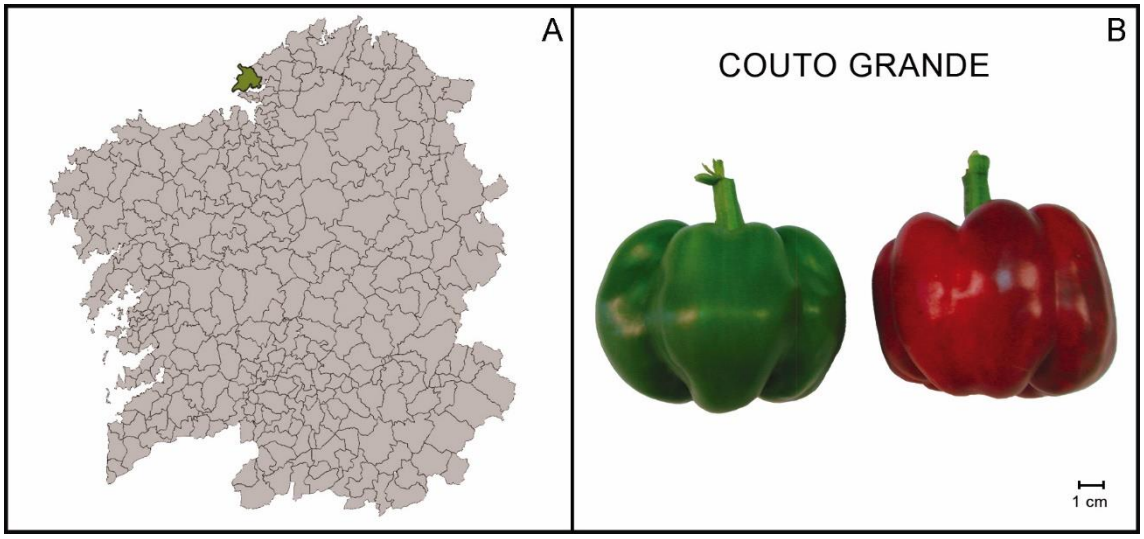

Figure 1.10. Ecotype Couto Grande. A: main production area in Galicia; B: Aspect of the fruits in immature stage (left) and ripe stage (right).

1.2. Material and methods

1.2.1. Fruit material

In this study the main Galician cultivars of pepper were selected to quantify their content in capsinoids. Capsaicinoids also were included in the experiment as controls of the process. The selected cultivars were Padrón, Couto, Arnoia, Branco Rosal, Oimbra, Mougan, Piñeira, Punxin, Vilanova and Couto grande.

The work of Estrada *et al*. (2000) showed a variation in capsaicinoids content during pepper development. Based on this, capsaicinoids content as well as its analogues content was measured in immature and ripe fruits from each cultivar. Before

processing the fruit, pictures of a couple of pepper fruits were taken for each cultivar and were showed previously in the introduction.

All the analysed fruits were collected in plantations of the research centrum "Centro de Investigaciones Agrarias de Mabegondo" (Galicia, Spain).

On the other hand, all the documents with the description of ecotypes designated as Protected Designation of Origin or as Protected Geographical Indication take into account the presence or absence of capsaicinoids in the commercial stage. In order to know the capsaicinoids content of two ecotypes typically sold in supermarkets of A Coruña city, three different bags of different commercial Padrón pepper and Couto pepper were bought in supermarkets randomly selected. Each pepper bag belongs to a different commercial brand. Then, to guarantee the anonymity of those commercial brands, the samples were named as Padrón 1, Padrón 2, Padrón 3, Couto 1, Couto 2, and Couto 3.

1.2.2. Extraction of capsaicinoids and capsinoids

Firstly whole peppers were lyophilized and stored in a desiccator until they were analysed. Then they were homogenized using an Ultra-turrax at room temperature. 4 ml of acetone were added to 2 mg of powder from the homogenate. Then the mixture was incubated with stirring for 15 min and then filtered. Subsequently, a second incubation with 1 ml of ethyl acetate was carried out with the precipitate for 15 min. The supernatants of both incubations were mixed and evaporated till dryness on a rotatory evaporator at 36ºC with pressure between 240 and 556 mbar.

Once evaporated, samples were dissolved in 2 ml of ethyl acetate, filtered and stored at -80ºC untill they were analysed by high performance liquid chromatograph (HPLC).

1.2.3. Determination of capsaicinoids and capsinoids by HPLC-UV

The content of capsaicinoids and their analogues, specifically the capsinoids, were separated by HPLC and quantify with a PDA (photo diode array) detector. This analysis was carried out by Dr. Gerardo Fernández Martínez from Chromatographic Techniques Unit of the Research Support Services (SAI) from Universidade da Coruña.

The separation of different compounds was made using a Phenomenex Luna C18 column of 150 x 4.6 mm x 5 µm. The detection of the compounds was performed using PDA detector scanning between 220 nm and 250 nm. The quantification was carried out at 279 nm.

The mobile phase was an isocratic gradient formed by two solvents: A: pure water and B: pure methanol.

Time (min)	Solvent A	Solvent B
0	70%	30%
1	70%	30%
21	0%	100%
26	0%	100%
31	70%	30%

The components analysed are listed in table 1.1 as well as their retention time. Moreover the chromatograms and the absorption spectra for each standard are showed in figure 1.11 and 1.12, respectively.

Table 1.1. Retention time for synthetic standards used in this work.

Synthetic standard	Type of analogue	Retention time (min)
Capsaicin	capsaicinoid	5.18
Dihydrocapsaicin	capsaicinoid	6.82
Capsiate	capsinoid	15.57
Vanillyl nonanoate	capsinoid	16.06
Dihydrocapsiate	capsinoid	17.62

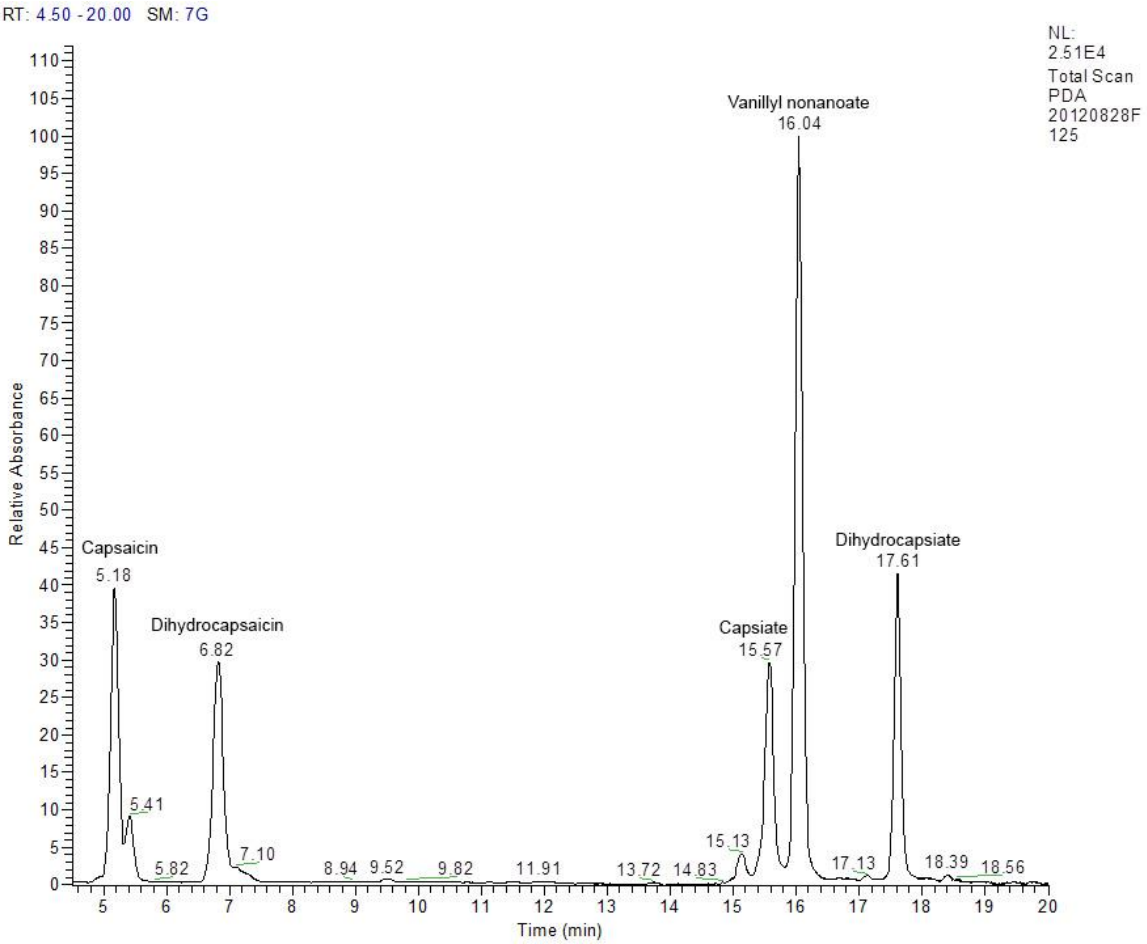

Figure 1.11. Chromatogram of all synthetic standards used in this study ordered by its retention time.

1.2.4. Determination of capsaicinoids and capsinoids by HPLC-MS

The content of capsaicinoids and capsinoids in commercial Herbón peppers and Couto peppers were separated by HPLC and quantified by mass-spectrometry. This analysis was carried out by Dr. Gerardo Fernández Martínez from Chromatographic Techniques Unit of the Research Support Services (SAI) from Universidade da Coruña.

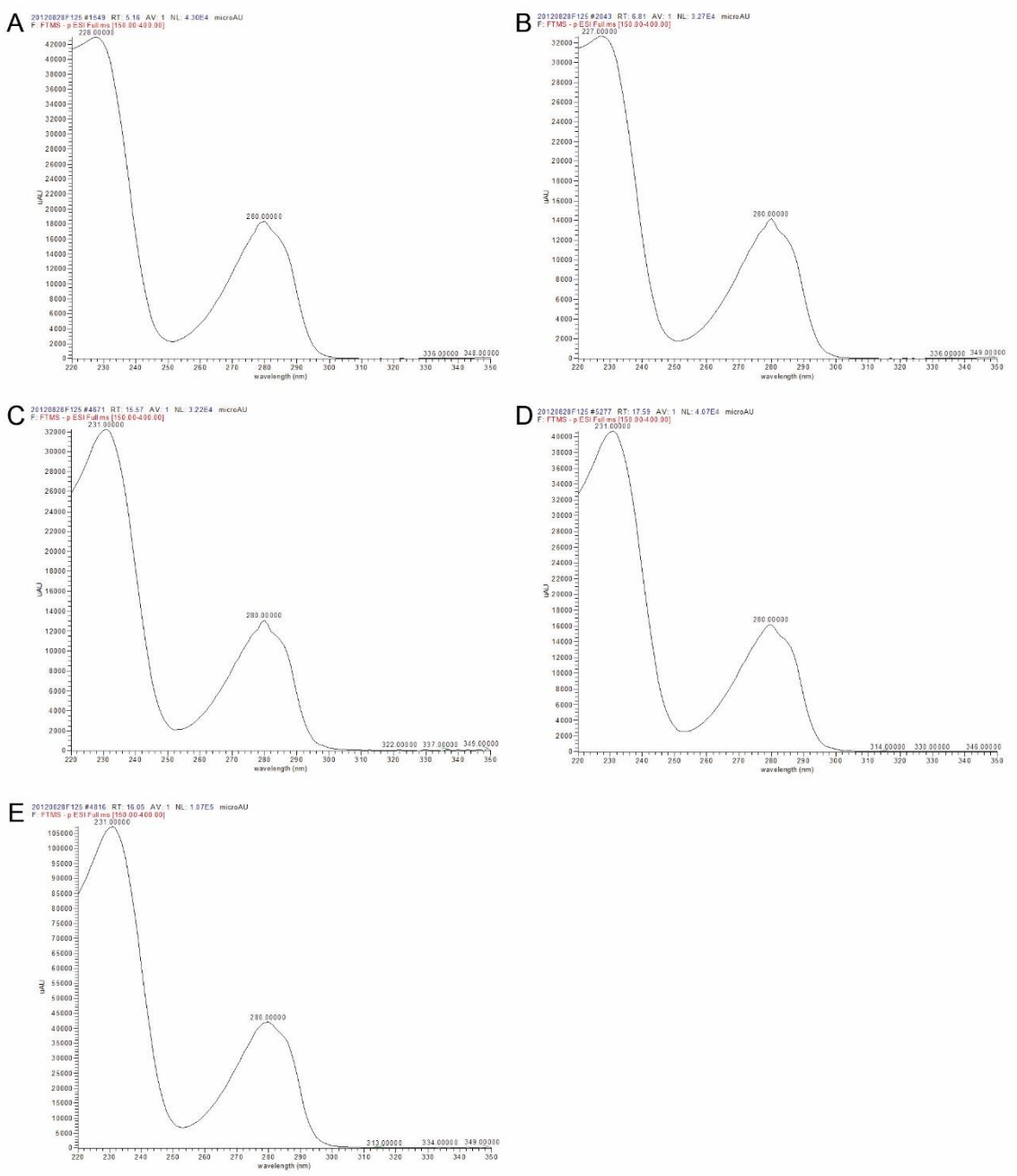

Figure 1.12. Absorption spectra for each standard (capsaicin (A), dihydrocapsaicin (B), capsiate (C), dihydrocapsiate (D) and vanillyl nonanoate (E)) used in this study.

The separation of different compounds was made using a Columna Kinetex 2.6u XB-C18 100A 100x2.10mm. The column temperature was 40ºC. The mobile phase was an isocratic gradient formed by two solvents: A: water acidified with 0.1% formic acid and C: acetonitrile acidified with 0.1% formic acid. The flow of mobile phase was 200 µl/min.

Time (min)	Solvent A	Solvent C
0	70%	30%
1	70%	30%
21	0%	100%
26	0%	100%
27	70%	30%
31	70%	30%

The components analysed are listed in table 1.2 as well as their retention time. 20 µl of each standard and of each sample was injected in the HPLC. Chromatograms of each standard are shown in figure 1.13. After that, samples were ionized by with an electrospray operating in positive mode. The scan range was 100-500 uma. The electrospray source conditions were ionspray voltage of 4kV, 300ºC of capillary temperature, 40 V of capillary voltage and 125 V of tube lens.

Table 1.2. Synthetic standards and their retention time used in samples of commercial peppers.

Synthetic standard	Retention time (min)
Capsaicin	9.28
Dihydrocapsaicin	10.47
Capsiate	n.i.
Dihydrocapsiate	n.i.

n.i.: not ionized

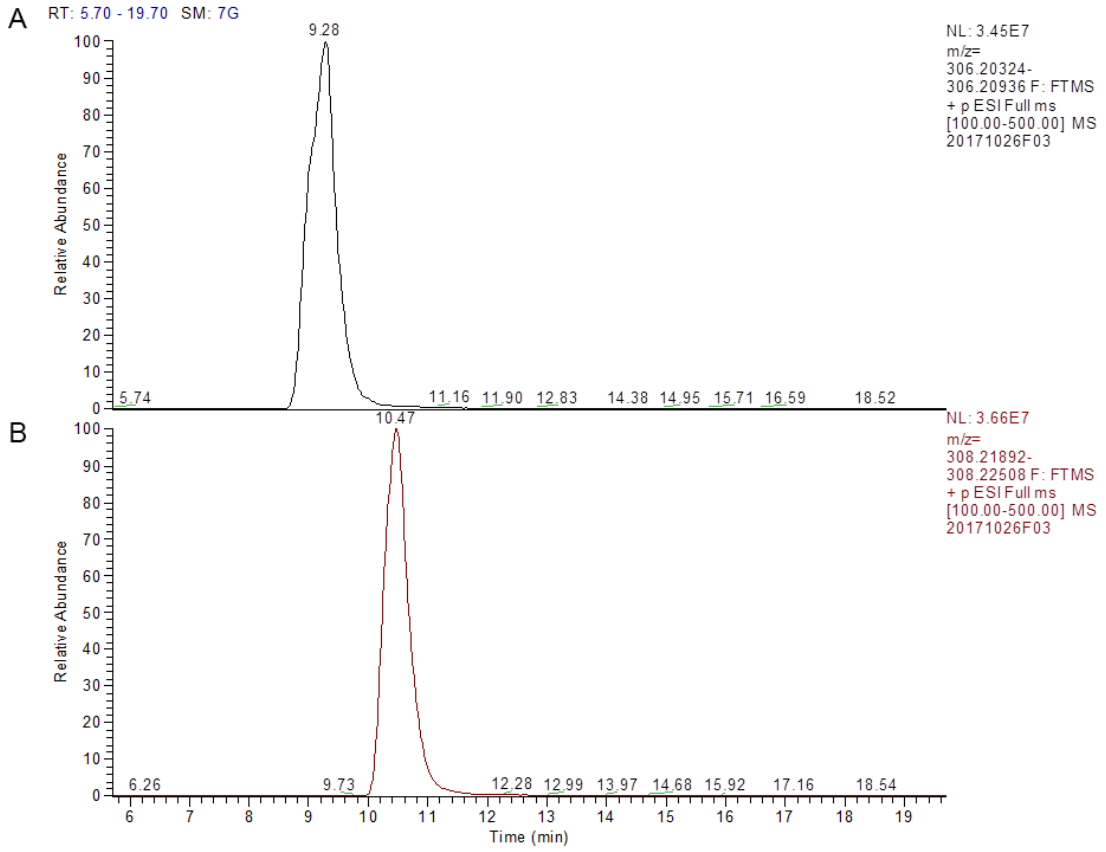

Figure 1.13. Chromatogram of the synthetic standards of capsaicin (A) and of dihydrocapsaicin (B) used to quantify the capsaicinoids content in commercial peppers, Padrón peppers and Couto peppers.

1.3. Results

The capsaicinoids and capsinoids content was analysed in ten Galician varieties of pepper in immature and mature stages.

Dihydrocapsaicin, capsiate and dihydrocapsiate could not be detected in any of the 10 pepper cultivars. Only the capsaicin was detected in varieties Padrón and Mougán in both ripe and immature stages. Mougán showed the highest capsaicin content (Table 1.3).

Estrada *et al*. (2000) detected an increase in capsaicinoids content with fruit development but we only observed a slight decrease in capsaicin in Padrón variety but no differences were observed in Mougán (Table 1.3).

Table 1.3. Capsaicin content detected in analysed Galician pepper.

Variety	Ripening state	Capsaicin (mg/g dry weight)
Padrón	Immature	0.50
	Ripe	0.40
Mougán	Immature	0.65
	Ripe	0.64

In order to further compare the capsaicinoids and capsinoids content among the different varieties of Galician peppers, we have done a similar process between commercial ecotypes Padrón and Couto. The results of these studies are shown in table 1.4. The content in capsinoids could not be measured because the synthetic standards, capsiate and dihydrocapsiate, did not ionize. Both capsaicin and dihydrocapsaicin synthetic standards did ionize but they could only be detected in Padrón. Padrón contained capsaicin and dihydocapsaicin but Couto did not.

Table 1.4. Capsaicin and dihydrocapsaicin content detected in commercial pepper of Padrón and Couto.

Variety	Capsaicin (µg/g dry weight)	Dihydocapsaicin (µg/g dry weight)
Padrón1	0.06	0.35
Padrón 2	4.39	4.96
Padrón 3	3.75	2.55
Couto 1	n.d	n.d
Couto 2	n.d	n.d
Couto 3	n.d	n.d

n.d.: not detected

1.4. Discussion

In this study we characterised the content of capsinoids in the most important Galician pepper ecotypes. Internal standards of the capsinoids, capsiate, dihydrocapsiate and vanillyl nonanoate were included, as well as capsaicin and dihydrocapsaicin as standards for capsaicinoids. The latter were added because pungent cultivars also synthesize capsinoids but in trace amounts (Han *et al*., 2013). In our samples, capsinoids were no detected. Similar results were obtained by Silvar & García-González (2017) and Coutinho *et al*. (2015). The samples of ten varieties of Galician peppers analysed did not contain any capsinoid or they are under the threshold of detection.

Capsinoids are present in *C. annuum*. It is possible to detect and quantify capsinoids in different pepper cultivars and species such as CH-19 Sweet (*C. annuum*), Himo (*C. annuum*), Zavory hot (*C. chinense*), Ají Dulce strain 2 (*C. chinense*) or Belize Sweet (*C. chinense*) (Kobata *et al*., 1998; Singh *et al*., 2009; Tanaka *et al*., 2009, 2010a, 2010b). Tanaka *et al*. (2009) analysed 35 pepper cultivars bellowing to 5 different species: *C. annuum, C. chinense, C. baccatum, C. pubecens* and *C. frutescens*. Tanaka *et al*. (2009) detected the highest content in capsinoids in the cultivar CH-19 Sweet, 5632.5 µg/g DW. On the other hand, other cultivars as Shima from the species *C. pubescens* and Charapita from the species *C. chinense* also had high amounts of capsinoids, 2056.6 µg/g DW and 1801.9 µg/g DW respectively.

In addition to capsaicinoids and capsinoids, pepper fruits can have other non-pungent analogues which are called capsiconinoids. The aromatic portion of these compounds derivates from coniferyl alcohol and they were found in *C. baccatum var. praetermissum* (Kobata *et al*., 2008). Tanaka *et al*. (2009) observed that some cultivars without capsinoids had capsiconinoids. This situation could explain the absence of capsinoids in our samples. Moreover, capsiconinoids have also been associated with pungent cultivars and they shared a similar pattern of capsaicinoid accumulation during fruit development (Tanaka *et al*., 2009). These could help us to find the best moment to collect fruits and to measure capsiconinoids. In fact, the consumption of both capsinoids and capsiconinoids is recommended in a diet. Thus, if Galician ecotypes of pepper had those compounds, their gastronomic interest would increase.

Capsaicinoids were detected and quantified in ecotypes Padrón and Mougán which are typically pungent Galician ecotypes. It has been already shown that Mougan cultivar is more pungent than Padrón cultivar (Taboada *et al*., 2010). Our results confirm the higher content of capsaicin in Mougan, although the difference is not so big as expected. In general, the growing conditions, such as temperature, light, water or fertilization, can alter the amount of capsaicin in fruits (Estrada *et al*., 1998, Estrada *et al*., 1999). We have observed the impact of growing conditions when we have analysed three replicates of commercial Padron pepper due to the different origin of production of each of the three samples. Even trying to keep controlled some of those parameters, it is possible that there was an alteration in levels of capsaicinoids. Bogusz *et al*., (2018) measured capsaicin and dihydrocapsaicin in fruits of *C. frutescens, C. chinense* and *C. baccatum* collected in two consecutive years. They observed changes depending on the collection year for both compounds (Bogusz *et al*., 2018).

In addition, levels of capsaicinoids vary in the fruits in the same maturity stage from a single plant (Kirschbaum-Titze *et al*., 2002; Mueller-Seitz *et al*., 2008). Owing to these variations in capsaicinoids content, the way of cultivation is important to keep the organoleptic properties of a specific ecotype. Moreover, a particular range can determine if a fruit belongs to certain ecotype. For example, D.O.P Herbón pepper establishes a maximum of capsaicinoids (0,114 mg/g DW) for the fruits in commercial stage (Council Regulation (EC) No 510/2006, 2009). None of our commercial samples reached that level.

Another parameter that can alter the level of these pungent compounds and their analogues is the moment of fruit harvest. In general, although the exact timing varies among cultivars, fruits start to accumulate capsaicinoids around 10-14 days after flowering although their levels are kept low. Then capsaicinoids levels moderately rise after 28 days, finding the highest amount 40-50 days after flowering. After that, they start to decrease (Contreras-Padilla & Yahia, 1998; Estrada *et al*., 2000; Barbero *et al*., 2014). A similar pattern was found for capsinoids and capsiconinoids (Yazawa *et al*., 1989; Tanaka *et al*., 2009; Tanaka, 2014). Therefore the levels of capsaicinoids vary depending on the moment of fruit harvest. In our samples, this ripening-dependent variation in capsaicinoids was absent in Mougan. In Padrón the level of capsaicin was moderately higher in immature fruit. This trend in decreased capsaicinoids in the ripe fruit is inverted in other Capsicum species. Bogusz *et al*., (2018) observed an increase in

both capsaicin and dihydrocapsaicin in *C. frutescens*, *C. chinense* and *C. baccatum* during fruit development.

In conclusion, the Galician pepper fruits analysed in immature stage and ripe stage did not have the capsinoids selected but capsaicinoids were detected in Padrón and Mougan. More analysis should be done to determine if the Galician pepper fruits could have capsiconinoids or only capsaicinoids.

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CHAPTER 2.

Properties of vanillyl nonanoate for protection of pepper plants against two pathogens.

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2.1. Introduction

Since agriculture began, farmers have had to cope with diseases caused by pathogens such as bacteria, virus or fungus. Controlling the impact of these attacks implies the use of preventive or therapeutic strategies (Maloy, 2005). The first group includes all those methods used before pathogen infection as, for example, establishment of quarantines of plants, fruits or natural materials. In the therapeutic strategies are grouped all those mechanism applied after a pathogen has infected the plant, being chemical control (fungicides) the most used (Maloy, 2005). However, the massive use of these compounds leads to the appearance of resistant isolates. This is the case of metalaxyl and mefenoxam, chemicals that are used worldwide in Phytophthora disease management (Parra & Ristaino, 2001). A similar situation can be found with fungicides used to control Botrytis (Fernández-Ortuño *et al*., 2015). Moreover, the latter is considered a "high risk" pathogen owing to its capacity to quickly develop resistance to the majority of fungicide classes (Konstantinou *et al*., 2015). Therefore, it is necessary to look for new effective compounds against these pathogens and particularly interesting is the development of products with less environmental impact. One option is the search of new fungicides based in molecules present in plants during their development or involved in plant-pathogen interaction. These compounds can be of plant origin, pathogen origin or even a result of the interaction of both (Oliveira *et al*., 2016). These compounds can have an antimicrobial effect, but they can induce plant defences instead, or show both properties.

In pepper fruits secondary metabolites were discovered to be related to both pungent sensation and reduction in Fusarium seed infection (Tewksbury *et al*. 2008). Moreover, the antimicrobial activity of those pungent pepper compounds has been known for a long time (Billing & Sherman, 1998; Ceylan & Fung, 2004; Cichewicz & Thorpe, 1996). These compounds are the so-called capsaicinoids and they are synthetized in the placental tissue of pepper fruits. At the structural level, in these compounds there are both an aromatic ring and an aliphatic chain. The aromatic moiety is formed by multiple transformations of phenylalanine via phenylpropanoid pathway and the aliphatic chain is originated from amino acids valine, isoleucine or leucine (Figure XIII, General Introduction).

Each part of the molecule shows antimicrobial activity, but it has also been reported that the molecule itself is able to strongly inhibit the growth of *Bacillus subtilis*

and delay it in *Escherichia coli* and *Pseudomonas solanacearum* (Molina-Torres *et al*., 1999). In yeast, the application of capsaicin induces response of osmotic stress genes and phosphatidylinositol and phosphatidylcholine synthesis, which are cell membrane components. This suggests that treatment with capsaicin causes osmotic stress and damage in the structure and fluidity of the cell membrane (Kurita *et al*., 2002). In fact, Aranda *et al*. (1995) found that capsaicin molecule was intercalated between the acyl chains of the membrane phospholipids and thus disturbed its behaviour.

Capsaicinoids also have demonstrated to possess antifungal activity that could be useful in the control of plant pathogens. Several fungi reduce their growth in presence of capsaicinoids, e.g. *Fusarium* sp., *Verticillium dahliae*, *P. capsici* and *B. cinerea* (Tewksbury *et al*., 2008; Veloso *et al*., 2014). Moreover, capsaicinoids have been reported to be able to induce resistance against plant pathogens (Song *et al*., 2013; Veloso *et al*., 2014). However, the pungency of capsaicinoids prevents their practical use as pesticides. Despite its great benefits, toxicological studies showed that capsaicinoids have low selectivity and high toxicity, which limits its application both in clinical practice (Arora *et al*., 2011) and in agriculture. Therefore, non-pungent compounds with the antifungal properties of capsaicinoids would be useful instead. Indeed, some pepper accessions contain non-pungent analogues to capsaicinoids, namely capsiconinoids and capsinoids (Díaz *et al*., 2004). These analogues have similar structure and biosynthesis pathway (Figure XIII, General Introduction). Because of these similarities, capsinoids have caused great interest as substitutes for capsaicinoids. Capsinoids are non-pungent and therefore less toxic, but they present many characteristics similar to these of capsaicinoids (Luo *et al*., 2011).

In the present work, we studied both in vitro and in vivo the properties of a capsinoid, vanillyl nonanoate (VNT), to control *P. capsici* and *B. cinerea*.

2.2. Material and method

2.2.1. Pathogen material

Phytophthora capsici Leon. isolate PC450 was provided by Frank Panabieres (INRA, France) and grown in V8 agar (Silvar *et al*. 2005) at 24°C and darkness. The

zoospores were obtained as described in Silvar *et al*. (2005). In the case of pepper inoculation, *P. capsici* cultures were grown on PDA (potatoe dextrose agar).

Botrytis cinerea Pers:Fr. isolate B05.10 was provided by Dr. Jan van Kan (Wageningen University, The Netherlands) and grown in tomato-PDA (Díaz *et al*. 2002) at 24°C and darkness. The conidia were collected by flooding the Petri dishes with sterile distilled water and filtering the suspension as described in Díaz *et al*. (2002). In the case of pepper inoculation, *B. cinerea* cultures were grown on PDA.

2.2.2. Plant material

Pepper plants of Capsicum annum L. cv. Padrón were used 21–27 days after sowing.

Before sowing, seeds were disinfected in a commercial bleach (sodium hypochlorite) solution at 1% during 30 min. Then, seeds were rinsed with tap water and sown in vermiculite. Vermiculite was watered with Hoagland nutrient solution: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0,5 μM $CuSO_4$, 0,5 μM H_2MoO_4,20 μM EDTA and 20 μM $Fe(NH_4)2(SO_4)$ (Hoagland & Arnon, 1950). Two weeks later, plants were transferred individually to pots filled with a mixture of potting soil and perlite (3:1 v/v). After this, plants were irrigated with tap water.

Plants grew in a growth chamber with 16 h light at 25°C and 8 h of darkness at 18°C.

2.2.3. Effect of VNT and their precursors on the germination of the spores of pathogens

The tested compounds were vanillyl nonanoate (VNT), a synthetic capsinoid, vanillyl alcohol (VOH), precursor of VNT aromatic portion, and nonanoic acid (NNA), precursor of VNT aliphatic chain. The compounds were dissolved in ethyl acetate in the case of *P. capsici* assays and acetone in *B. cinerea* assays. Different concentrations (0.1 mM, 0.25 mM, 0.5 mM and 1 mM) were prepared for each compound. As a control, the corresponding solvent was used.

Germination tests were carried out using excavated microscope slides. 25 µl of each solution were pipetted on the slide and let to dry. Immediately after evaporation of the solvent, 25 µl of spore suspension were pipetted on the slide. Spore concentration depends on the pathogen tested. For *P. capsici* the concentration was adjusted to 105 zoospore/ml and zoospores were encysted by shaking them in a vortex during 1 min. On the other hand, *B. cinerea* conidial suspension was adjusted to 10^6 conidia/ml and supplemented with glucose 10 mM.

All the slides were incubated in a growth chamber with high humidity, 24°C and darkness. The incubation time was 2 h for *P. capsici* and 5–6 h for *B. cinerea*. To stop spore germination 25 µl of lactophenol blue were added to each slide. Then, the percentage of germinated spores was scored: 100 spores were checked per treatment and experiment. Moreover, the length of the germ tube was measured in 20 spores per treatment and experiment. A spore was considered as germinated when its germ tube was 1.5 fold higher than spore diameter.

Four independent experiments were performed for each compound and for each pathogen.

2.2.4. Application of vanillyl nonanoate treatment to plants

Before inoculation, pepper plants were treated with 150 µM vanillyl nonanoate (VNT) dissolved in 0.1% dimethyl sulfoxide (DMSO) (Veloso *et al*. 2014). VNT treatment was applied 24 h before inoculation by spraying 5 ml on each secondary leaf. Plants treated with 0.1% DMSO were used as the control. At least two independent experiments were done for each pathogen

2.2.5. Inoculation and determination of symptoms

2.2.5.1. *Phytophthora capsici*

Pepper plants were inoculated with plugs of *P. capsici* grown in PDA. This culture was grown for 4–5 days at 24°C and darkness. Two plugs of 5 mm diameter were placed on each leaf avoiding the main veins. Plants were introduced in a wet chamber. Symptom evolution was followed for 2 days. The symptoms were determined using two parameters: percentage of expanding lesions 24 h after inoculation and

severity index 48 h after inoculation. An expanding lesion is a lesion whose diameter is bigger than 5 mm. The scale to measure severity index ranges from level 0 to level 4 based on affected leaf surface: 0, no symptoms, 1, 1–25%, 2, 26–50%, 3, 51–75% and 4, 76–100% affected leaf surface (Figure 2.1).

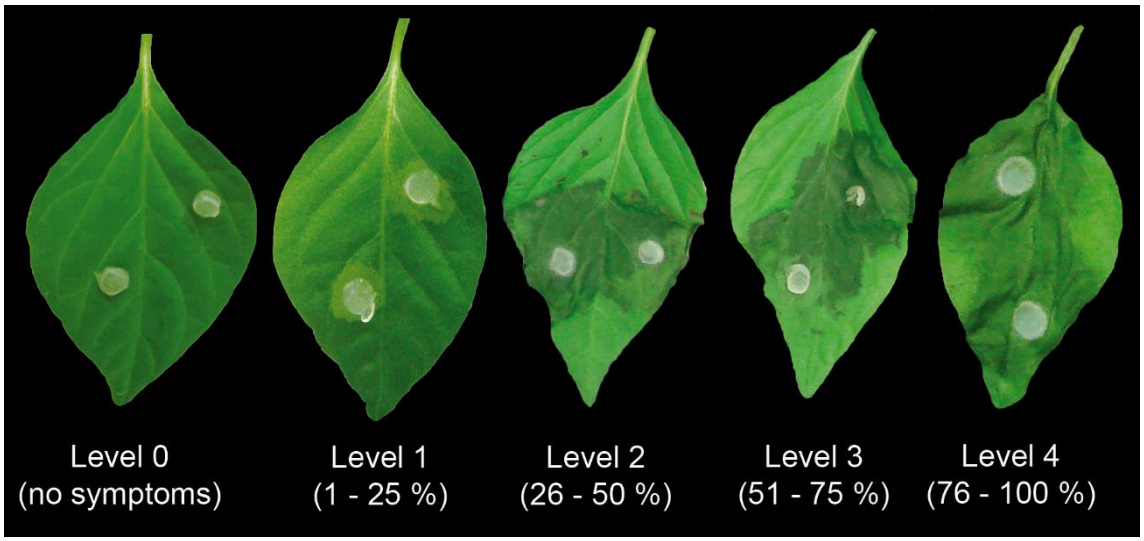

Figure 2.1. Scale of affected leaf surface used to calculate the percentage of severity.

2.2.5.2. *Botrytis cinerea*

The inoculation with this pathogen was performed with plugs of 5 mm of diameter from a 4 days old PDA culture. Plugs were placed on leaf surface avoiding main veins. Plants were introduced in a wet chamber with low light.

Disease evolution was followed after 1 day by determining the percentage of expanding lesions and the diseased area. An expanding lesion is a lesion whose diameter is bigger than 5 mm. Disease area was calculated using the diameter of expanding lesions.

2.2.6. Effect of VNT on the expression of genes related to plant defence

Samples from pepper leaves (three plants per sample) were collected 24 h after VNT treatment. The samples were frozen with liquid nitrogen and stored at −80°C. Three independent experiments were done. The extraction and reverse transcription were carried out following the protocols of the BioRad AurumTM Total RNA Mini kit and the iScriptTM cDNA Synthesis Kit respectively. The cDNA samples were analysed with the Biorad iCyclerTM iQ System following the protocol described by Veloso *et al*.

(2014). The genes analysed were: *CaBPR1* (a PR1 gene), *CaBGLU1* (a β-1,3-glucanase gene), *CaSC1* (a sesquiterpene cyclase gene, an enzyme involved in phytoalexin biosynthesis) and *CaPAL1* (a phenylalanine ammonia lyase gene, involved in salicylic acid and phenylpropanoid biosynthesis). The constitutively-expressed actin gene (AY572427) was used as housekeeping reference gene. All the primers are listed in table 2.1

Table 2.1. Primers used in quantification of the expression of genes related to plant defence through real time qPCR.

Gene	Accession number	Reference	Primer		
			Name	Sequence	Amplicon
CaSC1	AF061285	Silvar *et al*. (2009)	*CaSCFW* *CaSCRV*	5'GCCTCCTGCTTCTGAATACC3' 5'TTAATATCCTTCCATCCCGACTC3'	312 bp
CaBPR1	AF053343	Gayoso *et al.* (2007)	PR1FW PR1RV	5'GTTGTGCTAGGGTTCGGTGT3' 5'CAAGCAATTATTTAAACGATCCA3'	301 bp
CaBGLU1	AF227953	Silvar *et al*. (2009)	*GLUFW* *GLURV*	5'ACAGGCACATCTTCACTTACC3' 5'CGAGCAAAGGCGAATTTATCC3'	226 bp
CaPAL1	KF279696	García *et al*. (2018)	*PAL1FW* *PAL1RV*	5'GTGGCACGATCACTGCCTCG3' 5'TGGTCCGTGAACTCGGGCTT3'	319 bp
CaACT	AY572427	Silvar *et al*. (2009)	*CaACTFW* *CaACTRV*	5'ATCCCTCCACCTCTTCACTCTC3' 5'GCCTTAACCATTCCTGTTCCATTATC3'	128 bp

The PCR reactions consisted of Biorad 1X iQ SYBR Green Supermix, 0.3 μM of each primer and 2.5 μL of cDNA for a 50 μL end volume reaction. The PCR program started with a 2 min denaturalization step at 95 °C followed for 40 cycles of amplification (95 °C for 20 s, 58 °C for 25 s and 72 °C for 50 s) and finished by an elongation step of 5 min at 72 °C. The data analysis was performed with the Biorad Optical System Software 3.0. The Ct values were processed by the Pfaffl Method to obtain the relative expression values (Veloso *et al*., 2014).

2.2.7. Statistical analysis

All the statistical analysis was performed using Statgraphics 5.1 and MS Excel. Results from spore germination were analysed with Kruskal-Walls test followed by post-hoc test according to Conover (1980). Data obtained in the inoculation assay with *B. cinerea* (expanding lesions and disease area) and data from bioassay with *P. capsici* (expanding lesions) were analysed statistically with t-Student test. The data of severity from *P. capsici* bioassay were analysed using a Chi-square test. Results from gene expression analysis were analysed by Kruskal-Wallis test

2.3. Results

2.3.1. Effect of VNT and their precursors on phytopathogenic fungi spores germination

It was tested whether VNT was able to inhibit spore germination of *P. capsici* and *B. cinerea*. To do that, two parameters (percentage of germination and germ tube length) were measured at several concentrations of VNT (0.1, 0.25, 0.5 and 1 mM).

In experiments performed with *P. capsici* both zoospore germination and germ tube length decreased as tested concentrations of VNT increased. In fact, the reduction of both parameters was higher than 50% at the maximum assayed concentration (1 mM) (Figure 2.2.A and 2.2.D). VNT precursors, VOH and NNA, were also assayed. In this way, we could find out if the observed VNT effect is due to the whole molecule or any of its precursors. The exposure of *P. capsici* zoospore to VNT precursors did not have the same effect that VNT (Figure 2.2.B, 2.2.C, 2.2.E and 2.2.F). Therefore, the whole VNT molecule is the responsible for inhibition of zoospore germination and germ tube growth.

When *B. cinerea* conidia were exposed to VNT, we did not observe any trend between VNT concentration and both percentage of germination and germ tube (Figure 2.3.A and 2.3.D). On the other hand, the exposure of *B. cinerea* spores to VOH had no effect on spore germination but induced germ tube length (Figure 2.3.B and 2.3.E). NNA did not alter the normal development of spore germination, except in the case of

the highest assayed concentration (1 mM) that was extremely toxic for *B. cinerea* (Figure 2.3.C and 2.3.F).

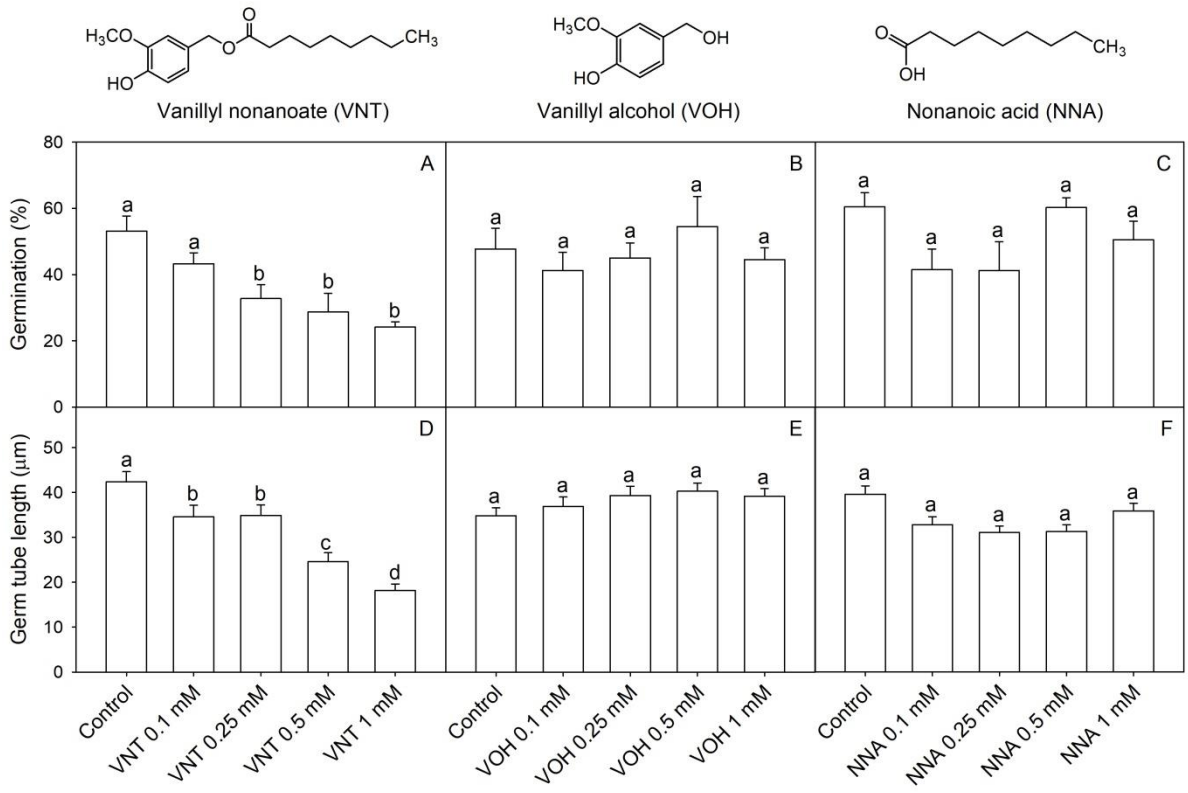

Figure 2.2. Effect of vanillyl nonanoate (VNT) and its precursors, vanillyl alcohol (VOH) and nonanoic acid (NNA), on *Phytophthora capsici* zoospore germination. A-C: effect of VNT (A), VOH (B) and NNA (C) on percentage of spore germination. D-F: effect of VNT (D), VOH (E) and NNA (F) on germ tube length. Four independent experiments were carried out for each compound. Data is the average ± SE. Different letters mean significant differences ($p<0.05$) in Kruskal-Wallis test followed by post-hoc according to Conover (1980).

2.3.2. Effect of VNT against *Phytophthora* blight

Once showing that VNT had antifungal activity against *P. capsici* in vitro, it was tested whether this compound also had the same capability in vivo. To do that, VNT was applied by spraying secondary leaves and these were inoculated with *P. capsici*. VNT treatment did not avoid the *P. capsici* infection but it reduced the percentage of expanding lesions (Figure 2.4.A). A reduction in symptoms was also observed at 48 h after inoculation. In figure 30.B it is shown how most of lesions of VNT-treated plants are classified in level 3 of disease severity while in control most of them are in level 4 (figure 2.4.B). This suggests that VNT delayed the growth of the lesion.

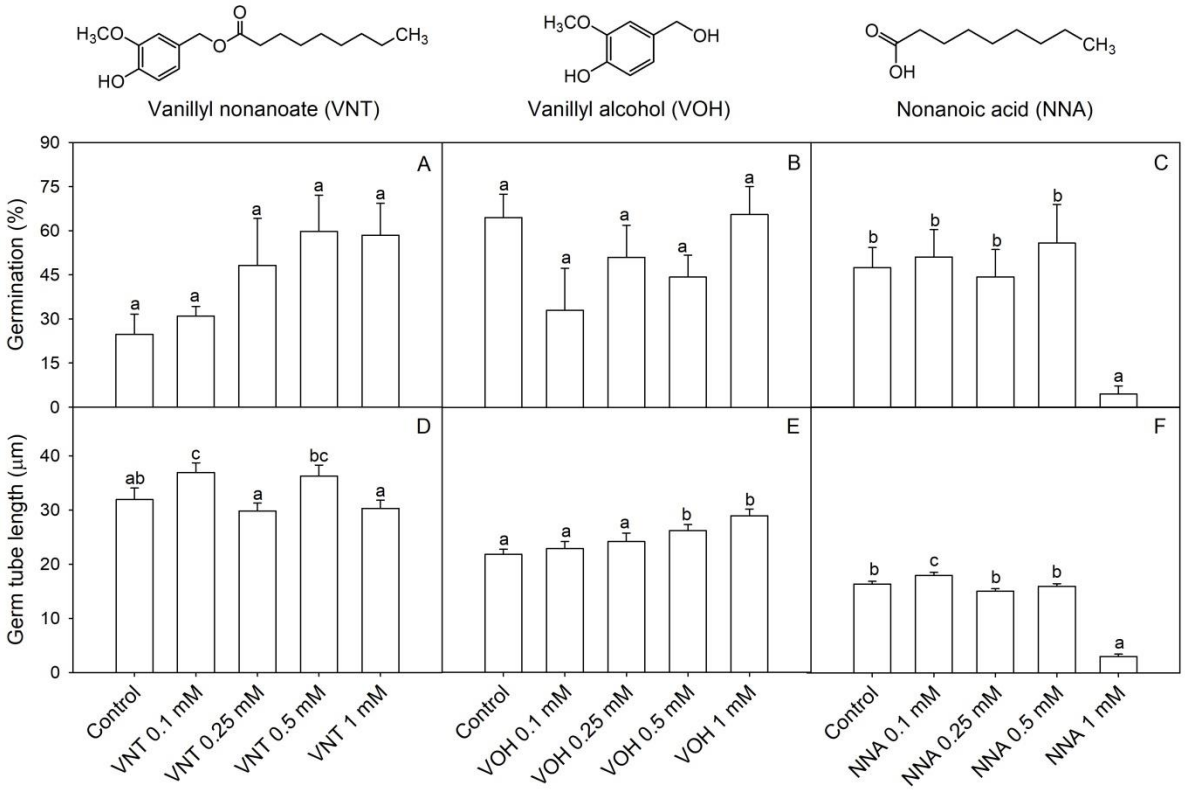

Figure 2.3. Effect of vanillyl nonanoate (VNT) and its precursors, vanillyl alcohol (VOH) and nonanoic acid (NNA), on *Botrytis cinerea* spore germination. A-C: effect of VNT (A), VOH (B) and NNA (C) on percentage of spore germination. D-F: effect of VNT (D), VOH (E) and NNA (F) on germ tube length. Four independent experiments were carried out for each compound. Data is the average ± SE. Different letters mean significant differences ($p<0.05$) in Kruskal-Wallis test followed by post-hoc according to Conover (1980).

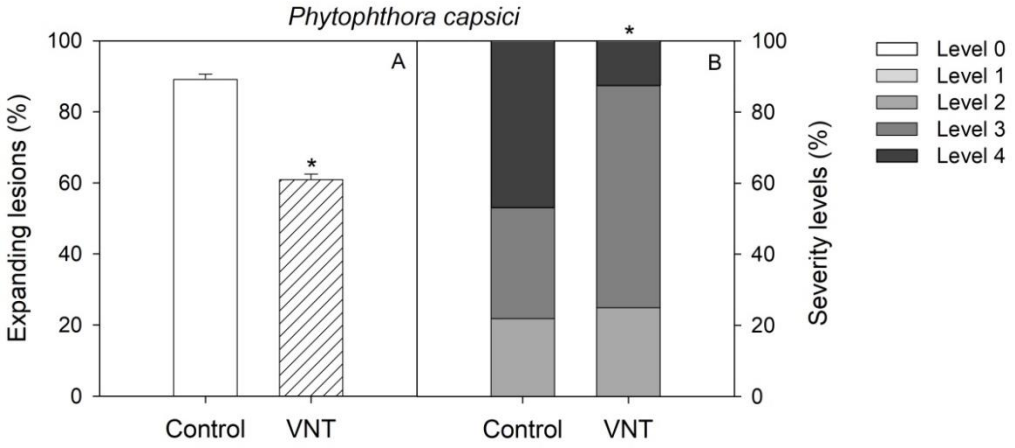

Figure 2.4. Effect of vanillyl nonanoate (VNT) on symptoms caused by *Phytophthora capsici* at local level. A: percentage of expanding lesions 24 h after inoculation. Two independent experiments were carried out (n=16). Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. B: Percentage of severity level 48 h after inoculation. Two independent experiments were

carried out (n=16). Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in Chi-square test.

2.3.3. Effect of VNT against *Botrytis* disease

In *vitro* assays showed that VNT had no effect on Botrytis spore germination. Notwithstanding, it was tested whether this compound had any effect in vivo. To do that, VNT was applied by spraying secondary leaves, then they were inoculated with *B. cinerea*. The VNT treatment reduced the percentage of expanding lesions but it did not abolish completely *B. cinerea* infection (Figure 2.5.A). Moreover, the disease area of expanded lesions was smaller in VNT-treated plants than control plants (figure 2.5.B).

2.3.4. Effect of VNT on pepper gene expression

VNT caused an increase in the expression of *CaBPR1* (Figure 2.6.A) but it was not statistically significant. However, VNT significantly increased the expression of *CaBGLU1*, *CaSC1* and *CaPAL1* (Figure 2.6.B, 2.6.C and 2.6.D), suggesting that the plant defences were activated.

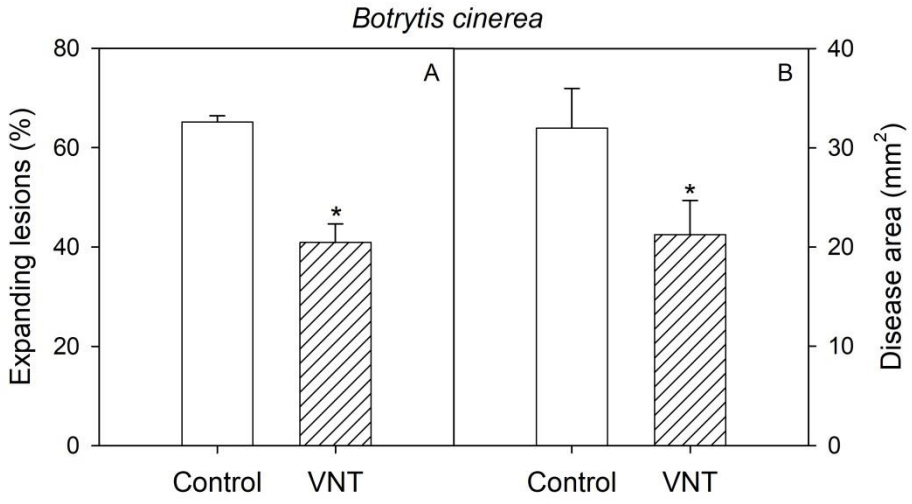

Figure 2.5. Effect of vanillyl nonanoate (VNT) on symptoms caused by *Botrytis cinerea* at local level. A: percentage of expanding lesions 24 h after inoculation. Two independent experiments were carried out (n=12). Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. B: Disease area 24 h after inoculation. Three independent experiments were carried out (n=18). Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test.

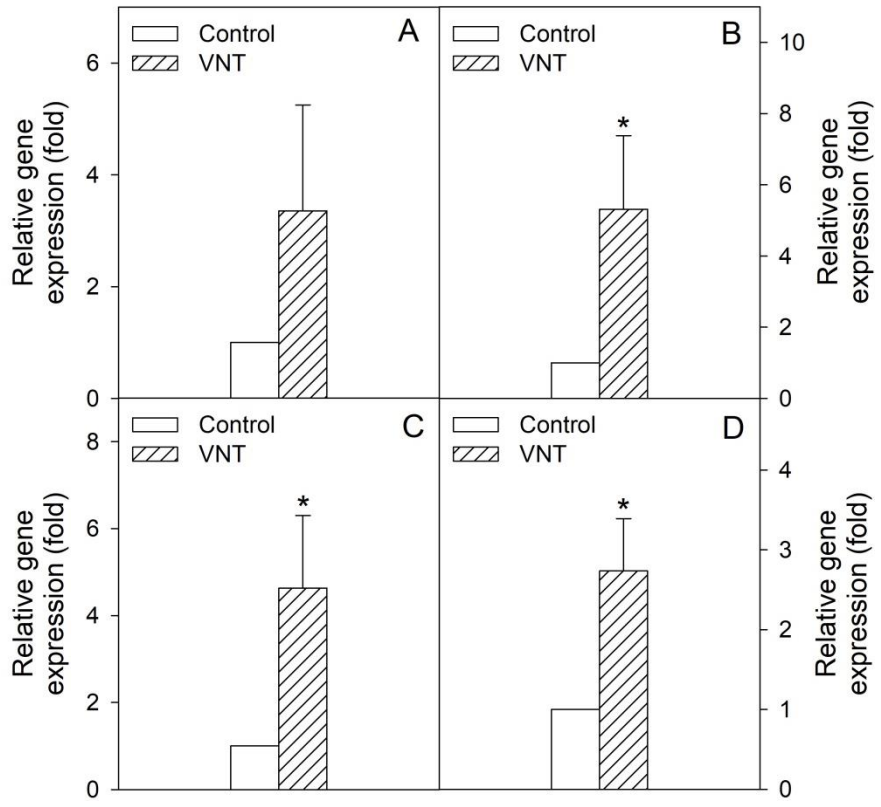

Figure 2.6. Effect of vanillyl nonanoate (VNT) on the expression of genes related to plant defence. A: *CaBPR1*. B: *CaBGLU1*, a β-1,3-glucanase. C: *CaSC1*, a sesquiterpene cyclase involved in phytoalexin biosynthesis. D: *CaPAL1*, a phenylalanine ammonia lyase. Three independent experiments were carried out (n=3).

2.4. Discussion

2.4.1. Effect of VNT on spore germination of *P. capsici* and *B. cinerea*

In this study we have checked if the synthetic capsinoid vanillyl nonanoate (VNT) has any fungistatic or fungicide effect on *P. capsici* and *B. cinerea* spores. To do that, we determined the percentage of spore germination and the length of germ tube from *P. capsici* and *B. cinerea*. As with capsaicin (Veloso *et al*., 2014), VNT caused no death of the spores of both pathogens at the assayed concentrations. VNT inhibited *P. capsici* zoospores germination but *B. cinerea* spores were not affected by the compound. However, 0.5 mM capsaicin reduced the mycelia growth of both pathogens: 63.7% in *P. capsici* and 35.9% in *B. cinerea* (Veloso *et al*., 2014). Despite capsaicin has higher antifungal activity than VNT, capsinoids also influence spore germination. Structurally VNT and capsaicin are very similar. However, there is a difference in the

type of bond in the linear moiety, amino in capsaicin and ester in VNT. This bond type plays an important role in pungency and maybe also in antimicrobial activity. Nevertheless, it is possible that modifications in other atom of carbon from aromatic ring increase the antimicrobial activity without altering the pungency degree. Something similar was studied by He *et al*. (2009) related with antinociceptive activity in capsinoid derivates. They studied how pungency is affected by the addition of various acyl chain length at C1 position or alkoxyl chain length at C4 position. Regarding to pungency all the derivates were no or slight pungent, that means that this type of alterations did not revert the pungency. However, alterations in acyl chain length at C1 position decrease the antinociceptive activity but this increase with elongation of alkoxyl chain at C4 (He *et al*., 2009).

Other modifications, such as the presence or absence of unsaturation in the phenolic portion, change the degree of antifungal activity of compound. The experiments carried out by Reddy *et al*. (2012) showed that the presence of double bond in the aromatic portion conferred good activity against *Candida albicans, Candida rugosa, Rhizopus oryzae, Aspergillus niger* and *Saccharomyces cerevisiae*. However, the absence of double bond showed a moderate antifungal activity and had no activity against *C. albicans* and *R. oryzae* (Reddy *et al*., 2012).

On the other hand, it was observed that the length of aliphatic chain also affects the antifungal activity of capsinoids. Reddy (2013) observed that the only synthetized capsinoids with short aliphatic chain showed good antifungal activity against *C. rugosa* and *S. cerevisiae*.

In spite of certain modifications in capsinoid molecular structure can improve in their antifungal activity, we have to keep in mind that not all microorganisms respond equally to the same compound. Our results point out a different biological response in *B. cinerea* (fungi) and *P. capsici* (oomycete). Strobel *et al*. (1997) observed a different effect of a leucinostatin A, a peptide produced by endophytic fungi *Acremonium* sp., in the growth of several pathogens. Its antifungal effect was stronger in *Pythium ultimum* (oomycete) than in *Sclerotinia sclerotium* (true fungus) (Strobel *et al*., 1997). Also Tanaka *et al*. (2014) observed differences in ID_{50} of *B. cinerea* and *Pythium aphanidermatum* exposed to four lipopeptides from *Bacillus amyloliquefaciens* strain SD-32 being bigger in second one than first one. Therefore, biological differences between oomycetes and fungi (Latijnhouwers *et al*., 2003) would explain the different

response of each kind of pathogen when exposed to an antifungal compound. One important dissimilarity between oomycetes and fungi is the composition of cell wall. In general the cell wall in oomycetes is made mainly by β-1,3-glucan but chitin is absent. Nevertheless, the cell wall from true fungi is enriched in chitin. In addition, there is another structural difference, in this case the plasma membrane. In oomycetes, this structure presents lipids with unusual structure and fat acid with long chain, which substitutes sterols in membrane of mycelia (Latijnhouwers *et al*., 2003). These differences in cell wall and membrane composition could explain the different response of *P. capsici* and *B. cinerea* to VNT. Moreover, VNT possibly modifies the hydrophobity of *P. capsici* spores, because the aliphatic chain of this compound could be inserted in zoospore membrane and then it could alter its germination capacity (Dohlemann *et al*., 2006).

VOH and vanillyl amide, which are the aromatic portion from VNT and capsaicin, respectively, showed less inhibitory effect than the aliphatic chain in *P. capsici*. VOH had no effect on *P. capsici* spores but vanillyl amide was able to inhibit 7.2% of its mycelial growth (Veloso *et al*., 2014). Vanillin, a precursor of both VOH and vanillyl amide, is also able to inhibit the growth of *Aspergillus ochraceus* at 1000 ppm and the growth of *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus parasiticus* at 1500 ppm (López-Malo *et al*., 1995). Fitzgerald *et al*. (2004) observed that *S. cerevisiae* is able to reduce vanillin, which inhibits its growth, to vanillyl alcohol, which has no antimicrobial activity. This conversion is advantageous for yeast but only at sub-MIC concentrations (1 mM). This suggests that yeasts are able to detox vanillin converting it into a non-toxic compound when in the medium there are concentrations that do not affect the cell integrity. This phenomenon seems like an acclimation process.

Concerning pathogen response to aliphatic chain, its inhibitory effect varies depending on the type of molecule and the pathogen. For example, percentage of germination of *P. capsici* zoospores was no altered by nonanoic acid (NNA) at the assayed concentrations. However, spore germ percentage of *B. cinerea* conidia was reduced drastically at NNA 1 mM. This reduction was no so strong compared with Aneja *et al*. (2005) assays in which they observed a 75% of reduction in *Crinipellis perniciosa* and *Moniliophthora rorei* spore germination at lower concentrations (0.09 and 0.92 µM, respectively) than the concentrations used in this study. What is more, 8-methyl-6-nonanoic acid, a capsaicin precursor, produce an 86% of inhibition on *P.*

capsici growth at 0.5 mM (Veloso *et al*., 2014), suggesting that methylation could have an important role in its toxic effect in comparison with NNA. On the other hand, mixing medium-chain fatty acids such as caprylic acid, pelargonic acid (nonanoic acid) and capric acid produced a 100% of inhibition on *Phytophthora infestans* at 100 ppm and on *B. cinerea* at 200 ppm (Liu *et al*., 2014).

The results obtained in this study show that the whole molecule of VNT is responsible of inhibition of *P. capsici* zoospore germination. However, the assays carried out with *B. cinerea* point to no antifungal effect.

2.4.2. Effect of VNT *in planta*

After checking the effect of VNT in an inhibition assay, the effect of this compound was also assayed *in planta* by applying the VNT to the plant and then inoculating the plant with *P. capsici* or *B. cinerea*.

After VNT treatment a reduction in symptoms was observed when plants were inoculated with *P. capsici*. These results together with in vitro assays support the idea that VNT has the capability to inhibit or slow down the *P. capsici* growth. It is possible that spraying VNT on leaves surface has a dual role: on one hand the VNT has an antifungal effect and on other hand VNT is inducing plant defences. Malo *et al*. (2017) observed that an extract of red fruits of *Capsicum frutescens* produced an inhibition over 75% in *Oidium* sp. spores. The fruits of this species of pepper have capsaicinoids and also capsinoids (Singh *et al*., 2009). Therefore, the effect of the extracts may be due to the joint participation of both capsaicinoids and capsinoids. There are other natural compounds with both activities in vivo and in vitro. An example is the essential oil extracted from *Metasequoia glyptostroboides* (Bajpai *et al*., 2010). Its application on melon plants hamper in 100% of cases the infection by *Xanthomonas campestris* pv. *vesicatoria* YK95-4 and *Xanthomonas* sp. SK12 and also produces inhibition in vitro (Bajpai *et al*., 2010). This behaviour is shared with fungicides used to fight against diseased caused by *Phytophthora* spp. A typical fungicide used in management of *Phytophthora capsici* is metalaxyl, which is highly effective inhibiting both the mycelial growth and sporangia formation, but is also able to reduce the symptoms *in planta* (Kim *et al*, 2000; Matheron & Porchas, 2000). However, in some cases the level of effectiveness is not the same in vitro than in vivo. Fosetyl-Al is the active compound of several fungicides used against *Phytophthora* sp. and has a strong impact in mycelial

growth and sporangium formation. Nevertheless, when the Phythophthora root rot and/or percentage of infected stem were quantified the reduction in symptoms was very low (Fenn & Coffey, 1984; Matheron & Porchas, 2000).

Both Metalaxyl and Fosetyl-Al share one particular characteristic: besides their fungicide action, both are able to induce the plant defences, that is, they induce resistance (Gozzo & Faoro, 2013). Malo *et al*. (2017) observed that PR1, PR2 and PR3 were induced after treating plants with an extract of red fruits of *Capsicum frutescens*, that is, the extract induced resistance in plants. As we stated above, these fruits contain both capsaicinoids and capsinoids, therefore VNT could be able to induce resistance

When plants were inoculated with *B. cinerea* a reduction in symptoms was observed, but this effect is completely opposite to the results of in vitro assays in which VNT do not inhibit the germination or even produced slight induction of spore germination. Therefore, the observed disease reduction is not due to a direct toxic effect of the compound on the fungus, but to the possibility that the VNT is generating some signal that induces plant defences. This is the case for other inducers such as BTH or BABA (β-amino-butyric acid). The application of these compounds induces plant defences (Görlach *et al*., 1996; Zimmerli *et al*., 2001; Buonaurio *et al*., 2002; Šašek *et al*. 2012). The reduction in *P. capsici* infection might be also, in part, due to these VNT-induced plant defences besides the direct VNT-toxic effect. Indeed, at least three plant genes involved in resistance to pathogens (*CaBGLU1*, *CASC1* and *CaPAL1*) were induced by VNT. *CaSC1* is involved in the biosynthesis of capsidiol, a phytoalexin (Back *et al*., 1998). *CaBGLU1* is ethylene and jasmonate responsive (Choi & Hwang, 2015), and *CaPAL1* is involved in salicylic acid biosynthesis (Kim & Hwang, 2014; Choi & Hwang, 2015). This induction suggests the involvement of the three plant hormones salicylic acid, ethylene and jasmonic acid, in the pepper response to VNT. Intriguingly, *CaBPR1*, that is responsive to the three hormones (Choi & Hwang, 2015), was apparently induced by VNT, but differences were not significant. Next chapters will clarify the signalling triggered by VNT and will address whether VNT treatment has the capacity to induce other the plant defences.

In summary, our results suggest that capsinoids could be useful in the control of plant diseases. Because pure VNT is expensive, the use of crude extracts of a non-pungent pepper containing capsinoids could be an alternative.

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CHAPTER 3.

Vanillyl nonanoate induces systemic resistance and plant defenses in pepper plants.

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3.1. Introduction

Plant diseases are a threat for crop production, and pepper (*Capsicum* spp.) is not an exception. Pepper yield is affected by several diseases, and three examples are Phytophthora root rot, Botrytis rot and Verticillium wilts (Silvar *et al*., 2005a; Polat *et al*., 2018). These diseases, especially Phytophthora root rot and Botrytis rot, can be controlled with fungicides, but fungal resistant strains appear in the field (Barchenger *et al*., 2018; Polat *et al*., 2018). Therefore, the search for alternative strategies of pathogen control is necessary. Recently we have reported the ability of vanillyl nonanoate (VNT) to control these two pathogens when applied and challenge inoculated in the same organ (leaves), that is, a local effect (García *et al*., 2018). VNT showed some antioomycete activity in vitro, but no antifungal activity against *Botrytis cinerea*. On the other hand, VNT was able to induce locally PR genes, related to plant defense, suggesting the role of induced resistance in the VNT mode of action. However, the systemic response to VNT in the plant as well as the involvement of other plant mechanisms to cope with the pathogen in addition to PR proteins was unexplored so far. One such mechanism is the reinforcement of the cell wall as well as the activation of the biochemical defences as phytoalexins and PR genes.

Cell wall together with cuticle is the first defensive line of the plant. Two types of cell wall can be distinguished: primary cell wall and secondary cell wall. The first one is flexible and expandable and is present when the cell is growing. Its composition consists in microfibrils of glucan, pectin and hemicellulosic polysaccharides and glycoproteins (Pogorelko *et al*., 2013). Opposite to this, secondary cell wall is formed when cells stop to expand or start their differentiation process. It is characterized to be more strengthened because it has lignin and lower amounts of pectins and xyloglucan (Pogorelko *et al*., 2013; Carpita *et al*., 2015).

In the past, it was thought that the functions of cell wall were just the maintenance of cell shape, the resistance of internal turgor pressure and the regulation of cell and plant growth, plant morphology and diffusion through the apoplast. However, the cell wall is involved in signalling processes such as cell to cell communication, maintenance of cell wall integrity, plant development and plant defence (Pogorelko *et al*., 2013).

Plant cell wall has multiple roles in plant defence. Per se, it is a physical barrier that can make difficult pathogen penetration but also it can confine it and limit its growth. Because of its composition, it is also a reservoir of antimicrobial compounds (Sattler & Funnell-Harris, 2013; Miedes *et al*., 2014). Even during pathogen attack the cell wall is still helping in plant defences because the pathogen tries to cross this barrier degrading it. This process generates fragments called Damage-Associated Molecular Patterns (DAMPs) that plant cell senses triggering the PTI (Miedes *et al*., 2014). Among the processes that are activated during defensive response, there is the cell wall reinforcement which helps to stop the pathogen penetration.

Plants strengthen their cell wall depositing certain polymers as lignin or lignin-like phenolic compounds (Carpita *et al*., 2015). The term lignin is used to designate all those components formed during the oxidation and polymerization of 4-hydroxyphenylpropanoids (Vanholme *et al*., 2010). The lignin is built with hydroxycinnamyl alcohols, namely coniferyl alcohol, sinapyl alcohol and ρ-coumaryl alcohol (Vanholme *et al*., 2010; Sattler & Funnell-Harris, 2013). Those alcohols are synthesized following the phenylpropanoid pathway and then oxidatively polymerized in hydroxyphenol- , guiacyl- or sinapyl- units. Finally these units will be incorporated to the lignin (Vanholme *et al*., 2010; Sattler & Funnell-Harris, 2013).

Peroxidases are the enzymes which catalyse an electron oxidation reaction using hydrogen peroxide as electron acceptor and a metal in their active centre (Almagro *et al*., 2009; Shigeto & Tsutsumi, 2016). Because peroxidases are coded by a multigenic family, these enzymes are involved in numerous physiological processes such as lignification, plant defence, development or germination. They are also involved in synthesis of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) which are signals in plant defence (Almagro *et al*., 2009; Shigeto & Tsutsumi, 2016).

Both ROS and RNS participate in the activation of the immune response involves such as the production of biochemical defences (Agrios, 2005). The most important inducible biochemical defences are phytoalexins and PR proteins. Phytoalexins are antimicrobial compounds that start to accumulate once the pathogen attacks the plant or after a treatment with an elicitor (Hammerschmidt, 1999). They are

considered one of the initial biochemical barriers at local level (Maldonado-Bonilla *et al*., 2008).

In the Solanaceae family several bicyclic sesquiterpenoids with a defensive role have been isolated. Rishitin, capsidiol, lubimin, phytuberin, and phytuberol are the most important phytoalexins with bicyclic sesquiterpenoid nature in this group of plants. Capsidiol is the main phytoalexin produced by pepper and tobacco (Chappell & Nable, 1987). The biosynthesis of capsidiol begins with the cyclation of farnesyl diphosphate into 5-epi-aristolochene by sesquiterpene cyclase. Subsequently, 5-epi-aristolochene is dihydroxylated to produce capsidiol (Maldonado-Bonilla *et al*., 2008). Several studies have demonstrated that the synthesis of this phytoalexin is correlated with pathogen attack or with elicitor treatment at both local and systemic level (Chappell & Nable, 1987; Bonilla-Maldonado *et al*., 2008).

Besides phytoalexins, PR proteins are also important inducible biochemical defences. PR proteins are "proteins that are not detectable in healthy tissues (or only at basal concentrations), but for which accumulation at the protein level has been demonstrated upon pathological conditions and related situations in at least two or more plant–pathogen combinations" (Sels *et al*., 2008). These proteins are "pathogenesis-related" because they are strongly induced during infection or related situations including treatment with inducers and wounds (Van Loon & Van Strien, 1999; Sels *et al*., 2008).

PR proteins were first discovered in tobacco leaves infected with tobacco mosaic virus although afterwards they were found in other species (Van Loon, 1999; Van Loon & Van Strien, 1999). The biochemical nature of the different PR proteins is very heterogeneous but they share characteristics such as low molecular weight, stability at low pH and resistance to proteases. According to their isoeletric point, PR proteins can be classified in acidic or basic. In tobacco, acidic PR proteins are located in the extracellular space and basic PR proteins in the vacuolar compartment. In most of the plants, PR proteins can be found in leaves, stems, roots and flowers (Van Loon, 1999).

Initially, only five classes of PR proteins were described in tobacco according to biochemical and molecular techniques. Later on, new families were added to this classification getting to a total of 17 families based on amino acid sequence, serological

relationship, and/or enzymatic or biological activity (Van Loon & Van Strien, 1999; Sels *et al*., 2008). PR proteins have a different function but all of them are related with plant defense (Table 3.1).

Table 3.1. Functions of PR proteins families (modified from Sels *et al*., 2008)

Family	Function	Family	Function
PR-1	Antioomycete	PR-10	Ribonuclease-like
PR-2	β-1,3-glucanase	PR-11	Chitinase class I
PR-3	Chitinase (class I,II, IV,V,VI,VI)	PR-12	Defensin
PR-4	Chitinase class I,II	PR-13	Thionin
PR-5	Thaumatin-like proteins	PR-14	Lipid-transfer protein
PR-6	Proteinase inhibitor	PR-15	Oxalate oxidase
PR-7	Endoproteinase	PR-16	Oxalate oxidase-like
PR-8	Chitinase class III	PR-17	Unknown
PR-9	Peroxidase		

The aim of this chapter is to test if VNT induced systemic resistance against *Phytophthora capsici*, *Botrytis cinerea* and *Verticillium dahliae* in Padrón pepper. Also we proposed to determine if the lignin and peroxidases as well as some biochemical defenses (the *CABPR1* gene, the *CASC1* gene related to the biosynthesis of capsidiol and the enzymes β-1,3-glucanase and chitinase) are induced by VNT treatment at systemic level before and after *P. capsici* and *B. cinerea* attack.

3.2. Material and methods

3.2.1. Pathogen material

Phytophthora capsici Leon. isolate PC450 was obtained from Dr. Frank Panabieres (INRA, France). *P. capsici* maintenance required several inoculations in V8

agar medium (100 ml of eight vegetables puree, 1 g of $CaCO_3$, 7.5 g agar and 400 ml of distilled water). Periodically, when the pathogen grew slow and/or showed low virulence, *P. capsici* was inoculated in pepper plants and then re-isolated from them to restore its virulence and growth. This pathogen was grown at 24ºC and in darkness.

Botrytis cinerea Pers:Fr isolate B0510 was obtained from Dr. Jan van Kan (Wageningen University, The Netherlands). The conidia of this pathogen were stored at -80ºC in 75% glycerol and 5mM NaCl. To perform the assays 2 µl of this stock was sown in PDA-tomato (50 g of tomato leaves, 10 g of PDA, 2.5 g of agar and 350 ml of distilled water) or PDA alone. *B. cinerea* was grown at 24ºC and in darkness.

Verticillium dahliae Kleb isolate UDC53VD was obtained by our research group in a prospecting in Galicia in 1998 (Novo *et al*., 2006). *V. dahliae* maintance required several inoculations in PDA. This pathogen grew at 24ºC and in darkness.

3.2.2. Plant material and vanillyl nonanoate treatment

Seeds of *Capsicum annuum* L. cv. Padrón (obtained in our greenhouse facilities) were surface-disinfected in 1% commercial bleach for 30 min. Seeds were then washed before being sown in vermiculite. During the time from sowing to transplanting, plants were watered with nutrient solution (Hoagland & Arnon 1950). Two weeks after sowing, plants were transplanted to pots with a mixture of potting soil and perlite (3:1, v/v) and watered with tap water. One week and a half later, plants were treated with VNT and 24 h later they were inoculated. Unless otherwise stated, plants were grown at 25°C day/18°C night, under a 16 h photoperiod.

In all the assays, both cotyledons of pepper plants were infiltrated through the stomata with ca. 250 µl of 150 µM VNT in 0.1% dimethyl sulfoxide (DMSO) using a syringe (Veloso *et al*., 2014). Controls were infiltrated with 0.1% DMSO. In all the experiments, controls were maintained and inoculated in the same way as the VNT-treated plants. Plants were inoculated 24 h after induction (application of VNT), time point called “before inoculation” in the figures showed in results.

Part of the assays with *V. dahliae* required induction by the roots. To do that, roots were immersed in a solution with 150 µM VNT dissolved in 0.1% DMSO and incubated for 1 h in growth chamber. Then roots were rinsed and left in Hogland nutrient solution for 24 h. Controls were treated with 0.1% DMSO.

3.2.3. Inoculation and determination of symptoms

3.2.3.1. *Phytophthora capsici*

Zoospores were obtained as follows: the pathogen was grown for 5-6 days in V8 agar medium, then it was cut into small pieces and placed in sterile KNO_3 0.01 M. This culture was incubated for 5-6 days at 24ºC with constant light and shaking to favour the sporangia formation. After this time, zoospore liberation was induced incubating the culture 45 min at 4ºC and then 45 min at room temperature. The culture was filtrated through a sterile gauze and the filtrate with zoospores was used to prepare the inoculum. The zoospore concentration was calculated using the counting chamber Malassez. The zoospore suspension was diluted to 103 zoospore/ml and 5 ml from this suspension was applied in plant collar area. Before inoculation plants were flooded to facilitate zoospore movement. The development of the symptoms was followed daily for 5 days. To determine the severity of symptoms three parameters were used (hypocotyl rot, wilted leaves and plant dead) which had a maximum value of 3, 3 and 1 (table 3.2). The sum of these parameters is the severity index which has values between 0 and 7. Severity index was used to calculate the AUDPC (area under the disease progress curve) as described in Díaz *et al*. (2005).

Two independent experiments were carried out for evaluating symptom severity, measuring eight plants per treatment and experiment (n=16).

Table 3.2. Scale used to determine severity symptoms caused by *Phytophthora capsici*.

Parameter	Value	Meaning
Rot	1	One part of hypocotyl with lesion
	2	Between one and two parts of hypocotyl with lesion
	3	Almost all hypocotyl with lesion
Wilt	1	Few leaves are wilted
	2	More than 50% leaves are wilted
	3	All leaves are wilted
Dead	1	Plant is dead

3.2.3.2. *Botrytis cinerea*

The inoculation with the pathogen was through conidia suspension or plugs. The conidia were obtained from a 10 days old culture on PDA-tomato. To collect the conidia 15 ml of distilled water and 3 µl 5% of sterile Triton X-100 were added to the culture and then rubbed with sterile loop to release the conidia. Obtained conidia suspension was filtered through sterile filter of glass wool and centrifuged at 1500 xg for 5 min. The pellet was rinsed twice with distilled water. Finally the pellet was resuspended in a known volume of distilled water to determine concentration using a counting chamber Neubauer-improved.

The leaves of pepper plants were inoculated with 3 µl drops of 106 conidia/ml suspension which was made in Gamborg B5 medium supplemented with 10 mM glucose and 10 mM KH_2PO_4. Previously to the inoculation, the suspension was incubated 2 h at room temperature. After inoculation plants placed in a wet chamber incubated at room temperature.

In other experiments pepper plants were inoculated with mycelium plugs of 5 mm of diameter from a 4 days old culture on PDA. Plugs were placed on the leaf surface avoiding main veins. After inoculation plants placed in a wet chamber incubated at room temperature.

The development of the disease was monitored, measuring the diameter for each expanded lesion 48, 72 and 96 h after inoculation with drops and 24 and 48h after inoculation with plugs. A lesion is expanded when its diameter was equal or greater than 2 mm, when the inoculation was with drops, or 5 mm, when the inoculation was with plugs. Disease area was calculated using this diameter which was measured in the underside of the leaf.

In experiments with conidial inoculation, four independent experiments were carried out, with eight plants per treatment and experiment (n=32). In experiments with plug inoculation, five independent experiments were performed with eight plants per treatment and experiment (n=40).

3.2.3.3. *Verticillium dahliae*

Pepper plants inoculation with *V. dahliae* was carried out by root immersion in conidia suspension. The conidia were harvest from a culture in PDA with 28 days. To collect conidia 10 ml of distilled water were added to the culture and then rubbed with sterile loop to release the conidia. This suspension was filtered through sterile filter of glass wool and centrifuged at 1500 xg for 5 min. The pellet was resuspended in 10 ml of distilled water and the concentration was determinate using a Neubauer-Improved counting chamber.

The concentration of conidia suspension to inoculate the plants was adjusted to 10^6 conidia/ml with distilled water and then the plants roots were immersed for 45 min. Next plants were transplanted in pots containing soil:perlite 3:1 (v/v) previously autoclaved. After inoculation plants were placed in a growth chamber. The symptoms were measured weekly till 21 days after inoculation. The parameters measured were stem length, percentage of wilted leaves and dry weight at 21 days after inoculation.

At least two independent experiments were carried out for evaluating symptom severity with eight plants per treatment and experiment (n=32).

3.2.4. Assay for qPCR quantification of pathogen colonization

Roots were sampled 48 and 72 h after inoculation with *P. capsici* from both VNT-treated and control plants. Leaves were sampled 48 h after inoculation with *B. cinerea* from both VNT-treated and control plants. Each sample was a pool of roots from five plants or a pool of leaves from 8 plants. Total DNA extraction and *P. capsici* DNA quantification in pepper plants were carried out according to Silvar *et al.* (2005). *Botrytis cinerea* biomass was quantified according to Brouwer *et al*. (2003). Plant DNA quantification was carried out according to Gayoso *et al*. (2007). Primers used are listed in table 3.3. Pathogen colonization was calculated as a ratio of pathogen DNA and plant DNA. Four independent experiments were carried out.

Table 3.3. Primers used in pathogen biomass quantification through real time qPCR.

Gene	Organism	Reference	Primer		
			Name	Sequence	Amplicon
CaBPR1	*Capsicum annuum*	Gayoso *et al*. (2007)	PR1FW PR1RV	5'GTTGTGCTAGGGTTCGGTGT3' 5'CAAGCAATTATTTAAACGATCCA3'	301 bp
ITS regions	*Phytophthora capsici*	Silvar *et al*. (2005a; 2005b)	PCAPfw PCAPrv1	5'TTTAGTTGGGGGTCTTGTACC3' 5'CCTCCACAACCAGCAACA3'	452 pb
Actin	*Botrytis cinerea*	Brower *et al*. (2003)	AFP26 AFP27	5'TGGAGATGAAGCGCAATCCAA3' 5'AAGCGTAAAGGGAGAGGACGGC3'	272 pb

3.2.5. Extraction and determination of phenolics and lignin

Samples from pepper roots or leaves (five pooled plants per sample) were collected before inoculation (24 h after induction) and 8 hours after inoculation and stored at -80ºC. Soluble phenolics were extracted with 80% MeOH as described by Díaz *et al*. (2001). Extracts were centrifuged at 1400 xg for 10 min. Supernatants were analysed for soluble phenolics using Foling-Ciocalteau reagent according to Díaz *et al*. (2001). Pellets were stored in fresh MeOH to extract cell walls later. 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 (Johnson *et al.* 1961) with a modification: the incubation of cell walls with a mixture of acetyl bromide/acetic acid (1:3, v/v) was carried out at 50ºC for two hours (Hatfield *et al*. 1999). A minimum of hree independent experiments were carried out.

3.2.6. Extraction and determination of peroxidase activity

Samples from pepper roots or leaves (five pooled plants per sample) were collected before inoculation (24 h after induction) and 8 h after inoculation and stored at -80ºC. For enzyme extraction, samples were homogenized in a mortar at 4°C with 50 mM Tris HCl buffer pH 7.5, containing 0.05 g of polyvinylpolypyrrolidone (PVPP) per g of tissue and 1M KCl. The crude extract was centrifuged at 12,857 xg and 4°C for 20 min. The supernatant was collected and desalted using a PD-10 Sephadex G-25 column

(GE Healthcare). Aliquots of 1 ml were collected and stored at -80°C until further analysis.

Peroxidase activity was quantified using 4-methoxy-1-naphthol as substrate. The reaction mixture (1 ml) consisted of 930 µl of 50 mM tris-HCL buffer pH 7.5 at 25°C, 10 µl of 100 mM of 4-methoxy-1-naphthol, 50 µl of 6.6 mM H_2O_2 and 10 µl of the sample, previously centrifuged (1 min at 16060 xg). The change in absorbance was measured for 1 min at 593 nm. Activity was expressed in International Units (U)/mg protein. Four independent experiments were performed in the case of roots (*P. capsici*) and five in the case of leaves (*B. cinerea*).

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

3.2.7. Measurement of β-1,3-glucanase and chitinase activity

β-1,3-glucanase and chitinase activities were quantified using laminarin and glycolchitin respectively as substrate (Veloso *et al*., 2014). The reaction mixture consisted of 82.5µl of a solution 1% of laminarin or glycolchitin, 332.5 µl of 100 mM sodium acetate warmed at 37ºC and 85 µl of sample obtained in section 3.2.6. This mixture was incubated for 10 minutes at 37ºC and then kept in ice for other 5 min. Then 670 µl of 15 mM potassium ferricyanide in 0.5 M sodium carbonate was added and then boiled for 15 minutes. The absorbance was measured at 420 nm. The reference was prepared following the same steps but containing 85 µl of Tris HCl 50 mM and 1M KCl buffer pH 7.5 instead of sample. The enzyme activity was calculated as International Units (U)/ mg of protein. At least two experiments were made per enzyme and pathogen.

3.2.8. Evaluation of gene expression in *Capsicum annuum*

Samples from pepper roots or leaves (five pooled plants per sample) were collected before inoculation (24 h after induction) and 8 h after inoculation. The samples were frozen with liquid nitrogen and stored at -80ºC. The RNA extraction and the reverse transcription were carried out following the protocol of BioRad Aurum™ Total RNA Mini Kit and iScript™ cDNA Synthesis Kit respectively.

The cDNA samples were analyzed with the Biorad iCycler^{TM} iQ System following the protocol described in Veloso & Díaz (2012). *CaSC*, *CaPR1* and *CaBGLU1* genes (the first related to phytoalexin biosynthesis, the second and the third coding for PR proteins) were used to test systemic response. Furthermore, two peroxidase genes were studied; *CaPO1* and *CaPO2*.The constitutively expressed actin gene was used as reference gene (Silvar *et al*. 2008). Primers are listed in table 3.4. Details of qPCR reactions and data analysis are in Veloso & Díaz (2012). At least two independent experiments were carried out.

Table 3.4. Primers used to evaluate the several genes by real time qPCR

Gene	Accession number	Reference	Primer		
			Name	Sequence	Amplicon
CaSC1	AF061285	Silvar *et al*. (2008)	*CaSCFW*	5'GCCTCCTGCTTCTGAATACC3'	**312 bp**
			CaSCRV	5'TTAATATCCTTCCATCCCGACTC3'	
CaBPR1	AF053343	Gayoso *et al.* (2007)	*PR1FW*	5'GTTGTGCTAGGGTTCGGTGT3'	**301 bp**
			PR1RV	5'CAAGCAATTATTTAAACGATCCA3'	
CaBGLU1	AF227953	Silvar *et al*. (2008)	*GLUFW*	5'ACAGGCACATCTTCACTTACC3'	**226 bp**
			GLURV	5'CGAGCAAAGGCGAATTTATCC3'	
CaPO1	AF442386	García *et al*. (2015)	*CAPO1FW*	5' ACACTGGAAGCGTGAACAAT 3'	**333 bp**
			CAPO1RV	5' CAGCTTGCGCTAACATGAAC 3'	
CaPO2	DQ489711	Designed in this thesis	*CAPO2FW*	5' TAGCACTAGAAGACGTCGGT 3'	**233 bp**
			CAPO2RV	5' TAATCATGGCAGCAGCGAAA 3'	
CaACT	AY572427	Silvar *et al*. (2008)	*CaACTFW*	5'ATCCCTCCACCTCTTCACTCTC3'	**128 bp**
			CaACTRV	5'GCCTTAACCATTCCTGTTCCATTATC3'	

3.2.9. Statistical analysis

All the statistical analysis was performed using Statgraphics 5.1 Plus. The t-Student test was used to analyse data from bioassays with *P. capsici*, study of *B. cinerea* colonization, enzymatic activities measured in root samples, chitinase and β-1,3-glucanase activity measured in leaves samples and lignin and soluble phenolics

measured in root samples. Data obtained in bioassays with *B. cinerea* and *V. dahliae* (dried weight) were analysed using a Mann-Withney (Wilcoxon) test. Data from stem length and wilted leaves from *V. dahliae* bioassay were analysed using an ANOVA test followed by Student-Newman-Keuls test as a post-hoct test. Finally *P. capsici* colonization was analysed using a double via ANOVA test. Data of gene expression and peroxidase activity, soluble phenolics and lignin were analysed using a Kruskall-Wallis test.

3.3. Results

3.3.1. VNT protects pepper against *Phytophthora capsici*

Pepper plants were treated with VNT by cotyledon infiltration and inoculated with *P. capsici*. The application of VNT did not prevent *P. capsici* infection but plants treated with this compound showed less symptom severity than the control (Figure 3.1.A and 3.1.B). Moreover, the development of symptoms was delayed in VNT-treated plants in comparison with control plants.

The next step was to quantify the amount of pathogen inside the plant. Both checked time points showed that VNT-treated plants presented less pathogen colonization than control plants (Figure 3.1.C). The reduction in symptoms appearance and severity was correlated with a reduction in pathogen colonization.

3.3.2. VNT protects pepper against *Botrytis cinerea*

Pepper plants were treated with VNT by cotyledon infiltration and inoculated with *B. cinerea* through conidia suspension or plugs. As in the case of *P. capsici*, VNT treatment did not prevent the infection with *B. cinerea* regardless on the inoculation method used. However, the VNT treatment reduced the disease area both in leaves inoculated with plugs (Figure 3.2.A and 3.2.B) and spore suspension (Figure 3.2.C).

Pathogen colonization was quantified 48h after inoculation. VNT-treated plants showed less *Botrytis* colonization than control plants (Figure 3.3). Therefore, the reduction in disease was correlated with a lower pathogen biomass inside the plant.

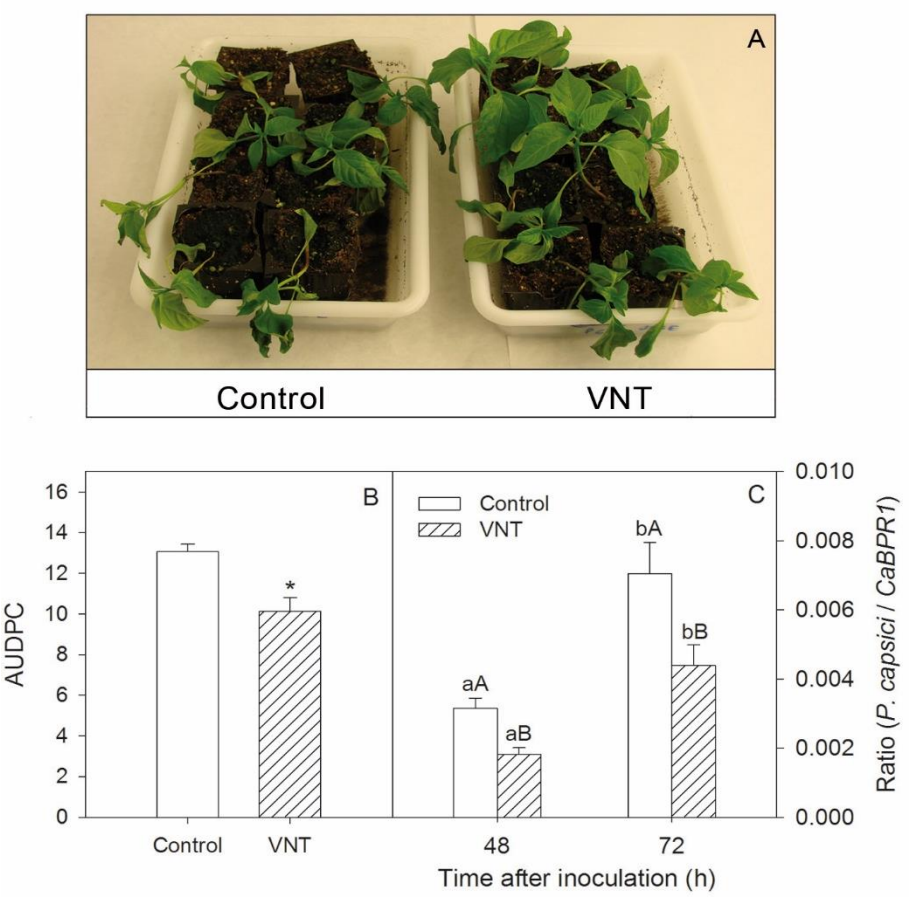

Figure 3.1. Vanillyl nonanoate (VNT) reduces symptoms and colonization by *P. capsici* in roots of pepper plants as a systemic effect. (A) Control and VNT-treated plants showing different degree of symptoms 4 days after inoculation. (B) Area under the disease progress curve (AUDPC); experiments were done twice, measuring eight plants per treatment and experiment (n=16). Data are mean ± SE. The asterisk (*) indicates statistical differences ($p<0.05$) in student t-test. (C) *P. capsici* colonization as the ratio between *P. capsici* biomass and *C. annuum* biomass (*CaBPR1*); experiments were done four times (n=4). Data are mean ± SE. Lower case letters indicate differences between different times after inoculation and upper case letters indicate differences between control and VNT in a two-way ANOVA test ($p<0.05$).

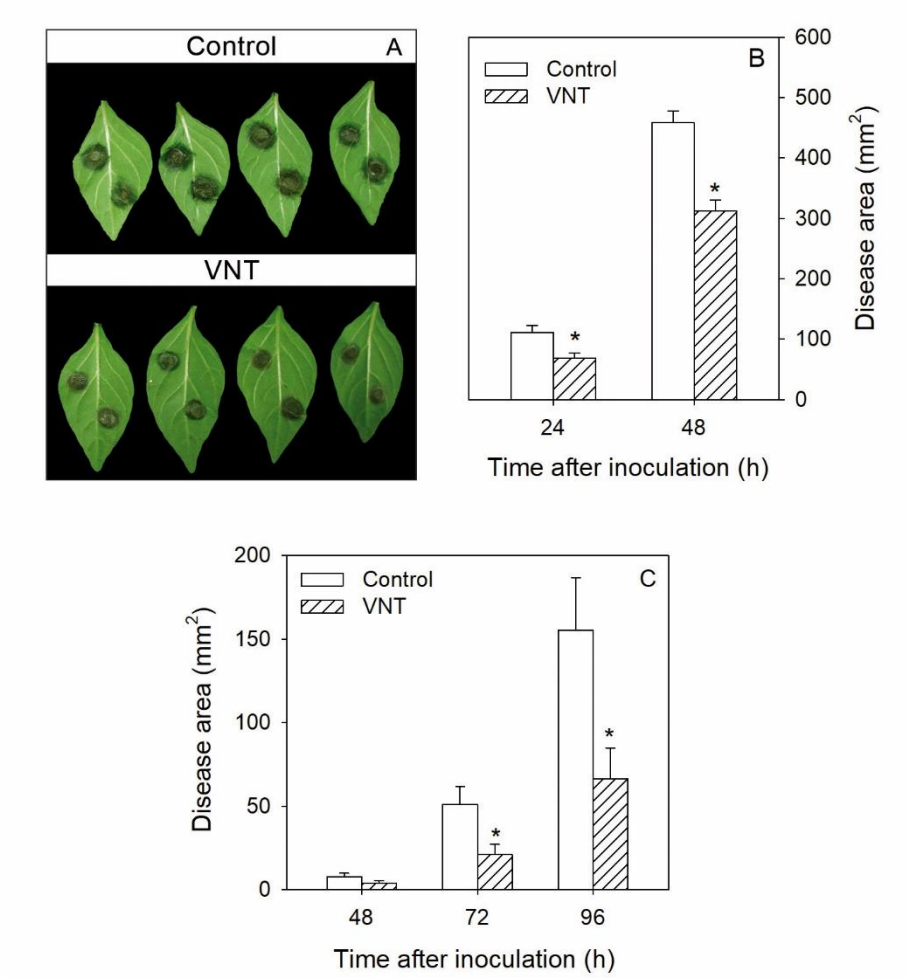

Figure 3.2. Vanillyl nonanoate (VNT) reduces symptoms by *B. cinerea* in leaves of pepper plants as a systemic effect. (A) Leaves from control and VNT-treated plants 48 hours after inoculation with plugs. (B) AUDPC in experiments carried out inoculating the plants with plugs. Five independent experiments were performed (n=40). Data are the average ± SE. The asterisk means significant differences ($p<0.05$) in Mann-Whitney (Wilcoxon) test. (C)AUDPC in experiments performed inoculating the plants with a conidia suspension. Four independent experiments were carried out (n=32). Data are the average ± SE. The asterisk means significant differences ($p<0.05$) in Mann-Whitney (Wilconxon) test.

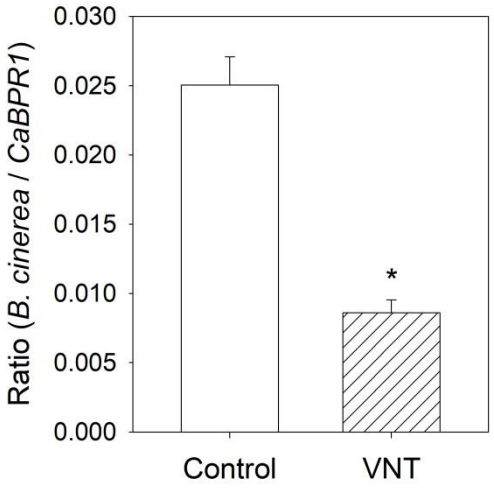

Figure 3.3. Vanillyl nonanoate (VNT) reduced colonization of *B. cinerea* 48 h after plug inoculation as the ratio between *B. cinerea* biomass and *C. annuum* biomass (*CaBPR1*). Two independent experiments were performed (n=2). Data is the average ± SE. The asterisk (*) means significant differences ($p<0.05$) on t-Student test.

3.3.3. VNT does not protect against *Verticillium dahliae*

Pepper plants were treated with VNT by cotyledon infiltration and inoculated with *V. dahliae*. None of the tested parameters show differences between control and VNT-treated plants (Figure 3.4.A and 3.4.C) except wilted leaves percentage at 21 days after inoculation (Figure 3.4.B).

This lack of protective effect could be due to the low volume of VNT applied to the cotyledons (< 1 ml) and also the long period between treatment and symptom appearance (more than one week). For this reason we applied the VNT directly to the roots. Nevertheless, also in this case no differences in symptoms were observed between control and treated plants (Figure 3.4.D, 3.4.E and 3.4.F).

These results point out that the assayed VNT concentration does not have any effect on Verticillium wilt in pepper. Besides, VNT had no direct toxic effect on the plant because uninoculated VNT-treated plants and control plants showed the same growth rate.

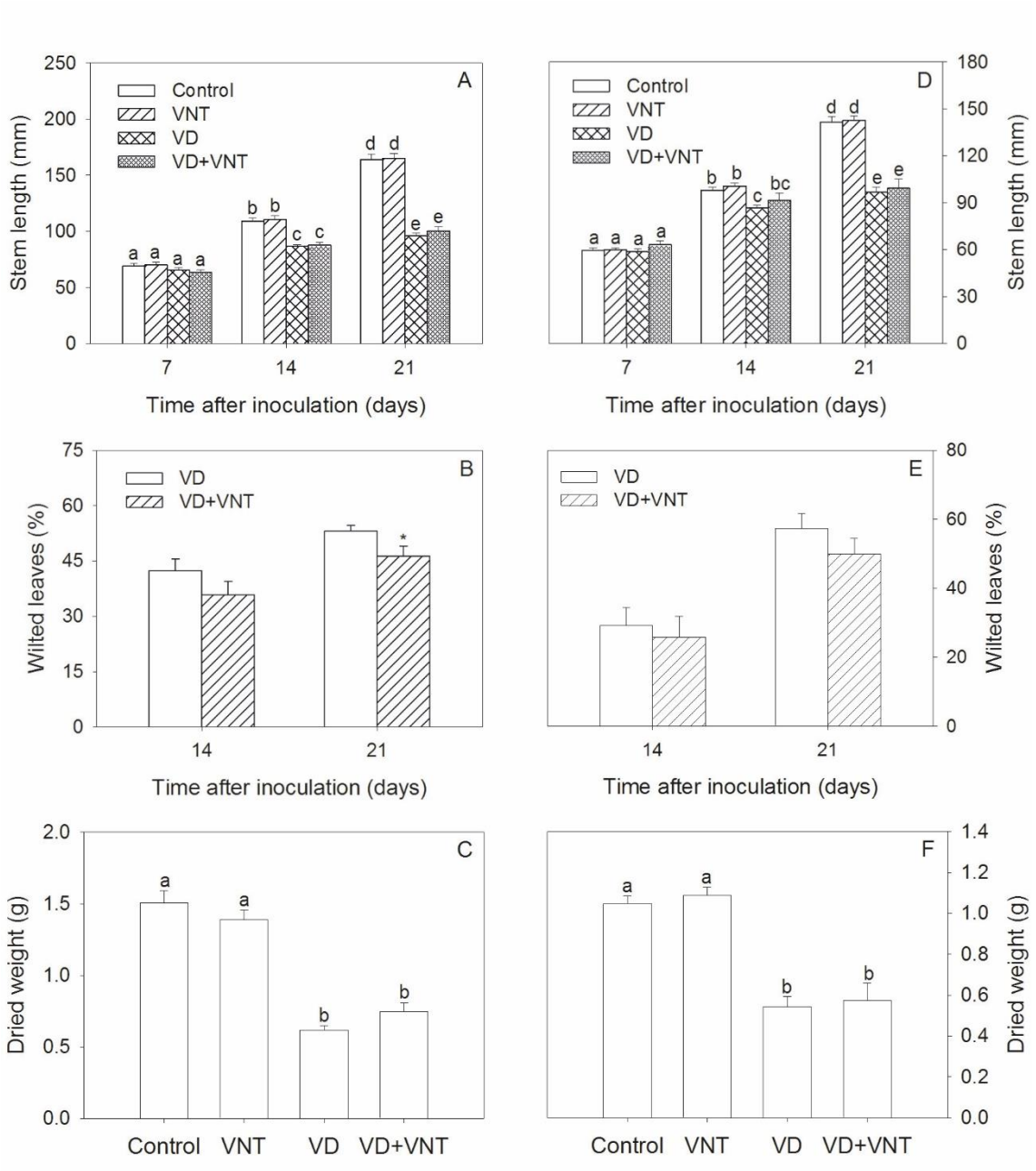

Figure 3.4. Effect of vanillyl nonanoate (VNT) applied by cotyledons (A-C) and by roots (D-F) of pepper on *Verticillium dahliae* (VD) infection. (A, D) Stem length. Data is the mean ± SE. Different letters mean significant differences ($p<0.05$) in ANOVA test followed by test post-hoc Student-Newman-Keuls. (B, E) Wilted leaves percentage. Data is the mean ± SE. The asterisks (*) means significant differences ($p<0.05$) in Mann-Whitney test. (C, F) Dried weight of plants at 21 days after inoculation. Data is the mean ± SE. Different letters mean significant differences ($p<0.05$) in ANOVA test followed by test post-hoc Student-Newman-Keuls. Data from graph F was transformed to obtain normality. Four independent experiments applying the treatment by cotyledons and two applying the treatment by roots were performed with 8 plants per treatment and experiment (n=32).

3.3.4. Systemic effect of VNT on lignification in roots inoculated with *Phytohthora capsici*

Three components involved in lignification were studied to determine the effect of VNT treatment on roots before inoculation (24 h after induction) and 8 h after *P. capsici* inoculation. Those components were: peroxidase activity (enzyme involved in the biosynthesis of lignin), soluble phenolics and amount of lignin and expression of two peroxidase genes (*CaPO1* and *CaPO2*). Both genes were selected because they respond to pathogens as well as they are involved inlignification (Do *et al*., 2003; Choi *et al*., 2007).

Before *P. capsici* inoculation, an increase of peroxidase activity was observed in VNT-treated plants (Figure 3.5.A). Moreover, this result was coincident with the increase in lignin at the same time (Figure 3.5.E). However, there were no differences in soluble phenolics (Figure 3.5.C).

After pathogen inoculation an increase on amount of lignin was also observed in VNT-treated plants compared to control plants (Figure 3.5.F) but there were no differences on peroxidase activity and soluble phenolics (Figure 3.5.B and Figure 3.5.D, respectively).

Regarding the effect of VNT treatment on the expression of two peroxidase genes, *CaPO1* increased its expression both before and after inoculation (Figure 3.6), whereas *CaPO2* increased only before inoculation (Figure 3.6).

3.3.5. Systemic effect of VNT on lignification in leaves inoculated with *Botrytis cinerea*

The effect of VNT on leaves was also studied, but the chosen pathogen was *B. cinerea*. The same parameters studied in roots were measured in leaves at the same time points.

Before *B. cinerea* inoculation, there was an increase in lignin in the leaves of VNT-treated plants (Figure 3.7.E), whereas there were no differences neither in peroxidase activity nor in soluble phenolics (Figures 3.7.A and 3.7.B, respectively).

On the other hand, changes in all the parameters were observed after Botrytis inoculation. There was an increase in peroxidase activity (Figure 3.7.B) and lignin (Figure 3.7.F) in VNT treated-plants, but these plants showed a decreased in the levels of soluble phenolics (Figure 3.7.D).

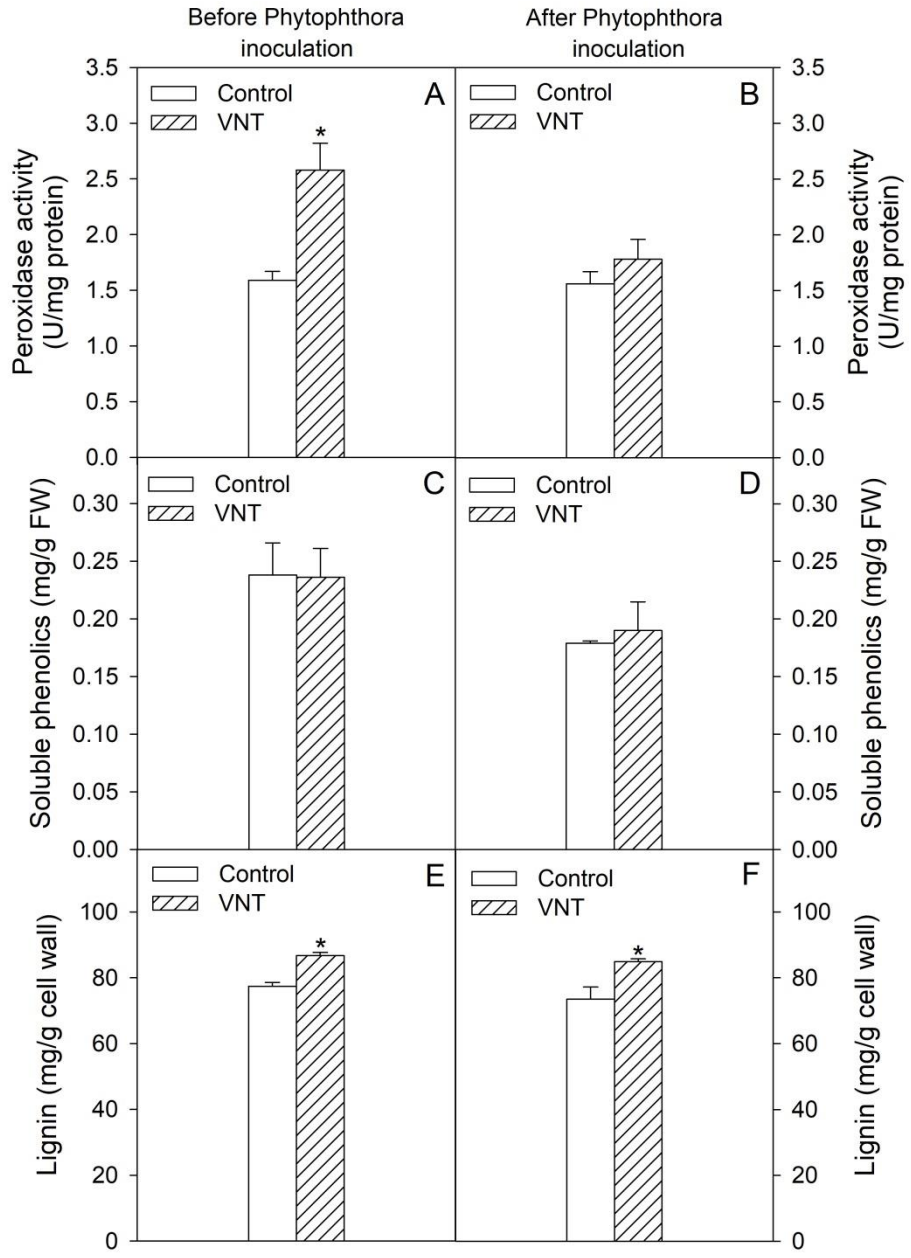

Figure 3.5. Systemic effect of VNT on three components involved in lignification process in roots before and after inoculation with *P. capsici*. (A-B): Peroxidase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. Four independent experiments were carried out. (C-D) Soluble phenolics. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. Four independent experiments were carried out. (E-F) Amount of lignin. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. Three independent experiments were carried out.

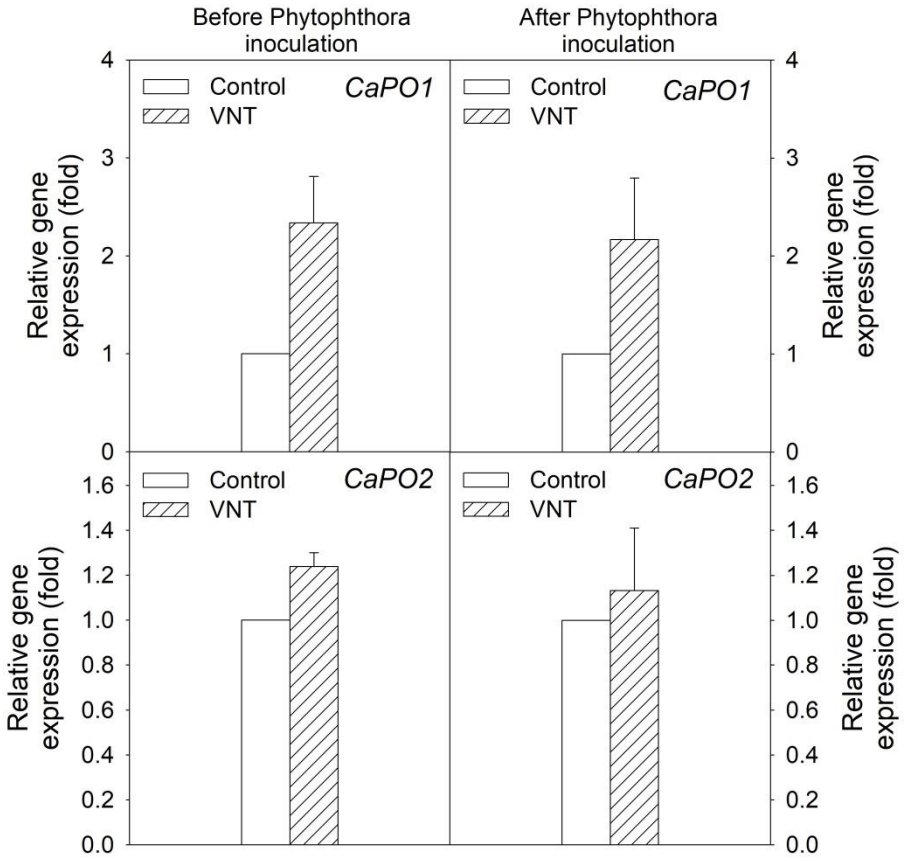

Figure 3.6. Systemic effect of vanillyl nonanoate (VNT) treatment on the expression of peroxidase genes *CaPO1* and *CaPO2* in the roots, before and after *P. capsici* inoculation. Data are the average ± SE of two independent experiments.

The effect of VNT treatment on gene expression of two peroxidases, *CaPO1* and *CaPO2*, was studied before inoculation and 8 h after inoculation with *B. cinerea*.

Before the inoculation, we were only able to detect and quantify the expression of *CaPO1* but not the expression of *CaPO2*. The levels of the expression of *CaPO2* were under the detection signal. However, the expression of both genes could be quantified after Botrytis inoculation. The VNT treatment induced the expression of *CaPO1* before and after inoculation (Figure 3.8). Also this treatment induced the *CaPO2* at 8 h after inoculation (Figure 3.8).

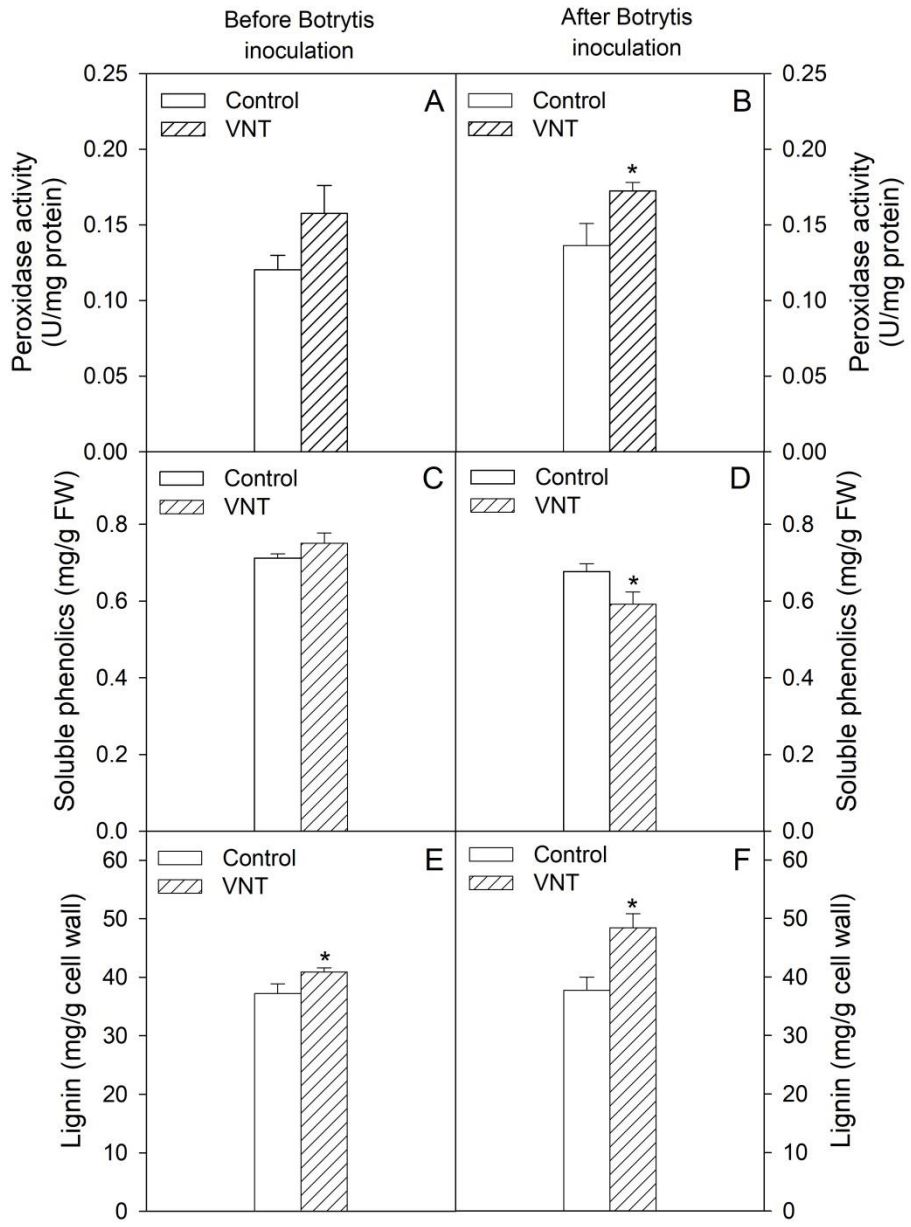

Figure 3.7. Systemic effect of vanillyl nonanoate (VNT) on three components involved in lignification process in leaves before and after the inoculation with *B. cinerea*. (A-B): Peroxidase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in Kruskall-Wallis test. Five independent experiments were carried out. (C-D) Soluble phenolics. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in Kruskall-Wallis test. Three independent experiments were carried out. (E-F) Amount of lignin. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in Kruskall-Wallis test. Three independent experiments were carried out.

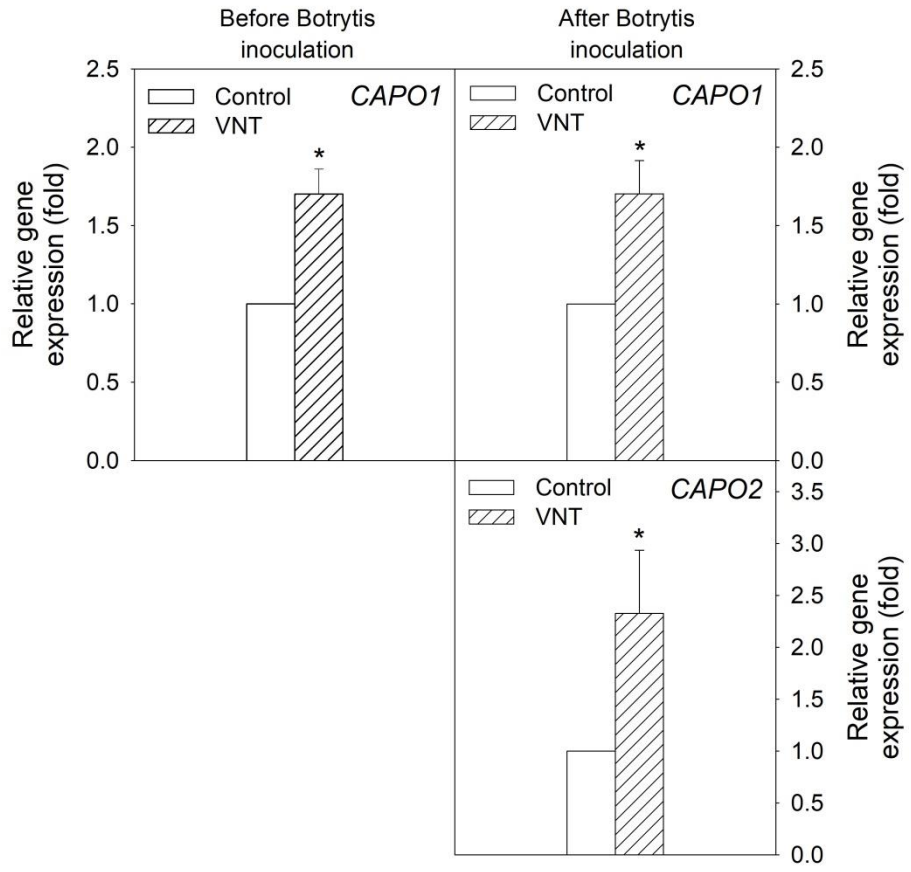

Figure 3.8. Systemic effect of VNT treatment on the expression of peroxidase genes *CaPO1* and *CaPO2* in the leaves, before and after *B. cinerea* inoculation. Data are the average ± SE. Between three and four independent experiments were carried out. The asterisk (*) indicates statistical differences ($p<0.05$) based on a Kruskal-Wallis test.

3.3.6. VNT induces biochemical defences in roots inoculated with *Phytophthora capsici*

The expression of *CaBPR1* (a basic PR1 protein) and *CaSC1* (a sesquiterpene cyclase responsible for capsidiol synthesis) was measured before (24 h after induction) and 48 h after inoculation. Previous work in our lab (Silvar *et al*., 2008, 2009; Veloso & Díaz, 2012; Veloso *et al*., 2014) demonstrated that there is a correlation between the expression of these genes and the induced resistant by different agents.

CaBPR1 expression was induced in VNT-treated plant after the inoculation with *P. capsici* but no differences were observed before the inoculation (Figure 3.9). However, *CaSC1* was induced both before and after Phytophthora inoculation (Figure 3.9).

Also we quantified the activity of β-1,3-glucanase and chitinase. However, no differences were observed in the activity of both enzymes due to the treatment except in β-1,3-glucanase before the infection (Figure 3.10).

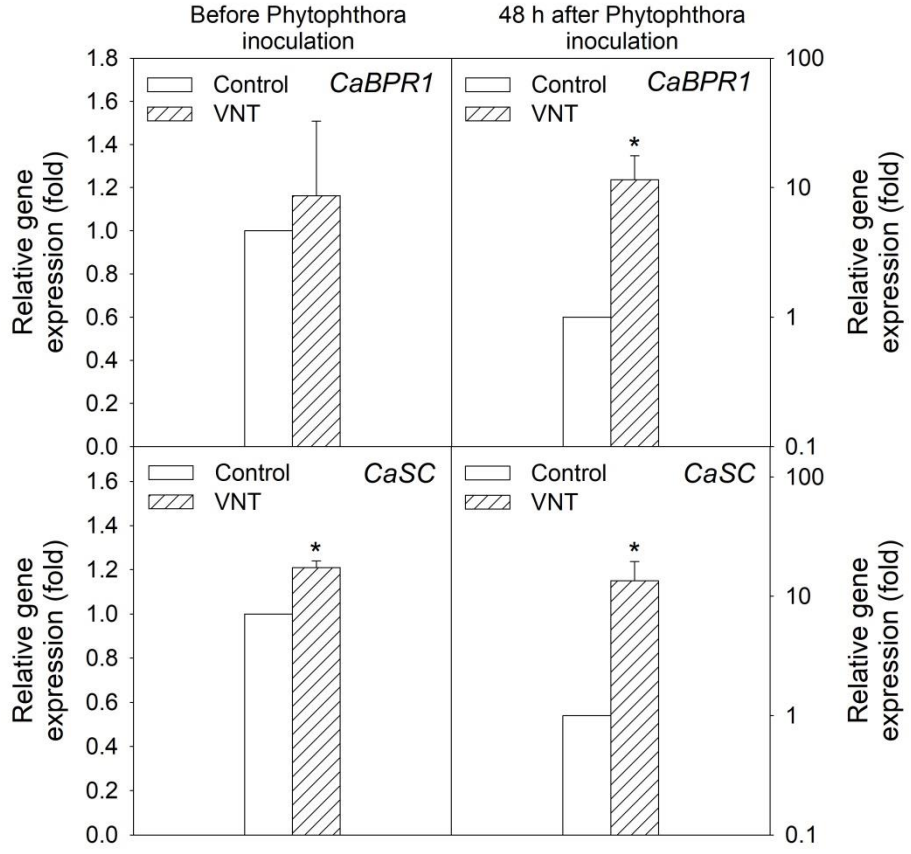

Figure 3.9. Systemic effect of vanillyl nonanoate (VNT) treatment on gene expression of *CaBPR1* (a PR1 proteins), and *CaCS1* (a sesquiterpeno cyclase) in roots of pepper before and 48 h after the inoculation with *P. capsici*. Data are the average ± SE. Between four and five independent experiments were carried out. Asterisk (*) indicates statistical differences ($p<0.05$) based on a Kruskal-Wallis test.

3.3.7. VNT induces an increase on biochemical defences in leaves before inoculation with *Botrytis cinerea*

The gene expression of two PR proteins, PR-1 and β-1,3-glucanase, was measured before (24 h after induction) and after Botrytis inoculation. Before inoculation, the expression of both genes was induced by VNT (Figure 3.11). However, after inoculation, there were no differences between control and VNT-treated plants (Figure 3.11).

In addition to β-1,3-glucanase gene expression, this enzymatic activity was measured at the same time points. Unlike gene expression, the activity of this enzyme did not show any differences due to VNT treatment or effect of pathogen (Figure 3.12.A and 3.12.B). Same result was observed in the activity of the enzyme chitinase (Figure 3.12.C and 3.12.D).

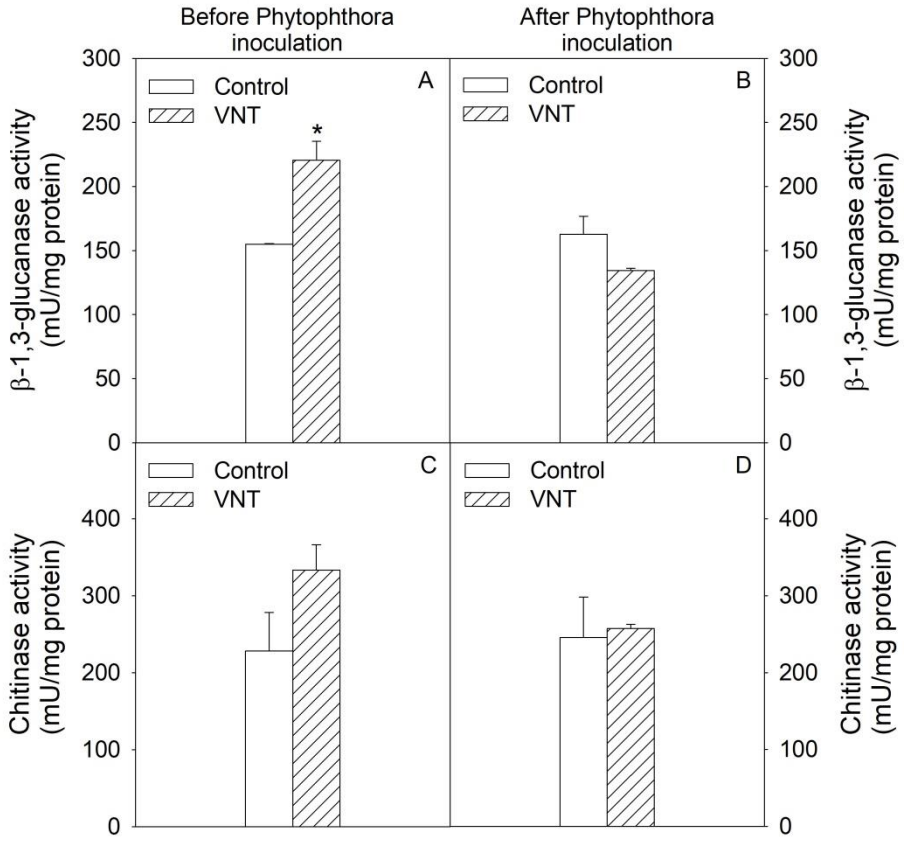

Figure 3.10. Systemic effect of vanillyl nonanoate (VNT) treatment on the activity of two enzymes involved in pathogen cell wall degradation in root before and after the inoculation with *P. capsici*. (A-B) β-1,3-glucanase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in a t-Student test. Four independent experiments were carried out. (C-D) Chitinase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in a t-Student test. Four independent experiments were carried out.

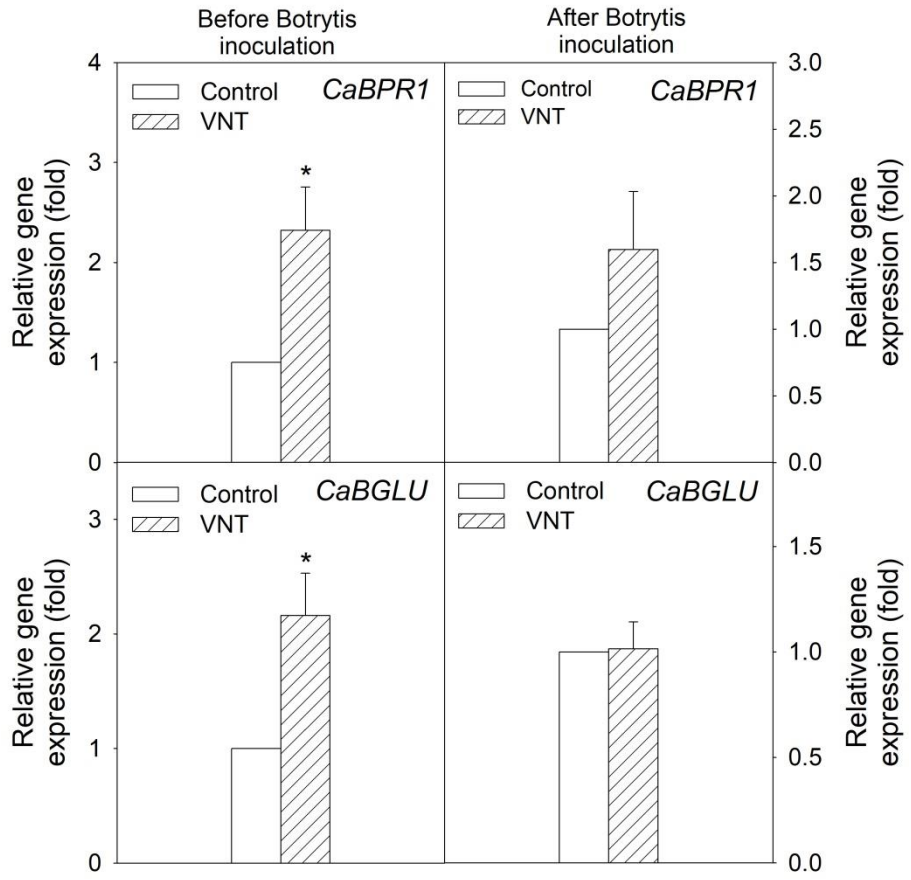

Figure 3.11. Systemic effect of vanillyl nonanoate (VNT) treatment on gene expression *CaBPR1* (a PR1 protein) and β-1,3-glucanase in leaves before and after the inoculation with *B. cinerea*. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in Kruskall-Wallis test. Between two and five independent experiments were carried out.

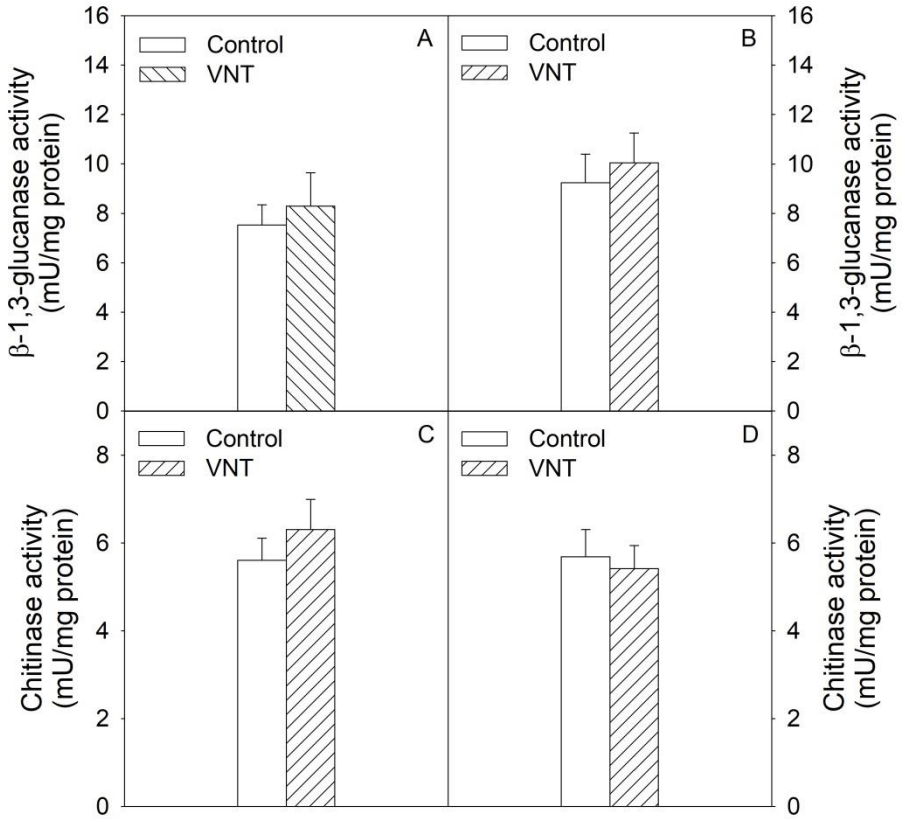

Figure 3.12. Systemic effect of vanillyl nonanoate (VNT) treatment on activity of two enzymes involved in pathogen cell wall degradation in leaves before and after the inoculation with *B. cinerea*. (A-B) β-1,3-glucanase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in a t-Student test. Four independent experiments were carried out. (C-D) Chitinase activity. Data are the average ± SE. The asterisk (*) means significant differences ($p<0.05$) in a t-Student test. Four independent experiments were carried out.

3.4. Discussion

In the previous chapter, we checked the antifungal effect of VNT both in *vivo* and in *vitro*. *In vivo* assays with *B. cinerea* and *P. capsici* showed that VNT to reduce the severity of the diseases in the place where the compound was applied (local effect), whereas in *vitro* assays showed inhibitory activity against *P. capsici* and no antifungal activity against *B. cinerea*. Moreover, it was shown that VNT induced local plant defences, as PR genes, suggesting a local induced resistance. However, there is also the possibility that a systemic response occurs. To address this hypothesis, in the present chapter we carried out systemic bioassays in which VNT was applied by infiltration in cotyledons and 24 h later secondary leaves or roots were inoculated. The studied

pathogens were *B. cinerea* for leaves and *P. capsici* and *V. dahliae* for roots. These assays demonstrated that VNT caused a reduction in the symptoms and the biomass of *B. cinerea* and *P. capsici*, but it had no effect on verticillium wilt. *V. dahliae* VD53 is a strongly virulent isolate. This means that the host inoculated with this isolate shows a high severity of symptoms hiding the low effect of an inducer. Because of this, it would be necessary to reduce the concentration of the inoculum of *V. dahliae*. This would reduce the severity of symptoms and, then, we could check if VNT induce resistance against this pathogen. However, the treatment of a pungent compound (capsaicin or N-vanillylnonamide) reduced symptoms under standard conditions of inoculation of *V. daliae* (Veloso *et al*., 2014). This suggests that the differences in molecular structure, this is, amide bond in capsaicinoids and ester bond in capsinoids could be important for induced defence against *V. dahliae*.

In the tests VNT was applied in a part of the plant (cotyledons) different from the organs where the pathogens were inoculated. Furthermore, the amount of VNT applied to the plant was very small (ca. 250 µl of a 150µM solution), therefore even in the case the compound could be translocated to other organs, its direct inhibition of the pathogen growth would be negligible. This implies that the observed protection is not caused by the toxicity of the compound on the pathogens, but to the possible generation of a plant response to VNT. Then VNT could generate a signal in cotyledons (treated organ, local level) that would induce plant defences at the systemic level. This is the case for other inducers such as BTH (benzothiadiazole) or BABA (β-amino-butyric acid). When these compounds are applied to the plant, they induce defences both at the systemic and local levels (Görlach *et al*., 1996; Zimmerli *et al*., 2001; Buonaurio *et al*., 2002; Šašek *et al*. 2012). Which mechanisms are involved in VNT-induced resistance? To address this question we performed further analyses of some parameters related to plant cell wall lignification as well as some biochemical defences.

As it was mentioned in the introduction, the cell wall represents the first barrier that pathogens have to face, and lignin is an important polymer in its composition. Lignin is not a static barrier, since the levels of this compound and its composition vary during the attack of a pathogen (Moura *et al*., 2010; Novo-Uzal *et al*., 2013; Zhao & Dixon, 2014). In this study the amount of lignin was quantified before and after *B. cinerea* and *P. capsici* inoculation, and an increase in lignin deposition was detected in both leaf and root cell walls of VNT-treated plants at both time points. At the leaf level,

a correlation between resistance to *B. cinerea* in pepper and abundance of lignin has recently been published (García *et al*., 2015). Moreover, in other plant species it has been demonstrated that lignin deposition plays an important role in the plant defence against this pathogen (Lloyd *et al.*, 2011; De Cremer *et al*., 2013). In fact, Arabidopsis mutants with impaired lignin biosynthesis such as *ref3-2*, which present reduced sinapate esters and guaiacyl and syringyl residues, or *fah1-2*, which present reduced sinapoyl malate, syringyl lignin and sinapoyl choline, showed an increase of susceptibility to *B. cinerea* (Lloyd *et al*., 2011).

Reinforcement of the cell wall also contributes to resistance induced against *P. capsici*, as demonstrated in the present work. Also Egea *et al*. (2001) observed a correlation between degree of lignification and genotype-specific resistance to *P. capsici*. These authors measured the amount of lignin in three pepper cultivars showing a different degree of resistance to this pathogen, Smith-5 (resistant), American (intermediate) and Yolo Wonder (susceptible). After inoculation they observed that the Smith-5 cultivar presented higher amount of lignin in comparison to the rest of cultivars (Egea *et al*., 2001). A similar result was observed by Vandana *et al*. (2014) in inoculated *Piper nigrum* cultivars that are susceptible or resistant to *P. capsici*, because they observed higher content of lignin in the roots of the resistant cultivar both before and after *P. capsici* inoculation. Khan *et al*. (2018) found that dirigent (DIR) proteins involved in lignification were expressed in pepper leaves as a response to *P. capsici* infection, and lignin increased as well with the inoculation. Moreover, silencing of one of the DIR genes (*CaDIR7*) made the leaves of the plant more susceptible to *P. capsici* (Khan *et al*., 2018). All these studies showed that high lignin amount in cell walls is correlated with an increase in plant resistance. Therefore the reinforcement of cell wall induced by VNT treatment might explain the reduction in symptoms and colonization observed during the bioassays with *B. cinerea* and *P. capsici*.

Several studies have demonstrated that the reinforcement of cell wall is correlated with an induction of the peroxidase activity and the phenylpropanoid pathway (Almagro *et al*., 2009). The peroxidases together with laccases catalyse the last step of lignin biosynthesis, that is, the oxidation of monolignols and polymerization (Novo-Uzal *et al*., 2013). Overall, peroxidase activity was induced in roots and leaves. At the root level, the induction in peroxidase activity as well as the expression of *CaPO1* and *CaPO2* were observed before inoculation (24 h after VNT treatment and

without pathogen), in coincidence with the induction in lignin. Similar correlation was observer in other pathosystems (Egea *et al*., 2001; Mandal & Mitra, 2007; Vandana *et al*., 2014; Abhayashree *et al*., 2016). Lignin biosynthesis consumes phenols, therefore phenylpropanoid pathway is usually activated to increase their amount in the plant (Candela *et al*., 1995; Baysal *et al*., 2005). In our study the amount of soluble phenolics showed no differences between control and VNT-treated plants at any time point. These results suggest that the production and consumption are balanced. This is the amount of soluble phenolics produced and the amount of soluble phenolics consumed is the same. Therefore, when we measured the total of soluble phenolics, we did not see differences.

In the leaves the situation was similar. A significant induction of peroxidase activity by VNT was observed 8 h after inoculation and the induction in lignin deposition started before inoculation. The absence of significant differences in peroxidase activity before inoculation is probably due to variability of the samples, but a trend of increase of peroxidase caused by VNT was also observed. Moreover, VNT induced the expression of *CaPO1* both before and after inoculation. Asselbergh *et al*. (2007) observed that peroxidase activity and lignin content were higher few hours after *B. cinerea* inoculation, a result that coincides with our observations 8 h after inoculation. Yang *et al*. (2018) also reported the increase of lignin deposition and peroxidases as a mechanism explaining the resistance induced in tomato by a cell wall degrading enzyme from *B. cinerea*. In our case, induction of peroxidase activity and lignin deposition after inoculation was accompanied by a decrease in the levels of soluble phenolics. This fact suggests that the consumption of phenolic compounds for lignin biosynthesis is faster than their production.

VNT also induced induce systemically plant defence genes as we previously observed at the local level (chapter 2). As in the previous chapter, we measured the expression of *CaSC1*, *CaBPR1* and *CaBGLU1*. The first encodes a cyclic sesquiterpene, an enzyme that is involved in the synthesis of phytoalexin capsidiol. It is considered a critical step in the synthesis of this phytoalexin (Veloso *et al*., 2014). The correlation between enzyme activity and capsidiol biosynthesis was showed by Mandujano-Chávez *et al*. (2000) in tobacco cell suspensions. Moreover, the measurement of the expression of this gene and the synthesis of capsidiol have the same trend, that means, high levels of capsidiol involves high *CaSC1* expression (Mialoundama *et al*., 2009). In this study, the expression of *CaSC1* was only measured in roots. The results showed that VNT

induced the expression of *CaSC1* meaning that capsidiol biosynthesis was stimulated. In fact, the accumulation of capsidiol is important in plant defence against *P. capsici* (Egea *et al*., 1996; Dunn & Smart, 2015). Moreover, Silvar *et al*. (2008) observed higher expression of *CaSC1* in resistant pepper SCM334 than susceptible cultivars.

Together with capsidiol, PR1 protein is important in defences against oomycetes. In fact, the function of PR1 was unknown till 2017, when Gamir *et al*. (2017) discovered its capacity of binding sterols, an important component of the cell wall. Gamir *et al*. (2017) studied the acidic protein PR1a from tobacco and the basic protein P14c from tomato. Gamir *et al*. (2017) observed that PR1 proteins acted sequestrating sterols and inhibiting pathogen growth. In fact, they observed that the plants infected with the oomycete *Phytophthora brassicae* accumulated more PR1 than those infected with the fungus *B. cinerea* (Gamir *et al*., 2017). In fact, Silvar *et al*. (2008) observed an increase of *CaBPR1* in resistant (SCM334) and moderately resistant (USDA PI210234) pepper cultivars inoculated with *P. capsici*. Also in this chapter we observed higher expression of *CaBPR1* in roots than in leaves.

In addition to PR1, we measured two enzymes involved in pathogen cell wall degradation: β-1,3-glucanase and chitinase. β-1,3-glucanases act on β-1,3-glucan and usually work together with chitinases which degrade chitin. These enzymes have two roles in plant defence: disrupting fungal cell wall and producing PAMPs (Balasubramanian *et al*., 2012). VNT induced the activity of β-1,3-glucanases only at 24 h after induction in roots. There were no differences in chitinases. In leaves, VNT induced the expression of *CaBGLU1* at 24 h after induction, but the activity of the enzyme did not show differences with the control. This gene is part of a multigenic family. When a protein BLAST is carried out, several sequences corresponding with other β-1,3-glucanases are obtained. This could explain the difference between gene expression and enzymatic activity. In this work only the expression of one gene from the multi-genic family was measured but the average enzymatic activity was measured. Also post-transcriptional regulation could also explain the difference between gene and enzymatic activity. This is, we observed induced expression of *CaBGLU1* but maybe the resulting protein is an isozyme with low activity.

To summarize, VNT is able to reduce the symptoms in systemic tissues and also the plant colonization by *P. capsici* and *B. cinerea*. This suggests that VNT induces the plant resistance against these pathogens. The defensive mechanisms involved are the

reinforcement of cell wall by lignin deposition and peroxidase activation in both roots and leaves. In addition, VNT treatment induced the expression of PR1 gene and biosynthesis of capsidiol gene and the activity of β-1,3-glucanase and chitinase in roots. The expression of *CaBPR1* and β-1,3-glucanase were also induced by VNT in leaves.

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CHAPTER 4.

Signalling involved in the resistance induced by vanillyl nonanoate in pepper plants.

4.1. Introduction

The activation of inducible defences requires the initial recognition of the pathogen by the plant. Upon recognition plant cells in the penetration point begin to produce signals that will activate defensive responses. Among the many signals orchestrating the defence response the reactive oxygen species (ROS) are considered the first players. The first ROS formed after pathogen recognition is the anion superoxide (O_2^-) but it is quickly degraded to hydrogen peroxide (H_2O_2) which is more stable (Torres, 2010). In general, ROS levels are balanced between production and degradation. This balance is broken during biotic or abiotic stress, when ROS levels increase rapidly. This phenomenon is known as oxidative burst and it is localized in the apoplast (Torres, 2010; Gilroy *et al.*, 2014).

There are several sources of ROS activated during PTI and ETI responses. Among them the main source for ROS production is the enzyme NADPH oxidase also known as "respiratory burst oxidase homologue" (RBOH) in plants. The importance of this enzyme in plant-pathogen interactions has been demonstrated in *Arabidopsis thaliana*, *Nicotiana tabacum*, *Nicotiana benthamiana*, *Oryza sativa* and *Solanum tuberosum* (Kadota *et al.*, 2015). In addition to NADPH, there are other enzymes that contribute to ROS production, such as cell wall peroxidases, diamine oxidases, oxalate oxidases, lipoxygenases and quinone reductases. The ROS production localizes not only in the apoplast but also in chloroplasts, mitochondria and peroxisomes (Torres, 2010; Camejo *et al.*, 2016). Several studies have suggested that the origin of ROS production may depend on the specific plant-pathogen interaction (Torres, 2010).

When the plant is able to recognize the pathogen attack and, finally, it can activate the defence machinery, the first step to initiate that process is the release of ROS. The accumulation of ROS is biphasic with two well characterized phases. The first phase occurs within minutes after pathogen recognition and it is unspecific, transient and of low amplitude. The second phase takes place hours after recognition and it has been observed to be sustained and of high amplitude (Torres, 2010; Camejo *et al.*, 2016). Normally the second phase is associated with the establishment of plant defences and HR (Torres, 2010).

The oxidative burst has multiple functions during pathogen challenge. ROS have been considered as antimicrobial due to their reactivity. ROS, specially the most

reactive forms, can directly kill the pathogen (Torres, 2010). On the other hand, ROS are used as signalling molecules by the plants to activate the defensive response. The reasons that make important these compounds in signalling are: rapid and dynamic control of ROS balance in individual cells and compartmentalization of ROS in several subcellular organelles that facilitates their control (Lehmann *et al.*, 2015).

Besides ROS, other factors that modulate the plant immune responses are the plant hormones, mainly SA, JA and ET although other hormones are also involved (Pieterse *et al*., 2012). The concentration of these hormones as well as their interaction with each other and timing provide the plant with a wide network to adequately process the stimuli and respond properly to the different kind of stresses (Pieterse *et al*., 2012).

It has been described that ROS triggers SA production after initial recognition (Veloso *et al.*, 2014a). SA is a major player in the plant response to pathogens. It is involved in many plant-pathogen interactions but it is typically associated with defence against biotrophs and the biotrophic phase of hemibiotrophs. It is the most important hormone in the establishment of SAR (Derksen *et al.*, 2013). SA is a phenolic compound synthetized in plastids by two main pathways: the isochorismate synthase (ICS) pathway and the phenylalanine ammonia-lyase (PAL) pathway. During a biotic stress, over 90% of SA production comes from ICS pathway (Kumar, 2014). However, in cases, in which PAL pathway was altered, the plant resistance was compromised (Pallas *et al*., 1996; Kim & Hwang, 2014). It has even been described that both pathways are needed for pathogen resistance (Shine *et al*., 2016).

SA can be accumulated as inactive form after being glucosylated, methylated, conjugated with amino acids or other chemical modifications. The first inactive form, glucosylated salicylic acid (SAG), is the most common storage form and it serves as a SA-reservoir for rapid release of SA during the immune response mediated by the enzyme SA β-glucosilase (Kumar, 2014).

NPR1 is a key regulator in the signalling downstream of SA. During pathogen attack NPR1 is released from oligomeric form and NPR1 monomers migrate to the nucleus (Hammond-Kosack & Jones, 2015). Once in the nucleus, NPR1 interact with the TGA family of transcription factors activating the expression of PR genes. WRKY transcription factors are also activated and have a role controlling SA signalling

pathway (Pieterse *et al*., 2012). Two paralogues of NPR1, NPR3 and NPR4 have been proposed to be receptors of SA (Veloso *et al*., 2014a).

JA belongs to the group known as oxylipins and it has been associated with insect and necrotroph attack (Derksen *et al*., 2013) and it has been regarded as the antithesis of SA. The synthesis of JA begins in the chloroplast with the liberation of α-linoleic acid from membranes and formation of cis-12-oxo-phytodienoic acid (OPDA) in which are involved the enzymes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Wasternack & Hause, 2013). Subsequently, OPDA moves to peroxisomes where is reduced to JA (Schaller & Stintzi, 2009). JA is conjugated with the amino acid isoleucine by the enzyme JAR1 (JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE1) to form (+)-7-iso-jasmonoyl-L-isoleucine (JA-ILE) that is considered as the active form of JA in higher plants. JA-ILE is formed when the plant is under stress (Pieterse *et al*., 2012; Wasternack & Hause, 2013).

During an attack, JA-ILE is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1) in the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI}. In absence of JA-ILE, the JA-responsive genes are repressed by JASMONATE ZIM (JAZ) proteins. The binding of JA-ILE to COI1 triggers the degradation of the JAZ repressor proteins leading to the transcriptional activation of JA-responsive genes (Pieterse *et al*., 2012; Hickman *et al*., 2016).

ET is a gaseous hormone that usually works synergistically with JA. ET is synthesized from methionine through the Yang cycle and the participation of enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and oxidase (ACO) (Wang *et al*., 2002).

ET is perceived by a group of receptors formed by ETR1 (ETHYLENE RESPONSE 1), ERS1 (ETHYLENE RESPONSE SENSOR 1), ETR2 (ETHYLENE RESPONSE 2), ERS2 (ETHYLENE RESPONSE SENSOR 2) and EIN4 (ETHYLENE INSENSITIVE 4) (Solano & Gimenez-Ibanez, 2013). In absence of ET, these receptors inhibit the protein EIN2 (ETHYLENE INSENSITIVE 2) through CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1). In presence of ET the repression over EIN2 gets released. EIN2 moves to the nucleus and activates the transcription factors EIN3 (ETHYLENE INSENSITIVE 3) and EIL1 (EIN3-LIKE 1) which activate the ET-

response genes (Solano & Gimenez-Ibanez, 2013). Moreover, EIN3, EIL1 and JAZ modulate the cross-talk between ET and JA (Pieterse *et al*., 2012).

These three hormones, SA, JA and ET, never act independently of each other, there is an intense cross-talk. They can be antagonistic or synergistic depending on the plant-pathogen interaction. Typically, JA and ET are synergists and both are antagonists of SA. On the other hand, some cases have been registered in which SA, JA or ET worked together but it depends on the plant-pathogen interaction. There are interactions in which a protein is induced by both SA and JA (Kim & Hwang, 2000; Derksen *et al.*, 2013). Also it has been observed that ET is able to induce PR1 in *Nicotiana glutinosa* and pepper (Kim & Hwang, 2000; Derksen *et al.*, 2013) and SA induced ACO in *N. glutinosa* (Derksen *et al.*, 2013). Moreover, the transcription factor EREBP1 (ETHYLENE RESPONSE ELEMENT BINDING PROTEIN 1) is an integration point between SA, JA and ET. Furthermore, the transcription factors CRP5 and CRP6 integrate the SA and JA signalling in Arabidopsis (Derksen *et al*., 2013).

The aim of this chapter was to study the role of ROS and hormones in the resistance induced by VNT in pepper.

4.2. Material and methods

4.2.1. Pathogen material and plant material

4.2.1.1. Pathogen material

Phytophthora capsici Leon. isolate PC450 was kindly provided by Dr. Frank Panabieres (INRA, France). *P. capsici* was grown in V8 agar (100 ml of eight vegetables puree, 1 g of $CaCO_3$, 7.5 g agar and 400 ml of distilled water) at 24ºC and darkness. Periodically, when the pathogen grew slow and/or showed low virulence, *P. capsici* was inoculated in pepper plants and then re-isolated from them to keep its virulence and growth.

Botrytis cinerea Pers:Fr isolate B05.10 was kindly provided by Dr. Jan van Kan (Laboratory of Phytopathology, Wageningen University, The Nederlands). To perform the assays, *B. cinerea* was grown in PDA medium in a constant temperature of 24ºC and in darkness.

4.2.1.2. Plant material

Pepper plants of *Capsicum annum* L. var. *annum* cv. Padrón were used 27 days after sowing.

Seeds were obtained in previous years and stored dry at 4 ºC. Before sowing the seeds were disinfected in 1% commercial bleach (sodium hypoclorite) solution during 30 min. Then the seeds were rinsed with water and sown in vermiculite. In this step, the seeds were watered with Hoagland nutrient solution: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2mM $NH_4H_2PO_4$, 1mM $MgSO_4$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM H_2MoO_4, 20 µM EDTA y 20 µM $Fe(NH_4)2(SO_4)$ (Hoagland & Arnon, 1950). Two weeks later, the plants were transferred individually to pots filled with soil:perlite (3:1 v/v) and watered regularly.

Plants were grown in a growth chamber with 16 h light at 25ºC and 8 h of darkness at 18ºC.

4.2.2. Inoculation and determination of symptoms

4.2.2.1. *Phytophthora capsici*

Pepper plants were inoculated with *P. capsici* by adding a zoospore suspension in the plant collar area. Zoospores were obtained from a plate in which the fungus was grown for 5-6 days in V8 agar medium. The plate was cut into pieces and placed in KNO_3 0.01 M liquid medium. This culture was grown for 5-6 days at 24ºC with constant light and shaking to stimulate sporangium formation. Zoospore release from the sporangium was induced by keeping the culture at 4ºC for 45 min and at room temperature for another 45 min. The culture was filtrated through sterile gauze obtaining the zoospores in the filtrate. A Malassez counting chamber was used to determine the zoospore concentration. This suspension was diluted to 10^3 zoospore/ml and 5 ml from this suspension was applied to the plant collar area. Plants were flooded before inoculation. Symptoms were evaluated for 5 days. To determine the severity of symptoms three parameters were used (hypocotyl rot, wilted leaves and plant dead) which had a maximum value of 3, 3 and 1 respectively (see table 4.1). The sum of these parameters is the severity index which has values between 0 and 7. Severity index was

used to calculate the AUDPC (area under the disease progress curve) following this equation (Madden & Campbell, 1990):

$$AUDPC = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where y is the severity value for observation number i, t is the number of days after inoculation that have passed until the time of observation and n is the number of observations.

Table 4.1. Scale used to determine severity symptoms caused by *Phytophthora capsici*.

Parameter	Value	Meaning
Rot	1	One part of hypocotyl with lesion
	2	Between one and two parts of hypocotyl with lesion
	3	Almost all hypocotyl with lesion
Wilt	1	Few leaves are wilted
	2	More than 50% leaves are wilted
	3	All leaves are wilted
Dead	1	Plant is dead

4.2.2.2. *Botrytis cinerea*

The inoculation with this pathogen was performed with plugs of 5 mm of diameter from a 4 days old PDA culture. Plugs were placed onto the leaf surface avoiding main veins. Plants were incubated in a wet chamber at room temperature.

Disease evolution was followed for 48 or 72 h depending on the experiment and the diameter of each expanded lesion was measured. A lesion was expanded when its diameter was equal or greater than 5 mm. Disease area was calculated using this diameter.

4.2.3. Vanillyl nonanoate treatment

Before inoculation pepper plants were treated with vanillyl nonanoate (VNT) 150 µM. This compound was infiltrated in both cotyledon through the stomata with ca. 250 µl of 150 µM VNT previously dissolved in 0.1% dimethyl sulfoxide (DMSO) (Veloso *et al*., 2014b).

Plants were transplanted in pots containing soil:perlite 3:1 (v/v) 2 weeks after sowing. During this time plants were watered with tap water. VNT treatment was applied 24 h before inoculation and plants were incubated in the growth chamber. Plants infiltrated with 0.1% DMSO were used as a control.

4.2.4. Treatment with inhibitors of ethylene perception, jasmonate biosynthesis and peroxide production

4.2.4.1. Inhibition of ethylene

Plants were treated with MCP (1-methylcyclopropene) before VNT treatment. MCP, an inhibitor of ethylene (Díaz *et al*., 2002), was applied to a final concentration of 200 ppb (Chmielowska *et al*., 2010). The plants were introduced in a sealed box, to guarantee the effect of the inhibitor. Plants without inhibitor treatment were used as a control. The duration of the treatment was 14 h. Subsequently the plants were treated with VNT and inoculated with *P. capsici* or *B. cinerea* as described in previous sections. At least two independent experiments were performed.

4.2.4.2. Inhibition of jasmonate synthesis

Before VNT treatment, plants were treated with ibuprofen, an inhibitor of the synthesis of jasmonic acid (Ren & Dai, 2012). This compound was dissolved in Hoagland solution at a final concentration of 0.1 mM and applied by irrigation. To avoid interferences between inhibitor and soil, plants were transplanted to perlite. Plants treated with Hoagland solution were used as controls. Treatment with ibuprofen was initiated 24 h before VNT treatment watering once per day till 48 h after Phytophthora inoculation. The plants were watered with Hoagland solution. Two independent experiments were performed.

4.2.4.3. Inhibition of hydrogen peroxide accumulation

Plants were treated with dithiothreitol (DTT) before VNT treatment. DTT is a reducing agent that scavenges the accumulation of hydrogen peroxide (Kováčik *et al*., 2010). This inhibitor was incorporated in Hoagland solution at a final concentration of 500 µM. It was administered by irrigation. To avoid interferences between inhibitor and soil, plants were transplanted to perlite. Plants treated with Hoagland solution were used as controls. Treatment with DTT was initiated 24 h before VNT treatment watering once per day with DTT during 3 days. Then plants were watered with Hoagland solution. The inoculation was carried out 24 h after VNT treatment. At least two independent experiments were performed.

4.2.5. Treatment with ethylene

Prior to inoculation, the plants were treated with ethylene with a final concentration of 1 ppm. Plants exposed to Purafil ™, an ethylene-absorbing compound were used as a control. In both cases, the plants remained in sealed boxes for 24 h before inoculation with *P. capsici*. Two independent experiments were performed.

4.2.6. Quantification of hydrogen peroxide in roots and leaves

The levels of hydrogen peroxide (H_2O_2) were quantified in roots and in leaves from plants treated with VNT and inoculated with *P. capsici* (roots) or *B. cinerea* (leaves). The methodology of Queval *et al*. (2008) was followed with some modifications.

Roots or leaves from five plants were collected at 0, 2, 4, 6 and 8 h after inoculation. In order to remove the substrate, roots were washed. The samples were homogenized with liquid nitrogen and 3 ml of 0.2 N HCl. Samples were centrifuged at 18514 xg for 5 min and 4°C and the supernatant was collected. A mix was made by adding 500 µl of the supernatant, 233 µl of 50 mM phosphate buffer pH 5.7 and 467 µl of 0.2 N NaOH. The mixture was centrifuged for 30 s at 16060 xg. This mixture constituted the sample. 100 µl from the sample was added to 1 ml of xylenol orange reagent and incubated for 30 min at room temperature. Subsequently the absorbance at 560 nm was measured. This determination was performed by triplicate for each sample and experiment.

A calibration curve with different concentrations of H_2O_2 was made to determine the concentration of H_2O_2.

4.2.7. Determination of hydrogen peroxide accumulation in leaves by DAB

The amount of H_2O_2 was quantified in leaves from plants treated with VNT and inoculated with *B. cinerea*. It was carried out according to Asselbergh *et al*. (2007).

Samples were analysed 8 h after inoculation. Four leaf discs of 13 mm of diameter were obtained from each plant and they were inoculated with *B. cinerea* mycelium discs. Then, they were incubated for 5 h in darkness and floating on distilled water. Subsequently the leaf discs were transferred to light conditions and the water was substituted by a solution of 3,3'-diaminobenzidine (DAB) (1 mg/ml) adjusted to pH 3.8.

To observe the DAB staining the leaves were bleached. The chlorophyll and other pigments were removed by incubating the disc in 80% ethanol for 10 min.

To take a picture, leaf discs were re-hydrated by incubation in 60% ethanol for 30 min and 30% ethanol for 30 min.

4.2.8. Evaluation of gene expression in *Capsicum annuum*

Gene expression was studied in roots and in secondary leaves from plants treated with VNT and inoculated with *P. capsici* or *B. cinerea*, respectively. Samples were analysed at 0, 8 or 48 h after inoculation depending on the tissue. For this purpose the roots or leaves of five plants were taken per treatment and per hour. The roots were washed in order to remove the soil. Samples were frozen in liquid nitrogen and stored at -80°C until RNA extraction. For that the samples were homogenized in liquid nitrogen. Then 60 mg of the resulting homogenate were taken for RNA extraction following the BioRad Aurum™ Total RNA Mini Kit protocol.

The amount of RNA present in the samples was quantified using a spectrophotometer (Helios γ, Thermo) and cDNA was synthesized following the protocol of the iScript™ cDNA Synthesis Kit by a reverse transcription PCR (RT-PCR). The obtained product was used to study the gene expression by real time quantitative PCR (qPCR). This step was carried out by Dr. M.ª Fernanda Rodríguez Fariña of the Molecular Biology Unit of the Research Support Services (SAI) from

Universidade da Coruña. The cDNA samples were analysed with the Biorad iCycler™ iQ System. The gene expression of four genes was evaluated: phenylalanine ammonia-lyase 1 (*CaPAL1*), 1-Aminocyclopropane-1-carboxylate synthase (*CaACS*), 1-Aminocyclopropane-1-carboxylate oxydase (*CaACO*) and allene oxide synthase (*CaAOS*). Actin (*CaACT*) was used as housekeeping gene (Veloso *et al*., 2014b). The primers used for amplification are shown in Table 4.1.

Primers for the genes *CaPAL1*, *CaACS* and *CaAOS* were designed from sequences of *C. annuum* available in NCBI (National Center for Biotechnology Information) database and using the software: Primer-BLAST from NCBI, Reverse Complement from Bioinformatics.org (http://www.bioinformatics.org/sms/rev_comp.html) and ClustalW2 - Multiple Sequence Alignment from EMBL-EBI (European Bioinformatics Institute).

The specificity of the primers was checked by standard PCR and qPCR of genomic DNA and cDNA. The PCR program started with a 2 min denaturation step at 94°C followed by 40 amplification cycles (each cycle consisting of 20 s at 94°C, 25 s at 58°C and 50 s at 72°C).

The conditions of qPCR were 2 min denaturation at 95°C followed by 40 amplification cycles (each cycle consisting of 20 s at 95°C, 25 s at 58°C and 50 s at 72°C).

Data analysis was performed using the Biorad Optical System Software 3.0. The efficiency and Ct (Cycle threshold) values obtained were calculated and processed by the Pfaffl method (2001) to obtain the relative expression values. This method defines the relative expression as:

$$Relative\ expression = \frac{E^{\Delta Ct_{target}\ (control-sample)}}{E^{\Delta Ct_{housekeeping}\ (control-sample)}}$$

where E is the efficiency and Ct is the number of cycles required to detect the amplicon signal.

At least two independent experiments were performed for each evaluated gene.

Table 4.1. 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
CaACS	X82265	Designed for this thesis	*CaACSFW* *CaACSRV*	5' TCTGCTTGCGTCAATGTTGTCTG 3' 5' TCCTCCACAGTTCCAATTCAGCA 3'	215 bp
CaACO	AJ011109	Carballeira, (2010)	*CaACOFW* *CaACORV*	5'CGCCACTCCATTGTG3' 5'TAGATTACTGCATCGCTTCC3'	152 bp
CaAOS	DQ832720	Designed for this thesis	*CaAOSFW* *CaAOSRV*	5'TGTCTACGAATCTCTCCGCA 3' 5' GGGACAAATTCTTCAGCCCT 3'	183 bp
CaACT	AY572427	Silvar *et al*. (2008)	*CaACTFW* *CaACTRV*	5'ATCCCTCCACCTCTTCACTCTC3' 5'GCCTTAACCATTCCTGTTCCATTATC3'	128 bp

4.2.9. Hormone quantification by liquid chromatography and ESI mass spectrometry

Different plant hormones were quantified in cotyledons and leaves from plants treated with VNT and inoculated with *B. cinerea*. Cotyledons and the first couple of secondary leaves were collected 0 h after inoculation and, also, samples inoculated and non-inoculated 8 h after inoculation. All the samples were immediately frozen in liquid nitrogen and stored at -80ºC until freeze drying. Then, they were homogenized and 50 mg were placed in a tube to be analysed according to Sánchez-Bel *et al*. (2018).

Samples were analysed by Victoria Pastor and Victor Flors (Metabolic Integration and Cell Signaling Group, Universitat Jaume I, Castellón, Spain).

For the hormonal extraction, 1 ml of Milli-Q water and internal standards containing a pool of abscisic 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 (2 mm of diameter) were added to each sample and then they were homogenized in a shaker for 3 min 30 Hz. Samples were centrifuged for 40 min at 15493.24 xg and 4ºC. 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 4ºC. Then the supernatant was evaporated in a Speedvac. The precipitated was resuspended in 1ml 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. Salicylic acid (SA), 4-hydroxybenzoic acid (4-HBA), jasmonoyl isoleucine (JA–Ile), oxo-phytodienoic acid (OPDA), abscisic acid (ABA), and IAA (indole acetic acid) were analysed in Acquity Ultraperformance Liquid Chromatography System (UPLC) coupled to triple quadrupole mass spectrometry (TQD) as described by Sánchez-Bel *et al.* (2018). 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 version 4.1 (Waters) software was used to process the data.

4.2.10. Statistical analyses

All the statistical analyses were performed using Statgraphics 5.1. ANOVA test followed by Student-Newman-Keuls test as a post-hoct test was used to analyse: H_2O_2 levels in roots, DTT assays, MCP and ibuprofen assays inoculating with *P. capsici* and quantification of 4-HBA and JA in leaves after *B. cinerea* infection. H_2O_2 levels in leaves, MCP assays inoculating with *B. cinerea* and of the rest of the measured hormones were analysed using Kruskal-Wallis test followed by post-hoc test according to Conover (1980). Also Kruskal-Wallis was used to analyse the expression of genes involved in hormone biosynthesis in root and leaves. Finally, t-Student test was used to analyse data obtained in the bioassay in which plants were elicited with ethylene.

4.3. Results

4.3.1. VNT increases hydrogen peroxide levels on roots infected with *Phytophthora capsici*

H_2O_2 acts as an early messenger in the immune response. To determine its participation in the response to VNT, H_2O_2 was measured during the first 8 hours after inoculation. A peak can be observed 4 h after inoculation in both control and VNT-treated plants (Figure 4.1). However, the intensity of the peak was higher in VNT-treated than in the control. Moreover, this signal was kept high till 6 h after inoculation by VNT treatment while the amount of peroxide in the control decreased until reaching pre-inoculation levels. At 8 h, both groups showed similar levels to the initial ones (Figure 4.1).

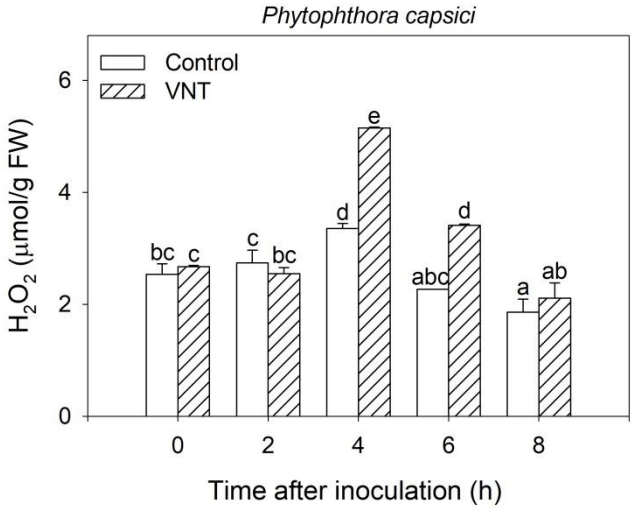

Figure 4.1. Effect of vanillyl nonanoate (VNT) on hydrogen peroxide levels in roots infected with *P. capsici*. Two independent experiments were performed. Data are the mean ± SE. Different letters mean statistical differences ($p<0.05$) in ANOVA test followed by Student-Newman-Keuls post-hoc test.

Since peroxide levels were higher in plants treated with VNT, bioassays were carried out treating the plants with the dithiothreitol (DTT) a few hours before VNT elicitation. DTT is an antioxidant that removes reactive oxygen species such as hydrogen peroxide.

During the assay, a reduction in symptoms was observed in plants treated with VNT (Figure 4.2). On the other hand, when plants were treated with DTT they showed higher AUDPC than non-treated plants. However, no differences were observed

between DTT-treated plants and plants treated with the combination of DTT and VNT (Figure 4.2). This indicates that DTT inhibits the VNT-induced resistance to *P.capsici* and points out that H_2O_2 is involved in the plant response to VNT.

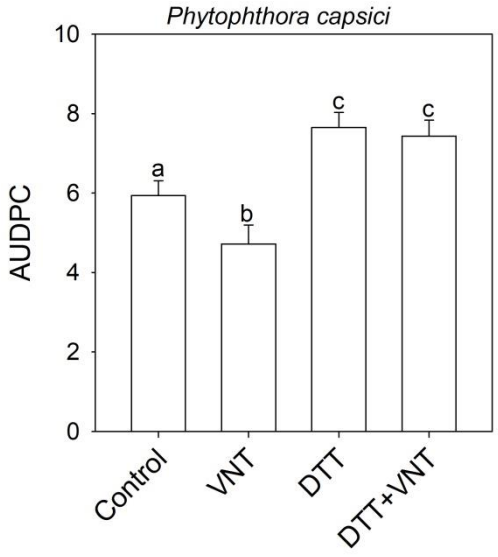

Figure 4.2. Effect of dithiothreitol (DTT) on vanillyl nonanoate (VNT)-induced resistance in plants infected with *P. capsici*. Four experiments were performed with 8 plants per group (n=32). The data are the mean ± SE. Different letters indicate statistical differences ($p < 0.05$) in an ANOVA test followed by Student-Newman-Keuls post-hoc test.

4.3.2. VNT increases hydrogen peroxide levels of secondary leaves infected by *Botrytis cinerea*

H_2O_2 levels were determined in the first 8 h after inoculation. A decrease in the levels of this molecule was observed after inoculation in all time points for plants not treated with VNT (Figure 4.3). However, VNT-treated plants showed an increment in H_2O_2 levels respect to the control 2, 4 and 6 h after inoculation with *B. cinerea* (Figure 4.3).

The accumulation of H_2O_2 was also studied in secondary leaves of VNT-treated plants by DAB staining.

In both groups a strong staining associated with the cutting was observed. Also a slight staining appeared in the veins. However, there was more staining at the inoculation site in VNT-treated plants compared with the control (Figure 4.4).

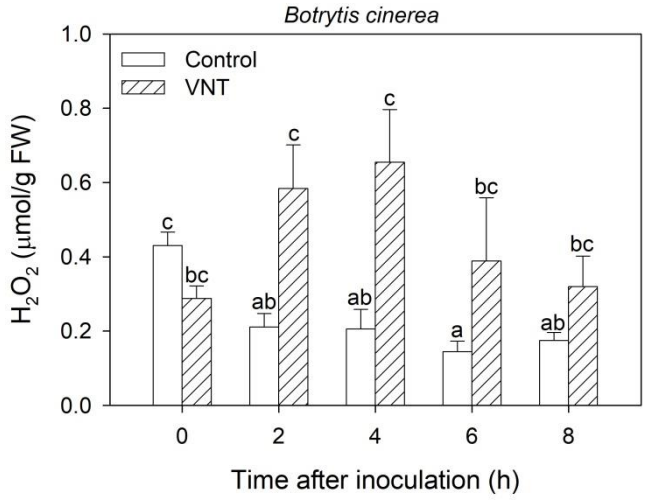

Figure 4.3. Effect of vanillyl nonanoate (VNT) on hydrogen peroxide levels after inoculation with *B. cinerea*. Four experiments were performed. The data are the mean ± SE. Different letters indicate statistical differences ($p < 0.05$) in a Kruskal-Wallis test followed by the post-hoc test according to Conover (1980).

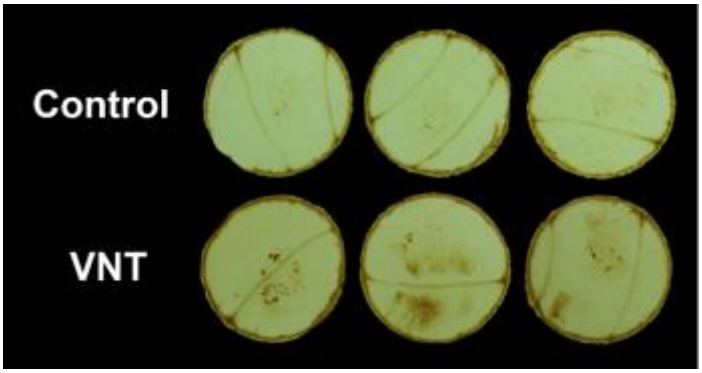

Figure 4.4. Hydrogen peroxide accumulation on secondary leaf discs of plants treated with vanillyl nonanoate (VNT) 8 h after inoculation with *B. cinerea*.

Because H_2O_2 levels were also increased by VNT in secondary leaves, the plants were also treated with DTT a few hours before VNT elicitation in order to know if ROS is important in leaves as well as it was in roots.

During the assay, a reduction on symptoms was observed in plants treated with VNT (Figure 4.5). On the other hand, the double treatment (DTT+VNT) caused an increased in the disease area. The disease area of DTT+VNT-treated plants was equal to the disease area of control plants (Figure 4.5). This indicated that DTT abolished the VNT effect suggesting that H_2O_2 is involved in VNT-induced resistance.

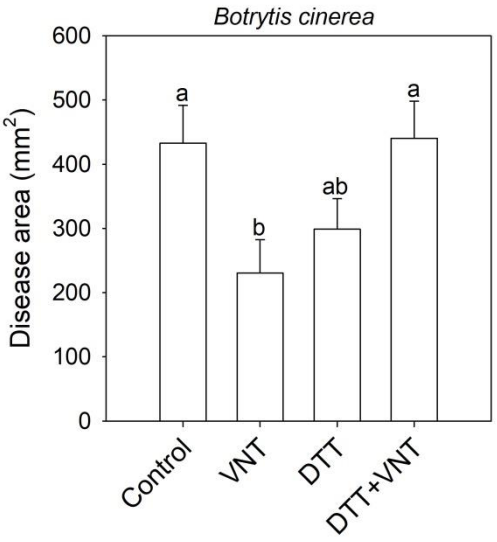

Figure 4.5. Effect of dithiothreitol (DTT) on vanillyl nonanoate (VNT)-induced resistance in plants inoculated with *B. cinerea*. Two experiments were performed with 6 plants per group (n=18). The data are the mean ± SE. Different letters indicate statistical differences ($p < 0.05$) in an ANOVA test followed by Student-Newman-Keuls post-hoc test.

4.3.3. VNT increases the levels of SA and ET biosynthetic genes in roots infected with *Phytophthora capsici*

The effect of VNT on defence hormones was evaluated by studying the expression of several genes involved in their biosynthesis: *CaPAL1* (SA), *CaACO* (ET) and *CaAOS* (JA).

The gen *CaPAL1* encodes the enzyme phenylalanine ammonia-lyase which is involved in SA biosynthesis and it also respondes to VNT treatment and pathogen attack (Kim & Hwang, 2014; García *et al*., 2018). The expression of this gene was not altered by the VNT treatment itself before inoculation (Figure 4.6). However, when plants were inoculated with *P. capsici*, *CaPAL1* expression increased at both 24 h and 48 h after inoculation (Figure 4.6).

The gene *CaACO* codes for ACC oxidase which is the last enzyme in ET biosynthesis. This gene showed a slight increase in its expression after VNT treatment and without pathogen (Figure 4.6). When plants were infected, no differences were observed 24 h after inoculation. Nevertheless, 48 h after inoculation *CaACO* was strongly up-regulated in VNT-treated plants (Figure 4.6).

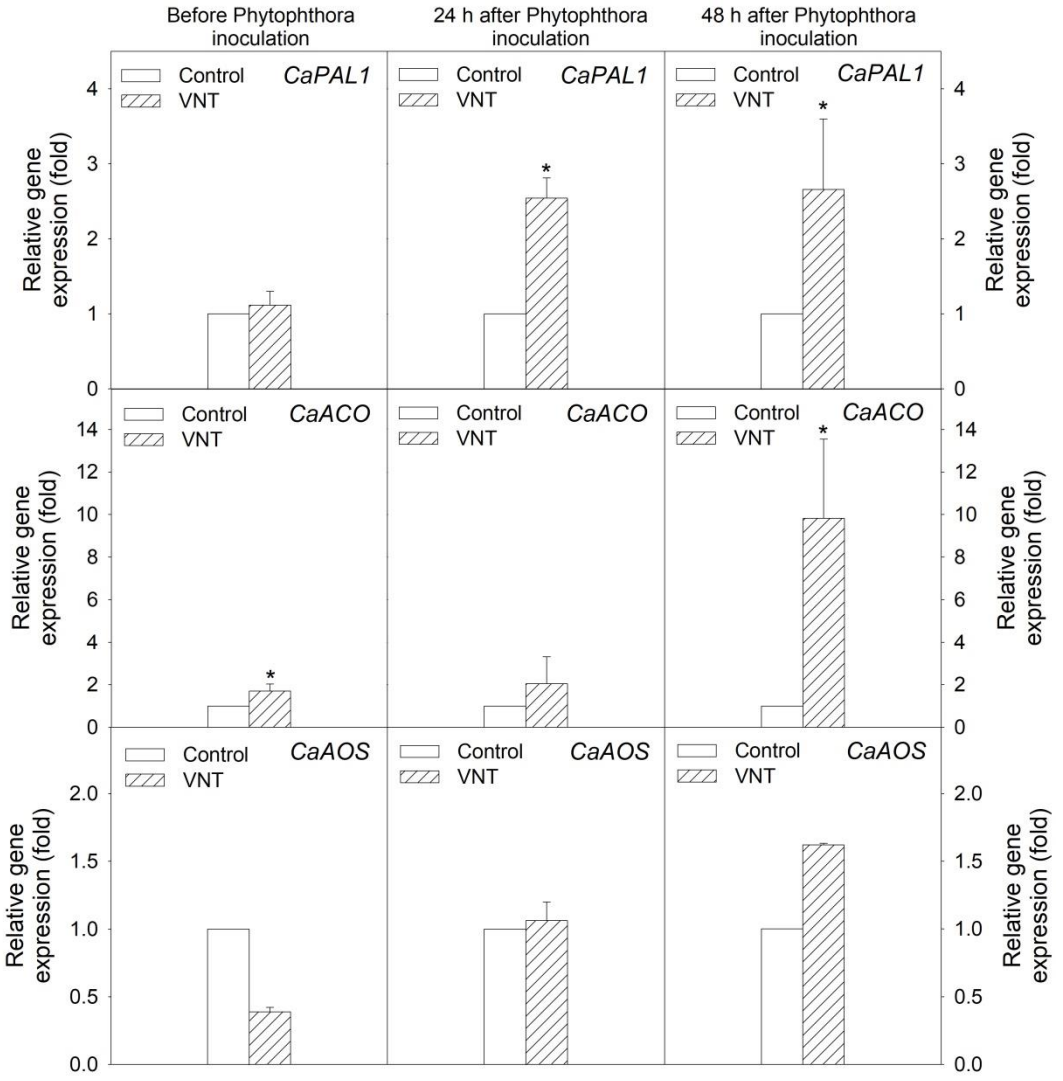

Figure 4.6. Effect of vanillylnonanoate (VNT) on the expression of genes involved in biosynthesis of hormones regulating the defence response in roots before (0 h after inoculation) and after (24 h and 48 h) *P. capsici* inoculation. In the first row of the figure is *CaPAL1* (phenylalanine ammonia lyase 1), a gene involved in SA biosynthesis. In the second row of the figure is *CaACO* (amino-cyclopropane-1-carboxylic acid oxidase), a gene involved in ET biosynthesis. In the third row of the figure is *CaAOS* (allene oxide synthase), a gene involved in JA biosynthesis. A minimum of two experiments and a maximum of six experiments were performed depending on the studied gene. Data are the mean ± SE. Asterisk (*) indicates statistical differences ($p < 0.05$) in a Kruskal-Wallis test.

Finally, the expression of the gene *CaAOS* involved in JA biosynthesis was measured. There were no statically significant differences but a trend can be observed. Before Phytophthora inoculation, this gene seemed to be down-regulated in VNT treated plants. When plants were inoculated, no differences were observed between VNT-treated plants and control 24 h after inoculation although *CaAOS* seems to be induced 48 h after inoculation (Figure 4.6).

These results suggested that VNT induced the biosynthesis of SA and ET

4.3.4. The inhibition of the ET perception reduces the effect of the VNT treatment against *P. capsici* and the treatment with ET protects against *P.capsici*

Given that an induction of the expression of *CaACO* was observed, the symptoms were monitored during infection with *P. capsici* in plants in which the perception of this hormone was blocked with 1-methylcyclopropene (MCP) (inhibitor of ethylene perception) before the elicitation with VNT.

During the experiment, VNT-treated plants showed fewer symptoms than the control (Figure 4.7.A). On the other hand, no differences were observed between the control plants and plants treated with MCP or double treatment (MCP+VNT) (Figure 4.7.A). These results indicate that MCP did not have an effect on the basal resistance but it can inhibit the response induced by the VNT. These results suggest the participation of ethylene in the activation of the VNT-induced defences against *P. capsici*.

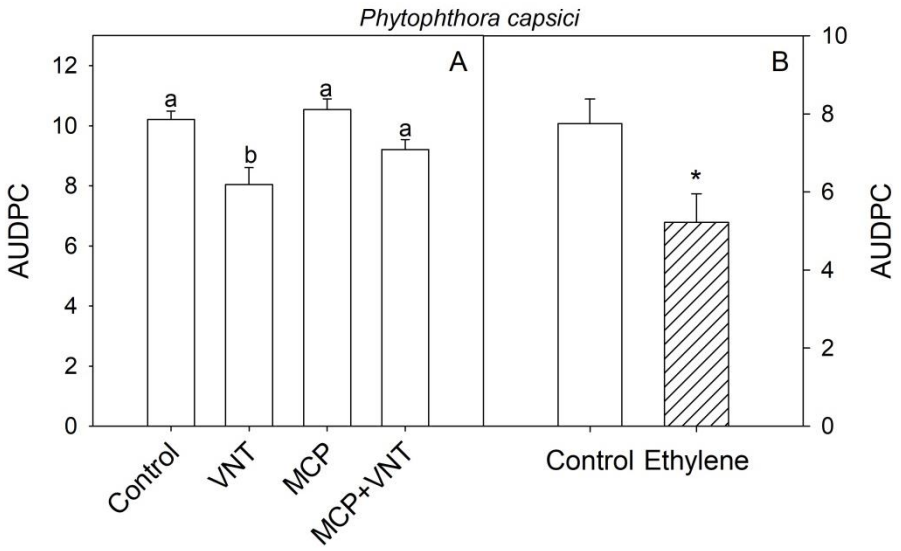

Figure 4.7. Effect of ethylene in the resistance induced by the vanillyl nonanoate (VNT). (A) Effect of the inhibitor MCP (1-methylcyclopropene) on resistance induced by the VNT. Two experiments were performed with 6 plants per group and experiment (n = 12). The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in an ANOVA test followed by a Student-Newman-Keuls post-hoc test. (B) Effect of ethylene on the symptoms caused by *P. capsici* in pepper. Two experiments were performed with 8 plants per group and experiment (n = 16). The data are the mean ± SE. The asterisk (*) indicates statistical differences ($p < 0.05$) in a t-Student test.

Both the expression of *CaACO* and the treatment with MCP reveal an active role of ethylene in VNT-induced defence. For this reason plants were treated with ethylene and the effect on the susceptibility to *P. capsici* was observed.

Treatment with ethylene caused a reduction in the symptoms caused by *P. capsici* compared to the control (Figure 4.7.B). These results confirm that ethylene induces resistance in pepper against *P. capsici*.

4.3.5. The inhibition of the jasmonate biosynthesis has no effect on the VNT treatment against *P.capsici*

On the other hand, the effect of ibuprofen, a jasmonate inhibitor, was studied during infection with *P. capsici*. In this way it could be confirmed if jasmonate has some role in the resistance induced by the VNT.

During the assay, the protective capacity of the VNT was again observed (Figure 4.8). Treatment with ibuprofen unexpectedly caused a reduction in symptoms in the same way as VNT did (figure 4.8). Also the treatment of ibuprofen and VNT in combination caused the same reduction than VNT and ibuprofen treatments along. There was no synergistic or additive effect of VNT and ibuprofen which could mean that they act through common signalling. VNT may have the same effect as ibuprofen by reducing jasmonate biosynthesis indicating a negative role of jasmonate in *P.capsici* resistance.

4.3.6. VNT treatment increses the expression of JA and ET biosynthetic genes in systemic leaves

We studied the effect of VNT on the expression of five genes involved in biosynthesis of the main defence hormones: SA (*CaPAL1*), ET (*CaACS*, *CaACO*) and ET (*CaAOS*).

The gene *CaPAL1* is involved in SA biosynthesis. *CaPAL1* did not show any difference with the control either before or after Botrytis inoculation (Figure 4.9). However, the genes *CaACS* and *CaACO*, involved in ET biosynthesis, increased their expression after treatment with VNT and before inoculation (Figure 4.9). Finally,

CaAOS, involved in JA biosynthesis, showed the same pattern than *CaACS* and *CaACO* (Figure 4.9).

These results point out that ET and JA are active hormones in VNT-induced resistance. In order to confirm these results MCP assays were carried out as well as hormone content was measured by HPLC.

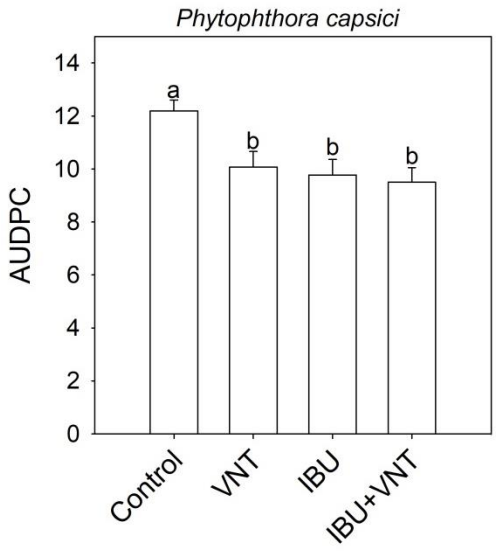

Figure 4.8. Effect of ibuprofen on induced resistance by vanillyl nonanoate (VNT). Two experiments were performed with 7 plants per group and experiment (n=14). The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in an ANOVA test followed by a Student-Newman-Keuls post-hoc test.

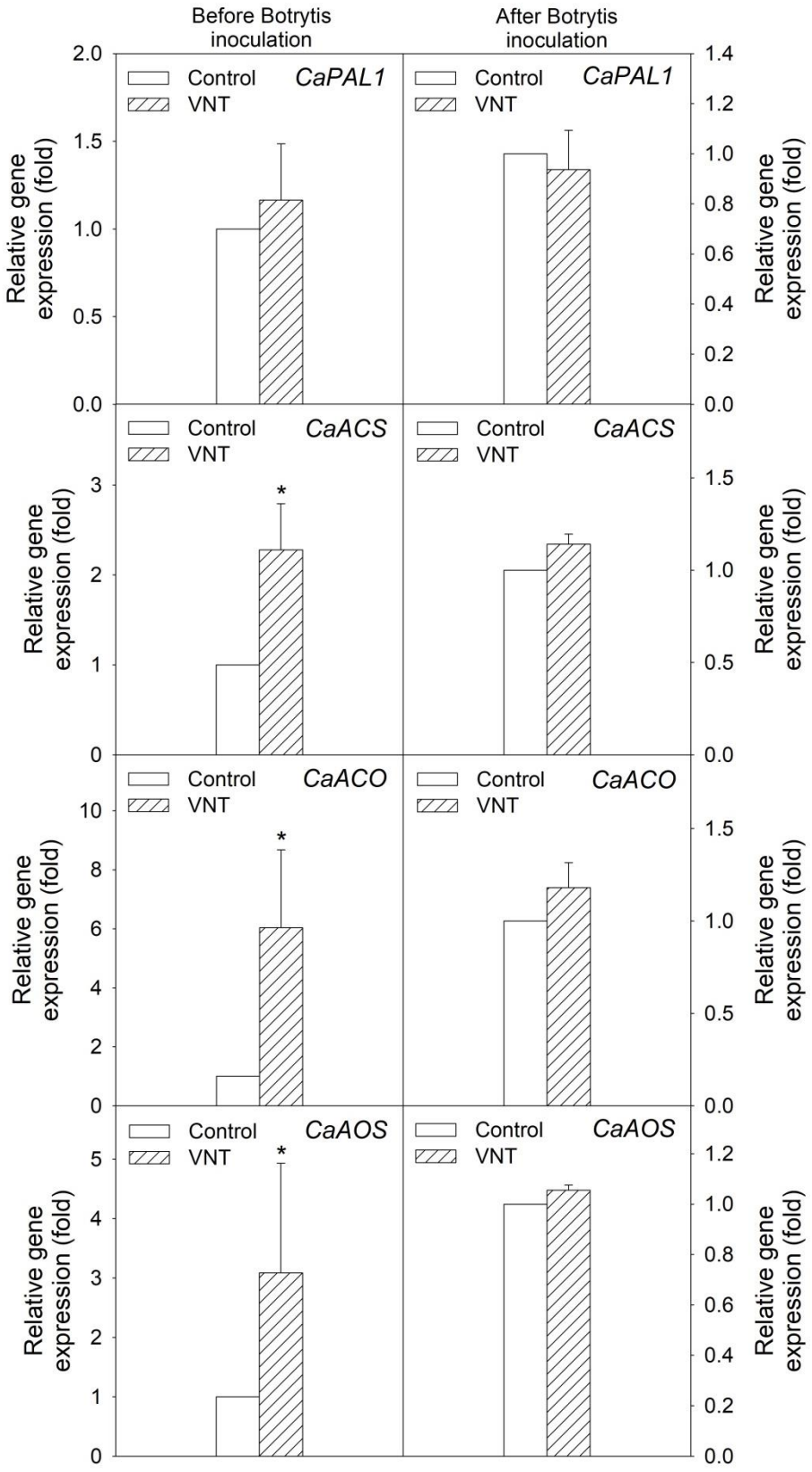

Figure 4.9. Effect of vanillyl nonanoate (VNT) on the expression of genes involved in biosynthesis of hormones regulating the defence response before and after Botrytis inoculation in leaves. In the first row of the figure is *CaPAL1* (phenylalanine ammonia lyase 1), a gene involved in SA biosynthesis. In the second (*CaACS*; aminocyclopropane-1-carboxylic acid synthase) and third row (*CaACO;* amino-cyclopropane-1-carboxylic acid oxidase) of the figure are the genes involved in ET biosynthesis. In the

fourth row of the figure is *CaAOS* (allene oxide syntase), a gene involved in JA biosynthesis. A minimum of two experiments and a maximum of five experiments were performed depending on the studied gene. Data are the mean ± SE. Asterisk (*) indicates statistical differences ($p < 0.05$) in a Kruskal-Wallis test.

4.3.7. Inhibtion of ethylene perception flips the effect of the VNT-treatment from inducing protection to inducing susceptibility

Because of the study of genes involved in hormone biosynthesis revealed ET as an active hormone, we performed an assay in which plants were treated with 1-methylcyclopropene (MCP), an inhibitor of ET perception.

Regarding to symptoms, VNT-treated plants consistently showed less disease area than control (Figure 4.10). There were no differences between MCP-treated plants and the control. On the other hand, we observed an increment in disease area when plant received the double treatment, MCP and VNT (figure 4.10). These results suggest that the absence of ET signalling produce a deleterious effect when plants are treated with VNT indicating ET is an important hormone in VNT-induced resistance against *B. cinerea*.

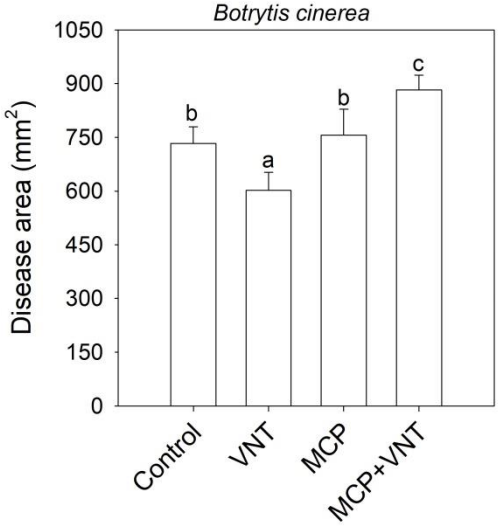

Figure 4.10. Effect of 1-methylcyclopropene (MCP) on induced resistance by vanillyl nonanoate (VNT) in leaves infected with *B. cinerea*. Five experiments were performed with between 5 and 8 plants per group (n=32). The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in a Kruskall-Wallis test followed by a post-hoc test according to Conover (1980).

4.3.8. VNT treatment decreases OPDA in cotyledons before the inoculation and JA in leaves after inoculation with *Botrytis cinerea*.

In addition we also quantified the content of some hormones by HPLC. The studied tissues were cotyledons and leaves 0 h after inoculation. Moreover, leaves were also collected 8 h after inoculation with *B. cinerea*. At the same time leaves non-inoculated were collected.

In cotyledons there were no differences related to SA content between VNT-treated plants and control (Figure 4.11). During the quantification of SA, it was observed the appearance of another component with similar SA structure. This compound appeared in both control and VNT-treated plants suggesting that it was not due to VNT treatment. This compound was identified as 4-hydroxybenzoic acid (4-HBA). There were no differences between control and VNT-treated plants (Figure 4.11).

It was also measured the hormone JA, its precursor 12-oxophytodienoic acid (OPDA) and the active form of JA in higher plants, jasmonate-isoleucine (JA-ILE). OPDA showed a reduction in VNT-treated plants compared to control. On the other hand, no differences were found in JA (Figure 4.11). The JA-ILE was not detected in any of the cotyledon samples.

Recently, it was observed that other hormones may have a role during a biotic stress. For this reason, ABA (abscisic acid) and IAA (indole acetic acid, auxins) were also measured. However, no differences between treatments were observed in both hormones (Figure 4.11).

These results suggested that VNT-treatment did not alter the hormone balance at local level in SA, 4-HBA, JA, ABA and IAA but VNT reduced the concentration of the JA precursor (OPDA).

JA, OPDA, ABA, IAA and JA-ILE were measured in true leaves before and after inoculation with *B. cinerea* (figure 4.12).

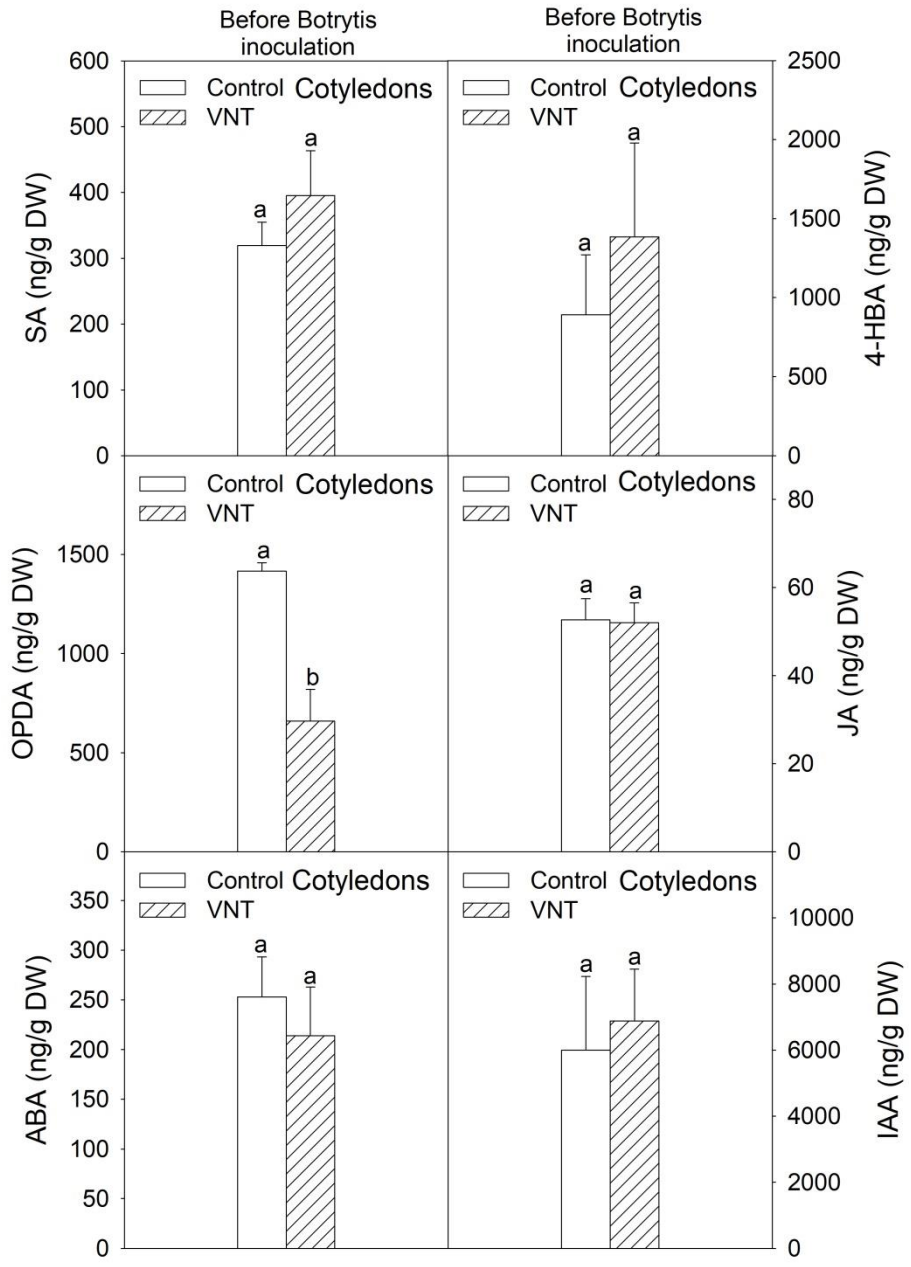

Figure 4.11. Effect of vanillyl nonanoate (VNT) on hormone levels in cotyledons. A minimum of three experiments and a maximum of four experiments were performed with 5 per group. The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in a Kruskall-Wallis test followed by a post-hoc test according to Conover (1980).

In true leaves, the VNT-treated plants showed no differences in OPDA before and after the inoculation with *B. cinerea* (Figure 4.12). There were no differences in JA content before Botrytis inoculation. However, the amount of JA after Botrytis inoculation was increased in Control+Bc and VNT+Bc comparing with control and VNT plants. Nevertheless, Control+Bc showed higher amounts of JA than VNT+Bc (Figure 4.12).

On the other hand, the conjugate JA-ILE was only detected in inoculated plants regardless of the treatment with VNT (Figure 4.12). No differences between VNT and control plants were found.

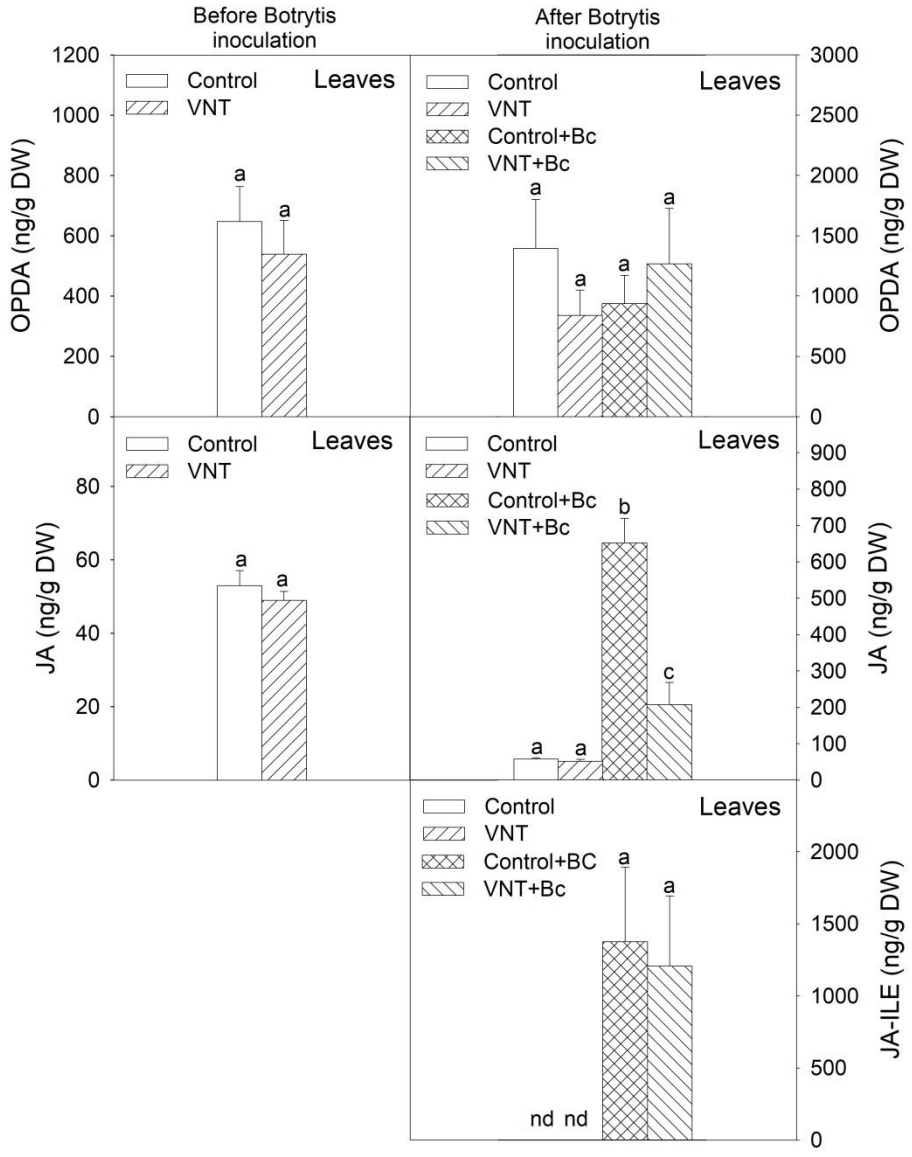

Figure 4.12. Effect of vanillyl nonanoate (VNT) on OPDA (12-oxophytodienoic acid), JA (jasmonate) and the conjugated JA-ILE (jasmonate-isoleucine) in leaves before and after inoculation with *B. cinerea* (Bc). A minimum of two experiments and a maximum of four experiments were performed with 5 plants per group. The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in a Kruskall-Wallis test followed by a post-hoc test according to Conover (1980) in all data except in JA after *B. cinerea* inoculation in which the statistical test was an ANOVA followed by a Student-Newman-Keuls post-hoc test.

The levels of ABA and IAA in leaves before and after Botrytis inoculation did not show any differences caused by VNT treatment before the inoculation (Figure 4.13).

After inoculation, either VNT treatment or presence or absence of Botrytis showed no differences in IAA concentration 8 h after inoculation (Figure 4.13). On the contrary, the presence of pathogen decreased the concentration of ABA in plants treated with VNT compared to the inoculated control. Moreover, the amount of this hormone in this group was equal to the concentration of non-inoculated plants 32 h after VNT treatment (Figure 4.13).

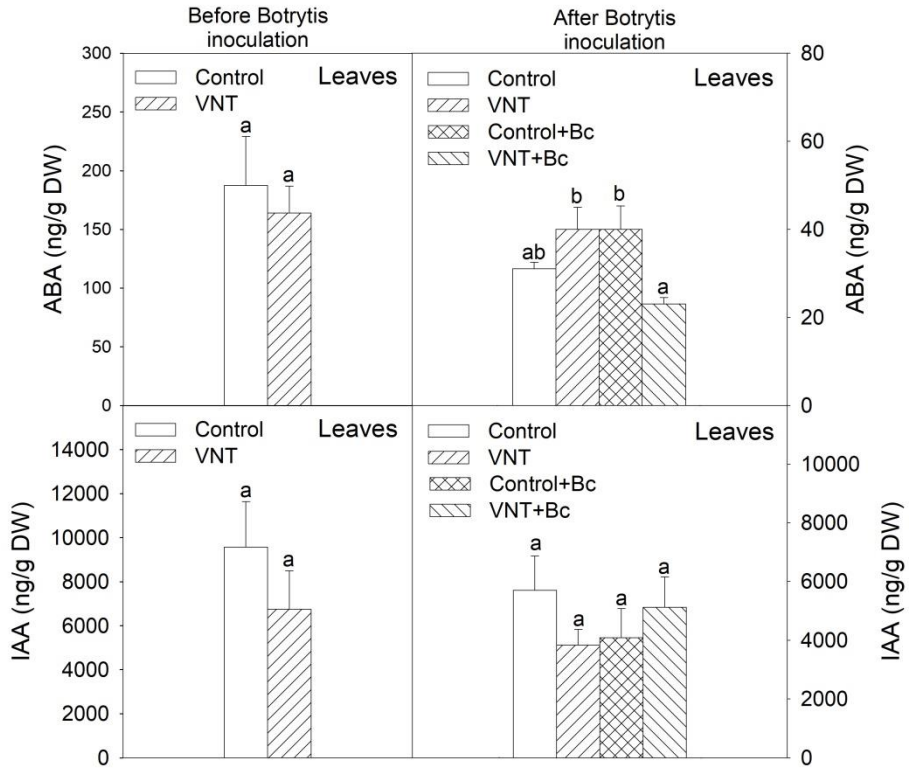

Figure 4.13. Effect of vanillyl nonanoate (VNT) on ABA (abscisic acid) and IAA (indole acetic acid) in leaves before and after inoculation with *B. cinerea* (Bc). A minimum of three experiments and a maximum of four experiments were performed with 5 plants per group. The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in a Kruskall-Wallis test followed by a post-hoc test according to Conover (1980).

4.3.9. VNT treatment decreased the levels of SA and increased the levels of 4-HBA

Regarding to SA in true leaves, the treatment with VNT did not show any differences before the inoculation with *Botrytis cinerea* (Figure 4.14). Similar results were found in plants inoculated with *B. cinerea* (in the figure 4.14 groups Control+Bc and VNT+Bc). Nevertheless, the plants that were not inoculated but treated with VNT

showed higher concentration of SA than control 32 h after VNT treatment (in figure 4.14 groups Control and VNT after Botrytis inoculation). This time point counts the time after VNT treatment (24 h) and also 8 h after the groups Control+Bc and VNT+Bc were inoculated. Moreover, inoculated plants showed the same SA concentration than VNT 8 h after inoculation (Figure 4.14).

In the same manner than in cotyledons, a peak of 4-HBA was observed. Before the inoculation, there were no differences between control and VNT (Figure 4.14). However, after inoculation the groups of VNT, Control+Bc and VNT+Bc had less amount of 4-HBA than control (Figure 4.14).

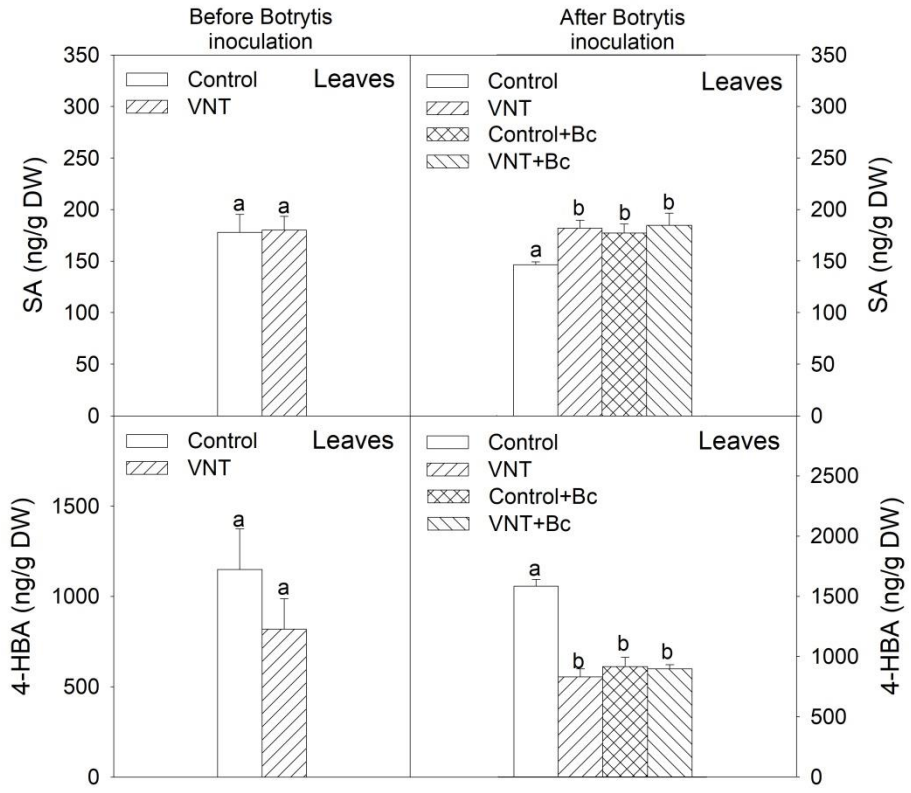

Figure 4.14. Effect of vanillyl nonanoate (VNT) on salycilic acid (SA) and 4-hydroxybenzoic acid (4-HBA) in leaves before and after inoculation with *B. cinerea* (Bc). A minimum of three experiments and a maximum of four experiments were performed with 5 plants per group. The data are the mean ± SE. Different letters indicate significant differences ($p < 0.05$) in a Kruskall-Wallis test followed by a post-hoc test according to Conover (1980) in all data except in 4-HBA after *B. cinerea* inoculation in which the statistical test was an ANOVA followed by a Student-Newman-Keuls post-hoc test.

4.4. Discussion

Reactive oxygen species (ROS) are composed mainly by hydrogen peroxide (H_2O_2), superoxide ion and hydroxyl radical (Neill *et al*., 2002). The alteration of the cellular homeostasis by the presence of some pathogen causes an increase of the ion flow at the cellular level as well as the production of these ROS (Lee & Hwang, 2005; Sharma *et al*., 2012). This increase in ROS levels is called oxidative burst, which is one of the most important defensive responses of the plant (Asselbergh *et al*., 2007). The relationship of ROS with plant resistance has been known since 1983, when Doke observed ROS production during an incompatible interaction between potato and *P. infestans* (Lehmann *et al*., 2015). ROS have several roles during plant-pathogen interaction. On one hand, H_2O_2 is toxic to the pathogen and, on the other hand, it plays a role in the plant defence participating in the cell wall reinforcement and also in signalling (Lehmann *et al*., 2015).

In this work the implication of H_2O_2 in VNT-induced resistance was studied in roots and leaves. The study in the aerial part was done inoculating with the necrotroph *B. cinerea* that kills the cells of the host to take its nutrients. In addition, this pathogen produces ROS during cuticle penetration (van Kan, 2006). Therefore, the production of ROS during oxidative burst would favour the plant infection by *B. cinerea* (Asselbergh *et al*., 2007). In fact, the application of antioxidant products, such as BHT (butylated hydroxytoluene) or ascorbic acid, caused a decrease in the symptoms by this pathogen (Elad, 1992). Similar results were observed in the present work by applying the ROS scavenger DTT. As it was mentioned in previous sections, DTT is a H_2O_2 scavenger and was used in order to know if this signal was involved in VNT-induced resistance. It was observed in our experiments that DTT-treated plants showed fewer symptoms than control, although this reduction was not statistically significant. This reduction is probably due to the change in redox status caused by DTT. When DTT is applied, the cellular redox status becomes reduced changing the activity of proteins like NPR1. It has been observed that the redox status modulates NPR1 change from oligomer form (oxidized status) to monomer form (reduced status). Then, the NPR1 conformation of DTT-treated plants would be in a monomer form allowing PR genes expression and, then, increasing the plant defence (Mou *et al*., 2003).

However, in the present work we have observed a decrease in the symptoms caused by *B. cinerea* associated with increased levels of H_2O_2 in plants. This situation has been observed in other cases as, for example, in the tomato mutant *sitiens*. This ABA-deficient mutant shows resistance to *B. cinerea* and a rapid accumulation of H_2O_2 in epidermal cells (Asselbergh *et al*., 2007). Also the oligogalacturonide treatment in Arabidopsis leaves reduces the symptoms caused by *B. cinerea* and triggers an increase in the production of H_2O_2 (Galletti *et al*., 2008). In addition, in this study the formation of H_2O_2 has been observed in the inoculated area by DAB staining. Stamler *et al*. (2015) using the same technique also observed an increase in H_2O_2 accumulation in leaves of pepper treated with an elicitor and infected with *P. capsici*. In addition, they could also observe that this accumulation was associated with the fungus penetration area (Stamler *et al*., 2015). In conclusion, ROS production does not always favour plant resistance because the outcome of *B. cinerea* infection will depend on the timing and intensity of the oxidative burst as well as the balance with other early signalling molecules such as nitric oxide (Pietrowska *et al*., 2015). Moreover the place of production seems to be involved in an intricate interaction that determines the result of the oxidative burst. In pepper, the production of ROS associated with SAR is a consequence of the activity of NADPH oxidase and extracellular peroxidases (Choi & Hwang, 2015). In fact, gene silencing by VIGS (Virus-Induced Gene Silencing) of the *CaPO2* gene, a peroxidase, confirms its involvement in systemic oxidative micro-splints (Choi *et al*., 2007). It should be noted that the previous results were obtained in works in which the pathogen was inoculated in the leaves, whereas our measurements were also done in the roots.

Unlike foliar level, the H_2O_2 accumulation during a defensive response at the root level is poorly studied. In spite of this, there are some cases where H_2O_2 accumulation in roots has been observed (for a review see Lehmann *et al*., 2015). In the present work an increase in the accumulation of H_2O_2 in the first hours after *P. capsici* inoculation was observed but it was greater in the plants treated with VNT. This increase could correspond to the moment when the plant recognizes the attack of *P. capsici* and generates an oxidative burst which results in an increase in response. In fact, treatment of plants with DTT (a reducing agent) suppressed the resistance induced by the VNT. As in this work, Knecht *et al*. (2010) observed an increase in H_2O_2 associated with an increase in resistance to *Verticillium longisporum* and *Rhizoctonia solani* in Arabidopsis plants expressing the GLP (Germin-Like Protein) protein from *Beta vulgaris*. This protein has superoxide dismutase activity or oxalate oxidase activity

which generates H_2O_2 (Knecht *et al*., 2010). However, it has been already mentioned above that ROS production as a result of plant-pathogen interaction may be related to resistance or susceptibility. Zhu *et al*. (2013) observed that inoculation of Arabidopsis roots with *Fusarium oxysporum* induced a gene family homologous to mammalian NADPH oxidases (*AtRboh*). Within this family, two of these genes (*AtRbohD* and *AtRbohF*) proved to be the most important during an incompatible interaction. However, plants with a mutation in *AtRbohD* showed resistance to *F. oxysporum*, unlike those with the mutation in *AtRbohF*, which developed more severe symptoms (Zhu *et al*., 2013).

Moreover ROS are early signals that can interact with and modulate other defensive molecules as hormones SA, JA and ET. One way to study the role of these signals is to analyse the expression pattern of genes regulated by these hormones. In Arabidopsis, there are unique markers for each of those hormones. For example, the PR1 gene is induced only by SA, but not by ET or JA, thus it is considered a SAR marker (Durrant & Dong, 2004). However, the situation is completely different in pepper. For example, the PR1 homologue of pepper, *CaBPR1,* is induced by SA, ET and JA (Choi & Hwang, 2015). For this reason, four genes that encode enzymes involved in the synthesis of those hormones were chosen: one gene for SA (*CaPAL1*), one for JA (*CaAOS*) and two for ET (*CaACS* and *CaAC*O).

The enzyme phenylalanine ammonia lyase (PAL) catalyses the first step of the phenylpropanoid pathway. Kim & Hwang (2014) studied the pepper PAL gene (*CaPAL1*) during biotic stress. They observed that *CaPAL1* was up-regulated and the SA accumulation as well as ROS burst was compromised in *CaPAL1*-silenced plants (Kim & Hwang, 2014). In fact, the authors proposed it as "a positive regulator of plant innate immunity" (Kim & Hwang, 2014). In this work, changes in *CaPAL1* expression have been detected after inoculation wit *P. capsici* indicating that SA might be involved in the response induced by VNT at the root level. Generally speaking, SA is associated with defence against biotrophs and the first stage of hemibiotroph infection (Derksen *et al*., 2013). Several studies using SA-induced defence compounds, such as ASM, BABA, or even SA itself have shown a reduction in plant susceptibility against *P. capsici* in pepper (Lee *et al*., 2000; Aijun *et al*., 2005; Baysal *et al*., 2005) and in other host as Arabidopsis (Wang *et al*., 2013).

On the other hand, the expression of the same gene (*CaPAL1*) was measured in true leaves before and after Botrytis inoculation but no differences were observed between treatments. However, we quantified the amount of SA in leaves and we observed an increase in these three groups of plants: VNT, Control+BC and VNT+BC. These data suggest that SA is not synthetized by the phenylpropanoid pathway. In Arabidopsis and tobacco, the SA production caused by a pathogen is associated with the isochorismate pathway. In fact, the mutation on isochorismate synthase strongly reduces SA accumulation (Wildermuth *et al*., 2001; Catinot *et al*., 2008). In the case of pepper, it is unclear which pathway is responsible for SA synthesis under a stress (Kim & Hwang, 2014). Another way in which plants accumulate large amounts of SA is in conjugate forms that are inactive as a form to control cytosolic SA levels (Dempsey & Klessig, 2017). These conjugates can be form by glycosylation (SA 2-O-β-D-glucoside (SAG), salicylate glucose ester (SGE)) or methylation (methyl SA (MeSA)). Also SA can be accumulated as 2,3-dihydroxybenzoic acid (2,3-DHBA) or 2,5-dihydroxybenzoic acid (2,5-DHBA) or SA-amino acids conjugates, such as salicyloyl-aspartate (SA-Asp), or 4-substituted benzoates conjugated with glutamate (Dempsey & Klessig, 2017). Therefore all forms are SA sources as well as an easy way to transport SA through the plant. Under pathogen attack, the plant can transform these compounds in SA instead of producing SA *de novo* what would entail a huge cost of energy and resources (Dempsey & Klessig, 2017). Thus, the increment of SA observed in this work could be due to the conversion of conjugated forms into SA instead of *the novo* synthesis.

When we were measuring SA in UPLC, we detected a peak which area varied depending on the treatment. In addition, it showed an inverted correlation with SA after infection. This is, when SA increased, this compound decreased and vice versa, when SA decreased, it increased. This compound was identified as 4-hydroxybenzoic acid (4-HBA). Initially, 4-HBA was classified as biologically inactive compound (Chen *et al*., 1993) but, lately, it was discovered that this compound is involved in synthesis of ubiquinone, shikonin and as a component of lignin (Okrent *et al*., 2009). Besides, this compound has been related with extracts of cell wall from infected roots and leaves in Arabidopsis (Tan *et al.*, 2004). Also SA together with 4-HBA were found in phloem fluids of infected cucumber (Smith-Becker *et al*., 1998). Moreover, it was able to improve the freezing tolerance in wheat Chinese Spring and drought tolerance in wheat Cheyenne (Horváth *et al*., 2007). All these cases suggest a role during a defensive

process. In fact, 4-HBA has been proposed as a regulator in SA synthesis through PBS3, member of GH3 family. PBS3 acts upstream of SA in early steps of defensive response. However, it was inhibited by low levels of SA and it showed greater affinity for 4-HBA. Therefore, under stress, PBS3 would lead to the conjugation of 4-HBA with glutamic acid which primes SA biosynthesis (Okrent *et al*., 2009). This could explain the relationship between 4-HBA and SA. The reduction in 4-HBA should be due to formation of conjugates what would cause the accumulation of SA.

On the other hand, we studied the role of JA quantifying the expression of gene *CaAOS*, using ibuprofen and also measuring the hormone and related compounds (OPDA and JA-ILE). The *CaAOS* gene codes for allene oxide synthase that acts on the substrate released by the enzyme lipoxygenase (Yan *et al*., 2013). In roots, the expression of this gene was apparently repressed by VNT, which implies a decrease in the synthesis of JA. Taking into account the situation, it can be said that the JA could be a negative regulator of the response to VNT in this part of the plant. In fact, when the plants were treated with ibuprofen, an inhibitor of JA synthesis, a reduction in symptoms was again observed in VNT-treated plants. However, no increase in resistance was observed when both treatments (ibuprofen + VNT) were applied. In the experiments carried out by Núñez-Pastrana *et al*. (2011) there was no increase in resistance in *Capsicum chinense* by spraying methyl jasmonate and inoculating with *P. capsici*. Nevertheless, the cultivar Serrano Criollo de Morelos 334 (SCM334), resistant to *P. capsici*, showed a rise in the production of JA when inoculated with this pathogen (Ueeda *et al*., 2006). These differences can be due to the plant organ used to study (roots in the present work and leaves in the assays of Núñez-Pastrana *et al*., (2011) and Ueeda *et al*. (2006)) as well as the plant cultivar and pathogen isolate. In fact, JA levels vary depending on developmental status, organ in which hormone has been measured, and environmental stimuli (Yan *et al*., 2013). On the other hand, it has been observed that H_2O_2 affects the amount of this hormone in the plant. JA increases its accumulation in response to wounds, but Noctor *et al*. (2015) observed that the amount of JA in the mutant *cat2*, deficient in catalase which is responsible of ROS elimination, was reduced but the amount of linoleic acid, a precursor of JA, was increased (Petrov & Van Breusegem, 2012; Noctor *et al*., 2015). This situation is similar to that found in this work, that is, there are high hydrogen peroxide levels in both mutant *cat2* and VNT-treated plants. This would explain the repression in *CaAOS* expression in roots of VNT-treated plants. The increase of hydrogen peroxide caused by the VNT would cause the

reduction of the synthesis of JA (reduction of *CaAOS*). This increase in ROS is transient, also the representation of *CaAOS*, which suggest the dependence of both events.

The picture in leaves is a bit different. The gene expression of *CaAOS* suggested that VNT-treated plants synthetize more JA than control. However, when it was quantified, the inoculated control showed higher amounts than inoculated VNT. In fact, the levels of the active form, JA-ILE, were similar in inoculated groups. In general, there is a correlation between *CaAOS* gene expression and JA amount, more expression implies more JA amount (Laudert & Weiler, 1998; Harms *et al*., 1995; Ziegler *et al*., 2001). Nevertheless, there is not always a correlation between JA synthesis and up-regulation of JA-responding genes. Harms *et al*. (1995) observed that an overexpression of AOS enhanced the synthesis of JA but the accumulation of this hormone did not increase the expression of wounded-responsive gene *pin2* (Harms *et al*., 1995). The formation of the JA active form (JA-ILE) is necessary for the activation of the JA-responsive genes. However, recently, it has been discovered that in *Marchantia polymorpha* the active form are the isomeric forms dinor-cis-OPDA and dinor-iso-OPDA because of a single-residue mutation in MpCOI1, the receptor of the JA active form (Monte *et al*., 2018). There is a tight regulation of the amounts of the active form that is produced. During a stress, the formation of JA-ILE is tightly regulated allowing only the formation of the minimum necessary amounts to activate the JA-responsive genes (Wasternack & Hause, 2013). In fact, Woldemariam *et al*. (2012) observed that less than 15% of the total JA was converted into JA-ILE in *Nicotiana attenuata.* These findings would explain that we did not observe differences in JA-ILE content. Because of the amount of JA-ILE is so regulated, it is possible that the VNT-treated plants and control plants used the same amount of JA-ILE and, for this reason, we did not see differences between these groups.

Once JA is synthetized, it can be metabolized by different pathways. One of them is the combination of JA with ACC (1-amino cyclopropane-1-carboxylic) by JAR1, enzyme responsible to conjugate JA with amino acids, what render JA and ACC after excision (Staswick & Tiryaki, 2004; Dar *et al*., 2015). Also JA can be transformed in methyl jasmonate (MeJA) which is able to trigger the antioxidant enzymes (Dar *et al*., 2015). This could explain the reduction in JA in VNT-treated plants. This is, after VNT treatment H_2O_2 enhance its levels what would unleash JA biosynthesis but only a

small portion is used to activate genes. As consequence, MeJA is metabolized from the rest of JA which is used as signal to activate antioxidant weapons. In control, H_2O_2 levels kept low, then the amount of JA was high.

To study the implication of ethylene we chose two genes *CaACS*, that encode the enzyme ACC synthase which is involved in the second last step of ET synthesis, and *CaACO* that encodes for the enzyme ACC oxidase, which catalyses the last step of ET synthesis. Normally the enzyme ACS is considered the limiting step in the synthesis of this hormone (Yoon, 2015). However, there are cases where ACO is the limiting step. An example is the case of *Rumex palustris*, in which ACC oxidase is a limiting step in ET biosynthesis when the plant is submerged (Vriezen *et al*., 1999). Likewise Van de Poel *et al*. (2012) observed how the limiting step changed from ACS to ACO as a function of tomato maturation stage. During the pre-climateric state, ACO activity increased while ACS activity decreased, which supports the idea that ACS is the limiting step. On the contrary, towards the end of the maturation of the fruit ACO activity decreases and ACS activity is maintained, which produces accumulation of ACC. This shows that ACO is the limiting enzyme in this phase and not ACS (Van de Poel *et al*., 2012). These examples suggest that the regulation of ACO is more complex than it is expected. Therefore, we studied both genes to know if ET was involved in VNT-induced resistance or not.

In the present work, an increase in the expression of *CaACO* was observed after treatment with VNT in leaves and roots. In addition, the effect of VNT disappeared when the perception of ethylene was blocked with MCP. Several papers show that ethylene participates in resistance against *P. capsici* and *B. cinerea*. Núñez-Pastrana *et al*. (2011) showed that ET treatment induces leaf resistance in *C. chinense* against *P. capsici*, what we also observed in the present work with *C. annuum* at root level. The treatment of tomato with ethylene reduced the lesions caused by *B. cinerea* (Díaz *et al*., 2002). Berrocal-Lobo *et al*. (2002) observed increased expression of ERF1 (Ethylene-Response Factor 1), an ethylene response gene, in Arabidopsis plants infected with *B. cinerea*. In fact, overexpression of ERF1 reduced the symptoms of infection with this pathogen (Berrocal-Lobo *et al*., 2002).

In literature, it is commonly established two types of response to a pathogen attack: dependent and independent of SA. However, this is not so simple. In last years, it was discovered that the other plant hormones (abscisic acid (ABA), auxin, cytokinins

and gibberellins), normally associated with plant growth, are also involved in regulation of defensive response (Denancé *et al*. 2013). For example, in plant defence against *B. cinerea*, it has been described cases in which the hormones SA, JA, ET and ABA regulate positively or negatively each other during the defence response (AbuQamar e*t al*., 2017). In this study we measured ABA and indolacetic acid (IAA) which did not show any significant differences after treatment with VNT. However, ABA levels were lower in VNT-plants after inoculation. The amount of ABA decreased due to the pathogen. It has been describe that ABA is a negative regulator of SA. Kusajima *et al*. (2017) observed that tomato plants treated with ABA do not accumulate SA even when they were subsequently treated with 1,2-benzisothiazol-3(2H)-one1,1-dioxide, a SAR inductor. Similar results were observed by de Torres Zabala *et al*. (2009) in the pathosystem Arabidopsis-*Pseudomonas syringae*. Similar to SA, Anderson *et al*. (2004) observed an antagonistic relationship between ABA and JA/ET. Arabidopsis plants treated with ABA showed a downregulation of *PDF1.2*, marker gene of JA. Therefore, based on these cases, it is necessary a reduction in ABA to activate defences by SA and JA.

To conclude, the signalling in plants is a huge and complicated network. The pathosystem used in studies determine which components of that web are activated or deactivated to overcome a pathogen attack. In our experiments, VNT seems to rely on the same components in both tissues: increase of ROS, increase of hormones SA, JA, and ET. However, the way that these players work in roots and leaves seems to be different. Therefore, further experiments are needed to get a deeper insight on the behaviour of each player.

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CHAPTER 5.

Evaluation of the protective effect of VNT on Arabidopsis against *Botrytis cinerea*.

5.1. Introduction

Capsaicinoids are secondary metabolites synthetized in placental tissue and they confer the pungent flavour to the pepper fruits. However, some pepper cultivars have non-pungent metabolites known as capsinoids or capsiconinoids being the most studied the first group (García *et al*., 2018). Capsinoids share pharmacological properties with capsaicinoids. For this reason, capsinoids could be substitutes of capsaicinoids because they are less toxic (Antonio *et al*., 2018).

Another feature described for capsaicinoids is their antimicrobial activity against bacteria and fungi (Cichewicz & Thorpe, 1996; Veloso *et al*., 2014). However, their properties to induce systemic resistance is even more interesting from a phytopathological point of view. Such property has been reported in Arabidopsis against *Pseudomonas syringae* and *Pectobacterium caratovorum* (Song *et al*., 2013), and in pepper against *Verticillium dahliae* and *Botrytis cinerea* (Veloso *et al.*, 2014).

In previous chapters of this thesis, we have proved that the synthetic capsinoid VNT induces resistance against *P. capsici* in roots and *B. cinerea* in leaves. Moreover, when we analysed different defensive parameters, we observed that both organs shared a similar response to the pathogen highlighting the induction of lignin deposition mediated by peroxidases and the involvement of different signalling components as ROS, SA, JA and ET.

Then, based on the knowledge about capsaicinoids as potencial phytosanitary compounds and the study developed in this project, we decided to test whether VNT was able to protect plant species that are not able to synthetize capsaicinoids or capsinoids. The lack of these secondary metabolites implies that the plant does not have the enzymatic components required for their synthesis. Then, the plant would not have the receptors to recognise the VNT and the induction of resistance would not be triggered.

To address this objective we have chosen the plant *Arabidopsis thaliana* for two reasons: first, there was a study in which Song *et al*. (2013) proved that capsaicin induces resistance in Arabidopsis plants against *Pseudomonas syringae* and *Pectobacterium caratovorum*, and, second, *A. thaliana* is a plant that has been thoroughly studied in phytopathological research and there is a lot of knowledge about its defensive pathways. Therefore, working with *A. thaliana* and *B. cinerea*, both model

organisms, might help in the understanding of the VNT-induced resistance in other hosts outside the *Solanaceae* family. For this reason the aims of this chapter were first to check if Arabidopsis plants treated with VNT respond to this chemical and show a reduction in the symptoms caused by *B. cinerea* and, after that, to study the physiological nature of the response, measuring lignin and biochemical defences and studying the involvement of plant hormones.

5.2. Material and methods

5.2.1. Pathogen and plant material

5.2.1.1. Pathogen material

Botrytis cinerea Pers:Fr isolate B05.10 was provided by Dr. Jan van Kan (Laboratory of Phytopathology, Wageningen University, The Nederlands). The conidia of this pathogen were stored at -80ºC in 75% glycerol and 5 mM NaCl. To perform the assays 2 µl of this stock was sown in malt extract agar or PDA plates. *B. cinerea* was grown in a constant temperature of 20ºC and darkness.

5.2.1.2. Plant material

Arabidopsis thaliana Columbia-0 (Col-0) and the mutants *NahG*, *npr1-1*, *jin4* and *ein3* were provided by the Laboratory of Phytopathology from Wageningen University (The Netherlands). *A. thaliana* seeds were sown in potting soil and incubated for 2 days at 4ºC. Then, they were transferred to a growth chamber and kept in darkness for 2 days. Since then, plants were grown under a photopheriod of 12 h of light and 12 h of darkness, 70% humidity, 21°C of diurnal temperature and 19°C of night temperature. Plants were watered with tap water.

5.2.2. Inoculation with *Botrytis cinerea* and determination of symptoms

The inoculation with *B. cinerea* was performed using conidia suspension or plugs. The conidia were obtained from a culture grown on malt extract agar at 20ºC in darkness for 3-4 days. Then the culture was incubated for 24 h under near-UV light (350-400 nm) to induce sporulation and immediately placed into darkness till 10th day.

To collect the conidia, 15-20 ml of sterile MilliQ water were added to the culture and then this was rubbed with a sterile loop. The suspension was filtered through a sterile filter of glass wool and the filtrate was centrifuged at 120 xg for 5 min. The pellet was resuspended with sterile MilliQ water. The concentration was determined using a Neubauer-Improved counting chamber.

Leaves of 5-6 weeks old *A. thaliana* plants were inoculated with 3 µl drops of 1.2% PDB (potato dextrose broth) with 10^6 conidia per ml. Three leaves per plant were inoculated. After inoculation plants were placed in a wet chamber and low light.

Alternatively plants were inoculated with plugs of 5 mm of diameter from a 4 days old culture on PDA. A plug was placed on the leaf surface avoiding the main vein. Three leaves per plant were inoculated. Plants were introduced in a wet chamber and low light.

Symptoms were measured 1 DPI (days post-inoculation), when plants were inoculated with plugs, or 3 DPI, when plants were inoculated with drops. To evaluate the symptoms three parameters were used: severity, incidence and disease area. Severity was calculated using a scale of severity that goes from level 0 (no symptoms) to level 4 (almost the whole leaf is affected) (Figure 5.1). Incidence was calculated counting the expanding and the non-expanding lesions and expressing the data as percentage. A lesion was expanded when the lesion was equal or greater than the point of inoculation. Finally, the disease area was calculated using the diameter of the expanded lesions.

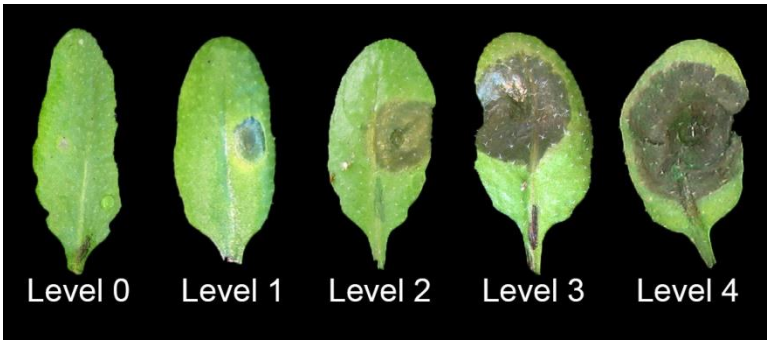

Figure 5.1. Scale of affected leaf surface used to calculate the percentage of severity.

The inoculation with plugs was used to check the effect of VNT in *A. thaliana* Col-0 and also to collect samples for the analysis of physiological parameters described below. The inoculation with drops of a conidial suspension was used to carry out the assays with the mutants as well as the corresponding wild type (Col-0) for comparison.

These last assays were carried out in the Botrytis group of Laboratory of Phytopathology of Wageningen University (The Netherlands).

5.2.3. Application of vanillyl nonanoate treatment to plants

24 h before inoculation, plants were treated with 150 µM VNT. This compound was applied by infiltration in 2 leaves from the bottom of the plant. VNT was dissolved in 0.1% DMSO (García *et al*., 2018). Plants infiltrated with 0.1% DMSO were used as a control. At least two independent experiments were carried out for each assay.

5.2.4. Determination of phenolic compounds

5.2.4.1. Phenols extraction

Phenols extraction was carried out in leaves at 0 and 8 h after inoculation. The methodology is described in Díaz *et al*. (2001).

For each experiment 3 leaves from five plants were taken per treatment and per time point. Each sample was homogenized in mortar with 2.5 ml of 80% methanol and incubated at 70ºC for 15 min. Then they were cooled and centrifuged at 1400 xg for 10 min. The supernatant was collected in a graduated tube. The precipitate was resuspended in another 2.5 ml of 80% methanol and re-centrifuged. The supernatant was collected and added to the previous supernatant and adjusted to 5 ml. The precipitate was preserved in pure methanol to measure lignin later (see section 5.2.5).

5.2.4.2. Phenolic compounds quantification

To quantify phenolic compounds we followed the protocol described in Díaz *et al*. (2001). 50 µl of the supernatant obtained in the extraction (see section above) were taken and mixed with 750 µl of water and 50 µl of Folin-Ciocalteu Reagent. This mixture was incubated for 3 min at room temperature. Subsequently, 150 µl of 20% Na_2CO_3 was added and was incubated at room temperature for 2 h. Finally absorbance was measured at 760 nm.

To determine the concentration of soluble phenolics, a calibration curve was made using different concentrations of gallic acid (0.01 mg/ml, 0.02 mg/ml, 0.05 mg/ml and 0.1 mg/ml). Three independent experiments were performed.

5.2.5. Determination of lignin in the cell wall

5.2.5.1. Preparation of cell wall samples

Cell walls were prepared using the technique described in Díaz & Merino (1998).

As it was mentioned before, the sample used to quantify the lignin was the pellet stored in pure methanol after the extraction of phenolic compounds (see section 5.2.4.1).

For the preparation of cell wall samples methanol was initially removed by centrifugation (1400 xg for 10 min) and then samples were rinsed with 10 ml distilled water. The supernatant was removed by centrifugation (1400 xg for 10 min) and the pellet was re-suspended in 6 ml of distilled water. The suspension was stirred for 1h. Then the samples were centrifuged for 10 min at 1400 xg and the supernatant was removed.

The precipitate was washed by adding water and centrifuging (1400xg for 10 min) three times. Then the precipitate was resuspended in 6 ml of a solution containing 0.5 M phosphate buffer pH 7.0, 5% ethanol and 0.02% protease (Pronase E) preincubated for 2 h at 37ºC. Subsequently this suspension was incubated 22 h at 37ºC. After this time the solution was removed by centrifugation (1400 xg, 10 min). The precipitate was washed three times with distilled water, three times with 96% ethanol and twice with absolute ethanol. Finally, the pellet was dried at 37ºC.

5.2.5.2. Lignin quantification

The abundance of lignin in cell walls was measured as it was described in Jonhson *et al*. (1961) with modifications according Hatfield *et al*. (1999).

Between 1 mg and 2 mg of cell walls were mixed with 1 ml of a solution composed of acetyl bromide and acetic acid in a proportion 1:3 (v/v). This mixture was

incubated at 50ºC for 2 h. Then the suspension was cooled in ice (5 min) and mixed with 0.9 ml of 2N NaOH, 5 ml of glacial acetic acid and 0.1 ml of 7.5 M hydroxylammonium chloride. After that, the mixture was filtered through a glass wool filter and the absorbance of the filtrate was measured at 280 nm. The blank was prepared in the same way but without cell wall sample.

The amount of lignin was calculated considering that 10 µg of lignin/ml is equal to an absorbance of 0.24 (Fry, 1988) and the result was expressed as a percentage of the control. Four independent experiments were performed.

5.2.6. Evaluation of gene expression in *Arabidopsis thaliana*

Gene expression was evaluated at 0 h and 8 h after inoculation. Three leaves from 5 plants were taken per treatment and per hour to perform the study. Samples were frozen in liquid nitrogen and stored at -80°C until RNA extraction. The samples were homogenized in liquid nitrogen. 60 mg of the homogenate were taken for RNA extraction following the BioRad Aurum™ Total RNA Mini Kit protocol.

Subsequently, the amount of RNA present in the samples was quantified using a spectrophotometer (Helios γ, Thermo) and the cDNA was synthesized following the protocol of the iScript™ cDNA Synthesis Kit.

The obtained product was used to study the gene expression by Real Time quantitative PCR (qPCR). This step was carried out by Dr. M.ª Fernanda Rodríguez Fariña from the Molecular Biology Unit from Research Support Services of UDC. The cDNA samples were analysed with the Biorad iCyclerTMiQ System. The gene expression of *AtPR1*, marker gene for SA, and *AtPDF1.2*, marker gene for JA/ET, was measured. *AtAkaG6* was used as housekeeping gene (van der Does *et al*., 2013). The primers used for amplification are shown in Table 5.1. Also several genes encoding peroxidases were evaluated. There are 73 genes of class III peroxidases in Arabidopsis, and we selected genes that were reported to show a change in their expression as response to a pathogen or to be involved in lignification. To do that we used the data present in AtGenExpress (https://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) available through TAIR (www.arabidopsis.org) as well as data published in different articles. In table 5.2 there is a liste of all the genes, the primers

and the criteria of selection. The primers were designed following the method described in chapter 4 section 4.2.10 and their specificity was checked by standard PCR.

The conditions of qPCR were 2 min of denaturation at 95°C followed by 40 amplification cycles (each cycle consisting of 20 s at 95°C, 25 s at 58°C and 50 s at 72°C).

Data analysis was performed using the Biorad Optical System Software 3.0. The efficiency and Ct (Cycle threshold) values obtained were calculated and processed by the Pfaffl method (2001) to obtain the relative expression values. This method defines the relative expression as:

$$Relative\ expression = \frac{E^{\Delta Ct_{target}\ (control-sample)}}{E^{\Delta Ct_{housekeeping}\ (control-sample)}}$$

where E is the efficiency and Ct is the number of cycles required to detect the amplicon signal.

At least three independent experiments were performed for each gene evaluated.

Table 5.1. Primers used to evaluate the expression of gene markers for the hormones SA and JA/ET by real time qPCR

Gene	Accession number	Reference	Primer		
			Name	Sequence	Amplicon
Aka G6	At1g13320	van der Does *et al*. (2013)	At1g13320 Fw At1g13320 Rv	5'TAACGTGGCCAAAATGATGC3' 5'GTTCTCCACAACCGCTTGGT3'	61
PDF1.2	At5g44420	Zhang & van Kan (2013)	PDF1.2 Fw PDF1.2 Rv	5'CACCCTTATCTTCGCTGCTC3' 5'GTTGCATGATCCATGTTTGG3'	175
PR1	At2g14610	Zhang & van Kan (2013)	qPR1FW qPR1RV	5'TCGTCTTTGTAGCTCTTGTAGGTGC3' 5'ACCCCAGGCTAAGTTTTCCC3'	227

Table 5.2. Primers used to evaluate the expression of genes encoding peroxidases by Real Time qPCR and criteria used to choose them.

Gene	Accession number	Primer			Criteria of selection	Reference
		Name	Sequence	Amplicon		
PRX4	At1g14540	At1g14540FW At1g14540RV	5'GCTCTCTCAGGAGCTCACAC3' 5'GCTCTCTCAGGAGCTCACAC3'	92	Response to pathogen (low)	TAIR
PRX17	At2g22420	At2g22420FW At2g22420RV	5'GACTTGGTTTCAGGGAGAGGG3' 5'CACCAGGTCTCCCAGATTGC3'	166	Involvement in cell wall lignification	Cosio *et al*. (2017)
PRX 21	At2g37130	At2g37130FW At2g37130RV	5'TCGTCCCAAGGTTTCGATGG3' 5'CGAATGTCCGGATCGGTAGT3'	92	Response to pathogen	TAIR
PRX25	At2g41480	At2g41480FW At2g41480RV	5'TGCATGTTACGTAATTGTCGTCT3' 5'TTTACCATCCAAAACAAACTCACT3'	89	Involvement in cell wall lignification	Shigeto *et al*. (2013)
ATP24a	At5g39580	At5g39580FW At5g39580RV	5'ATTGGTGTGAAGACGGGGAC3' 5'GCCCTTGAACAAGAGTTAGCC3'	120	Response to pathogen (low)	TAIR
PRX53	At5g06720	At5g06720FW At5g06720RV	5'ACGCGTTTAATCGACTTTTTCTCT3' 5'TTCCACTGATTGGTGCGGAA3'	87	Involvement in cell wall lignification	Barros *et al*. (2015)
AtPRX 67	At5g58390	At5g58390FW At5g58390RV	5'GAGGGGGTTCGAAGTGATCG3' 5'CCACCTAGGAGGAGAACGGA3'	120	Low response to pathogen	TAIR
PRX71	At5g64120	At5g64120FW At5g64120RV	5'ACCCAAAAGTGGGTTGATGA3' 5'ATCCAAACTAGGACCAAACTCA3'	126	Response to pathogen and involved in lignification	TAIR, Shigeto *et al*. (2013)

5.2.7. Quantification of total proteins and activity measurement of three enzymes involved in defence

5.2.7.1. Extraction of total proteins

Protein extraction was performed in leaves at 0 and 8 h after inoculation. Three leaves from five plants were taken per treatment and per time point.

Samples were homogenized in a mortar at 4°C with 50 mM Tris HCl buffer pH 7.5, containing 1M KCl and 0.5 g of PVPP (polyvinylpolypyrrolidone)/10 g of tissue. The crude extract was centrifuged at 12,857 xg and 4°C for 20 min. The supernatant was collected and stored at -80°C until further use.

5.2.7.2. Quantification of total proteins

The determination of the total proteins was carried out following the method described in Stoscheck (1990) using Bradford Reagent.

To determine the total proteins, 900 µl of Bradford Reagent was mixed with 50 µl of 1M NaOH and 10 µl of the extract obtained, previously centrifuged (1 min at 16060 xg). The mixture was adjusted to 1 ml with distilled water and incubated 30 min in darkness. Then the absorbance was measured at 590 nm. The blank was prepared identically but with water instead of sample. The protein concentration in the sample was calculated for a standard curve made with bovine serum albumin. Four independent experiments were carried out.

5.2.7.3. Measurement of peroxidase activity

Peroxidase activity was quantified using 4-methoxy-1-naphthol as substrate. The reaction mixture consisted of 930 µl of 50 mM Tris HCl buffer pH 7.5 at 25°C, 10 µl of 100 mM 4-methoxy-1-naphthol, 50 µl of 6.6 mM H_2O_2 and 10 µl of the sample, previously centrifuged (1 min at 16060 xg). This mixture was adjusted to 1 ml with distilled water. The change in absorbance was measured for 1 min at 593 nm. Activity was calculated in International Units (U)/mg protein and then it was expressed as percentage of the control. Four independent experiments were performed.

5.2.7.4. Measurement of β-1,3-glucanase and chitinase activity

β-1,3-glucanase and chitinase activities were quantified using laminarin and glycolchitin respectively as substrate (Veloso *et al*., 2014). The reaction mixture consisted of 82.5µl of 1% of laminarin or glycolchitin, 332.5 µl of 100 mM sodium acetate warmed at 37ºC and 85 µl of sample. This mixture was incubated for 10 minutes at 37ºC and then kept in ice for other 5 min. Then, 670 µl of 15 mM potassium ferricyanide in 0.5 M sodium carbonate was added and then the mixture was boiled for 15 minutes. The absorbance was measured at 420 nm. The reference was prepared following the same steps but containing 85 µl of Tris HCl 50 mM buffer pH 7.5 instead of sample. The enzyme activity was calculated as International Units (U)/ mg of protein and then it was expressed as percentage of the control. Four independent experiments were carried out for each enzyme.

5.2.8. Hormone quantification by liquid chromatography and ESI mass spectrometry

Different plant hormones were quantified in leaves from plants treated with VNT. Three leaves from eight plants were collected per treatment. All the samples were immediately frozen in liquid nitrogen and stored at -80ºC till being freeze-dried. After freeze-drying, they were homogenized and 50 mg were placed in a microtube to be analysed according to Sánchez-Bel *et al*. (2018).

Samples were analysed with the assistance of Victoria Pastor and Victor Flors (Metabolic Integration and Cell Signaling Group, Universitat Jaume I, Castellón, Spain).

For the hormone extraction, 1 ml of Milli-Q water and internal standards containing a pool of abscisic acid-d6 (ABA-d6), salicylic acid-d5 (SA-d5), dehydrojasmonic acid (hJA), and JA-Ile-d6, were added to each sample and incubated for 1 h in ice. Five glass beads (2 mm of diameter) were added to each sample and then they were homogenized in a shaker for 3 min 30 Hz. Samples were centrifuged for 40 min at 15493.24 xg and 4ºC. The supernatant was recovered and its pH was adjusted to 2.6 with 30% acetic acid. After that an extraction liquid-liquid was performed twice with 1.5 ml of diethyl ether. Both organic phases were gathered in another tube after a centrifugation of 3 min at 3689.4 xg and 4ºC. Then the supernatant was evaporated in a Speedvac. The pellet was resuspended in 1ml 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. Salicylic acid (SA), jasmonoyl isoleucine (JA–Ile), oxo-phytodienoic acid (OPDA), abscisic acid (ABA) were analysed in Acquity Ultraperformance Liquid Chromatography System (UPLC) coupled to triple quadrupole mass spectrometry (TQD) as described by Sánchez-Bel *et al*. (2018). 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 version 4.1 (Waters) software was used to process the data. Two independent experiments were carried out per hormone.

5.2.9. Statistical analysis

All the statistical analysis was performed using Statgraphics 5.1. The t-Student test was used to analyse data from initial disease areas with wild type and the quantification of hormones. Data from peroxidase activity, soluble phenolics, lignin, β-1,3-glucanase activity, chitinase activity and the gene expression of peroxidases, *PR1* and *PDF1.2* were analysed using Kruskall-Wallis test. Finally, data from incidence and severity with drop inoculation were analysed using a Chi-square test.

5.3. Results

5.3.1. VNT reduces grey mould symptoms in *Arabidopsis thaliana*

Arabidopsis Col-0 plants were treated with VNT and then inoculated with *B. cinerea*. This treatment did not block the infection because there were leaves with necrotic tissue. However, the disease area was lower in VNT-treated plants than in control (Figure 5.2).

5.3.2. VNT induces lignification in Arabidopsis

To determine the impact of VNT on lignification before and after inoculation with *B. cinerea*, we measured peroxidase activity, soluble phenolics, lignin and peroxidase gene expression. The enzyme peroxidase was chosen because it catalyses the lignin formation using soluble phenolics as supply source of units for lignin biosynthesis (Barros *et al*., 2015).

Before pathogen inoculation, 24 h after VNT treatment, only the activity of the enzyme peroxidase was induced by the treatment (Figure 5.3.A) but no differences were observed in either soluble phenolics or amount of lignin (Figure 5.3.C and 5.3.E).

On the other hand, when those compounds were evaluated 8 h after inoculation, the situation was different. In this case the activity of the enzyme showed no differences between treatments (Figure 5.3.B) but soluble phenolics decreased (Figure 5.3.D) and the amount of lignin increased in VNT-treated plants (Figure 5.3.F).

Peroxidase activity showed an increase before inoculation in VNT-treated plants. For this reason we decided to check the expression of several peroxidases genes. In table 5.3 there is a list of all the genes studied. PRX4, PRX25, ATP24a, PRX53 and PRX71 did not amplified in the samples but PRX17, PRX21 and AtPRX67 amplified in both times (before and after Botrytis inoculation). Only PRX17 was upregulated after VNT treatment but after inoculation there were no significant differences between groups (Figure 5.4).

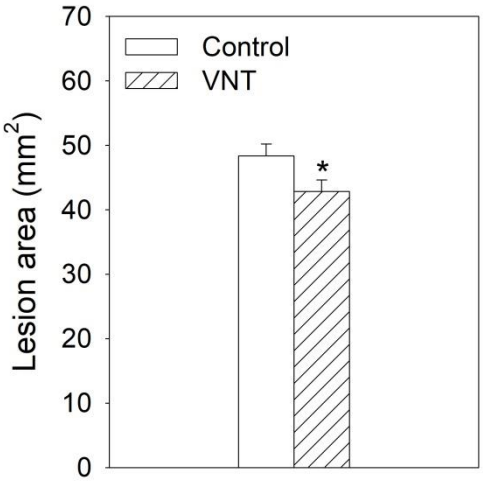

Figure 5.2. Effect of vanillyl nonanoate (VNT) on symptoms of grey mould in *Arabidopsis thaliana* Col-0. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in t-Student test. Three independent experiments with 21 plants per group and 63 leaves in total were performed.

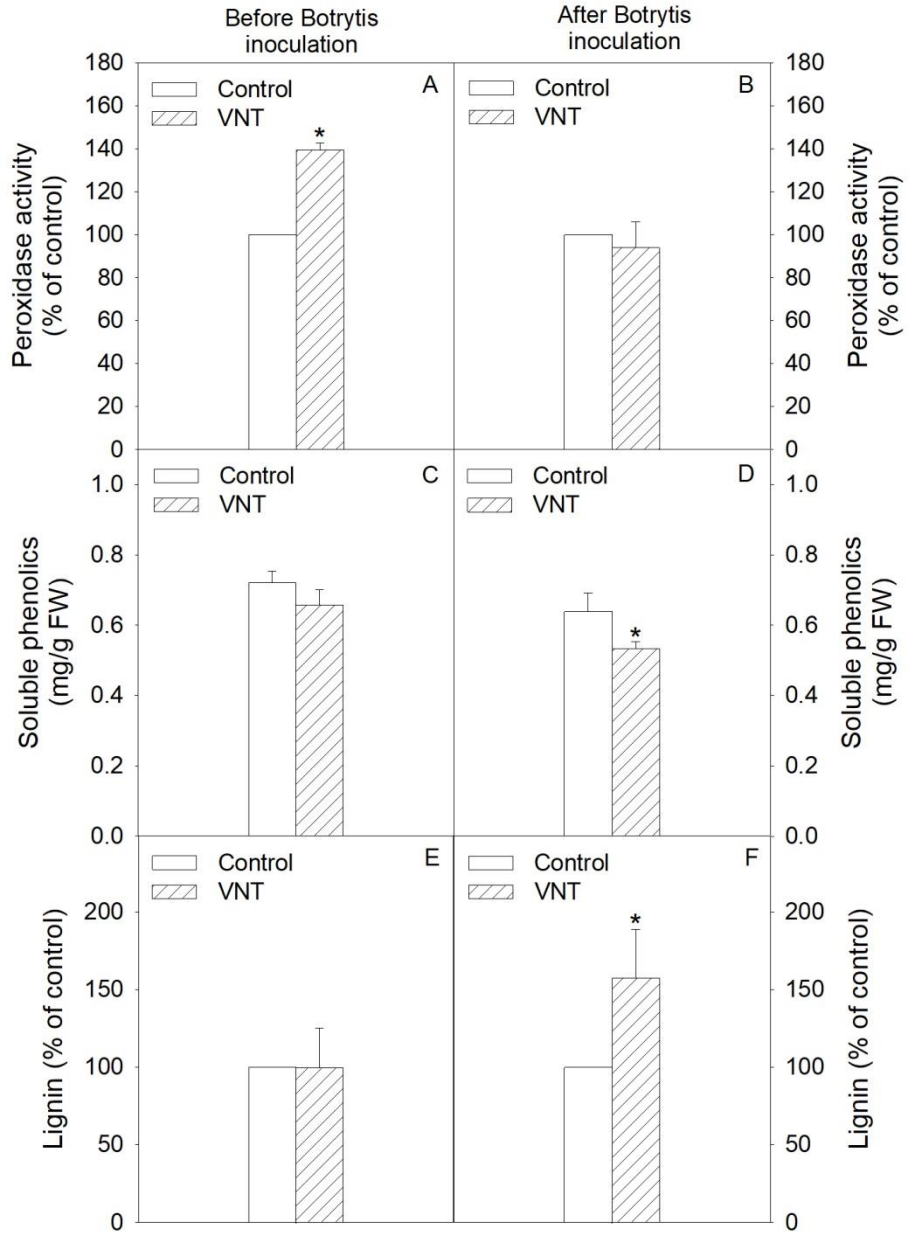

Figure 5.3. Effect of vanillyl nonanoate (VNT) on lignification before and after (8 h) *B. cinerea* inoculation. (A-B): Peroxidase activity. (C-D) Soluble phenolics. (E-F) Amount of lignin. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in a Kruskal-Wallis test. Three independent were carried out to measure the soluble phenolics and four independent experiments were performed to evaluate the peroxidase activity and the amount of lignin.

Table 5.3. List of peroxidase genes studied indicating if there was amplification, the effect of vanillyl nonanoate (VNT) and the effect of pathogen *Botrytis cinerea*.

Peroxidase gene	Accession number	Amplification	Effect of VNT	Effect of pathogen
PRX4	At1g14540	No	-	-
PRX17	At2g22420	Yes	Increase	No effect
PRX 21	At2g37130	Yes	No effect	No effect
PRX25	At2g41480	No	-	-
ATP24a	At5g39580	No	-	-
PRX53	At5g06720	No	-	-
AtPRX67	At5g58390	Yes	No effect	No effect
PRX71	At5g64120	No	-	-

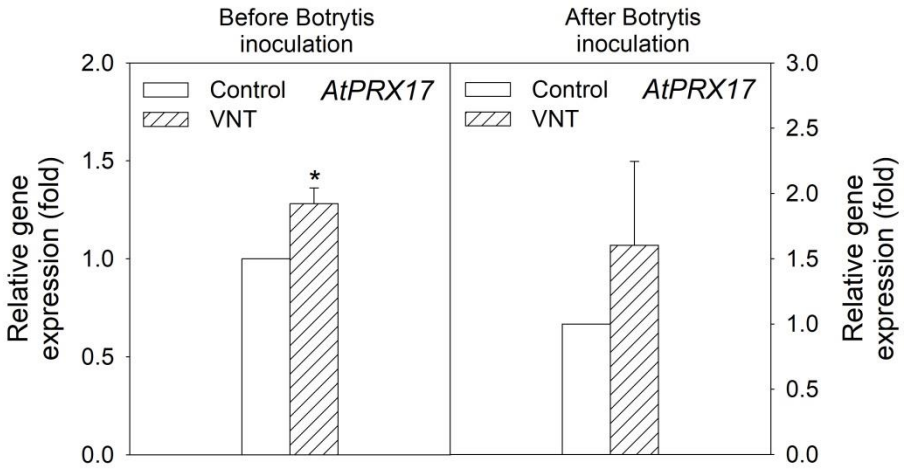

Figure 5.4. Effect of vanillyl nonanoate (VNT) on the gene expression of Arabidopsis peroxidase 17 (At2g22420) before and after (8 h) *Botrytis cinerea* infection. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in Kruskal Wallis test. Three independent experiments were performed.

5.3.3. Effect of VNT on activity of two enzymes involved in plant defence

The activity of β-1.3-glucanase and chitinase was quantified before and after *B. cinerea* inoculation, in plants previously treated with VNT and control plants.

Before the inoculation both enzymes showed an induction due to treatment with VNT (Figure 5.5.A and 5.5.C). However, there were no differences between groups in both enzymes after Botrytis inoculation (Figure 5.5.B and 5.5.D).

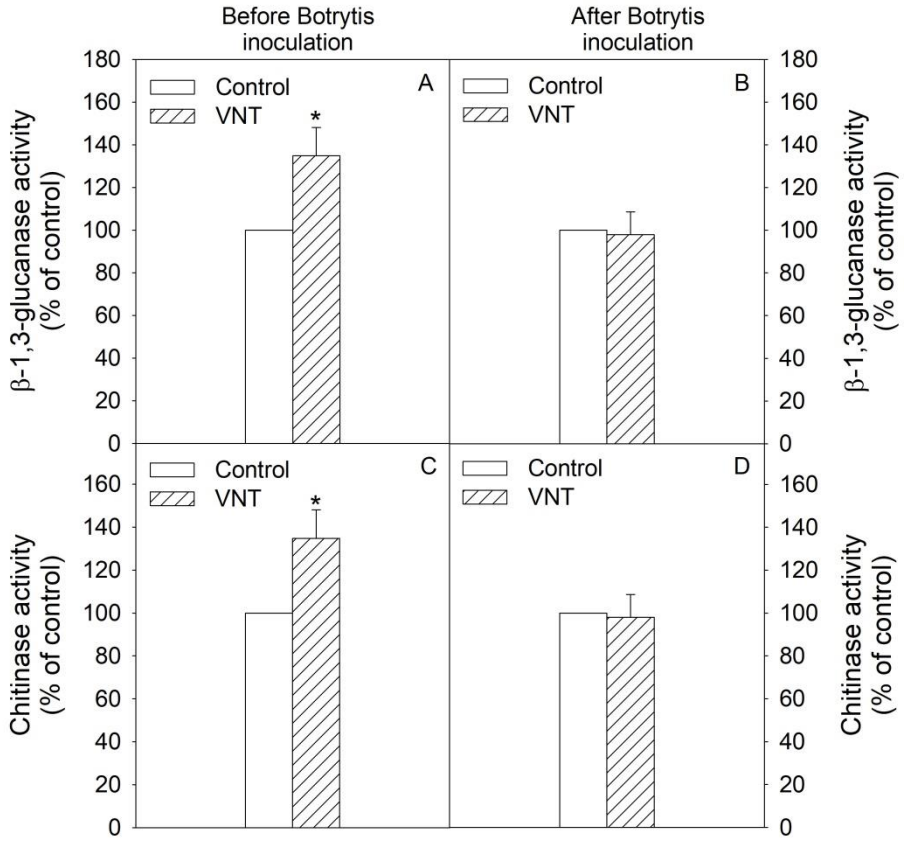

Figure 5.5. Effect of vanillyl nonanoate (VNT) on the activity of two enzymes involved in plant defence before and after (8 h) inoculation with *B. cinerea*. (A-B) β-1.3-glucanase activity. (C-D) Chitinase activity. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in a Kruskal-Wallis test. Four independent experiments were performed for each enzyme.

5.3.4. Effect of VNT on the main defence hormones

Four Arabidopsis mutants were chosen to test the role of three hormone (SA, JA, ET) pathways in the signalling associated to VNT. Those were *NahG*, defective in SA accumulation, *npr1-1*, defective in the induction of SAR, *jin4*, a MeJA-insensitive mutant, and *ein3*, an ET-insensitive mutant.

NahG plants showed the same incidence and severity in control and VNT-treated plants (Figure 5.4.D and 5.6.E). However, both incidence and severity were higher in *NahG* than wild-type (WT) (Figures 5.6.A and 5.6.B). This mutant had almost a 100% of leaves with expanding lessions and the severity of those lesions was classified mainly in levels 2 and 3 even reached the level 4 indicating a medium and strong infection. These results point to a SA involvement in VNT-induced resistance. To probe this hypothesis we tested the mutant *npr1-1*. Both incidence and severity did not show differences due to the VNT treatment (Figure 5.6.G and 5.6.H).

Regarding to mutant jin4, both indicence and severity showed no differences due to the VNT treatment (Figure 5.6.J). These results suggest a role of JA in the response to VNT.

Opposite to the rest of mutants, the treatment of *ein3* with VNT caused a decrease in expanding lesions (Figure 5.6.M) and lower severity (Figure 5.6.N), pointing to the lack of involvement of ethylene in the response to VNT.

In addition, the expression of *PR1*, marker gene for SA, and *PDF1.2*, marker gene for JA/ET, were measured in wild type before and after Botrytis inoculation. However, there were no statically significant differences in *AtPR1* and *AtPDF1.2* at the tested time points (Figure 5.7).

Because of the lack of correlation between the data from the mutants and the marker genes, we decided to quantify the hormones directly in the plant. SA, JA, JA-ILE, OPDA and ABA were measured in leaves 0 h before inoculation. This means that the samples were collected 24 h after VNT treatment.

SA showed no differences due to the treatment (Figure 5.8). However, JA and JA-ILE increased their amount after VNT treatment (Figure 5.8). In the same way as SA, OPDA had no significant differences due to the application of VNT (p-value = 0.125) but a trend can be observed (Figure 5.8).

Finally, ABA was also quantified on account of its role regulating the signal of SA, JA and ET (AbuQamar *et al.*, 2017). The treatment with VNT led to a decrease of the amount of this hormone (Figure 5.8).

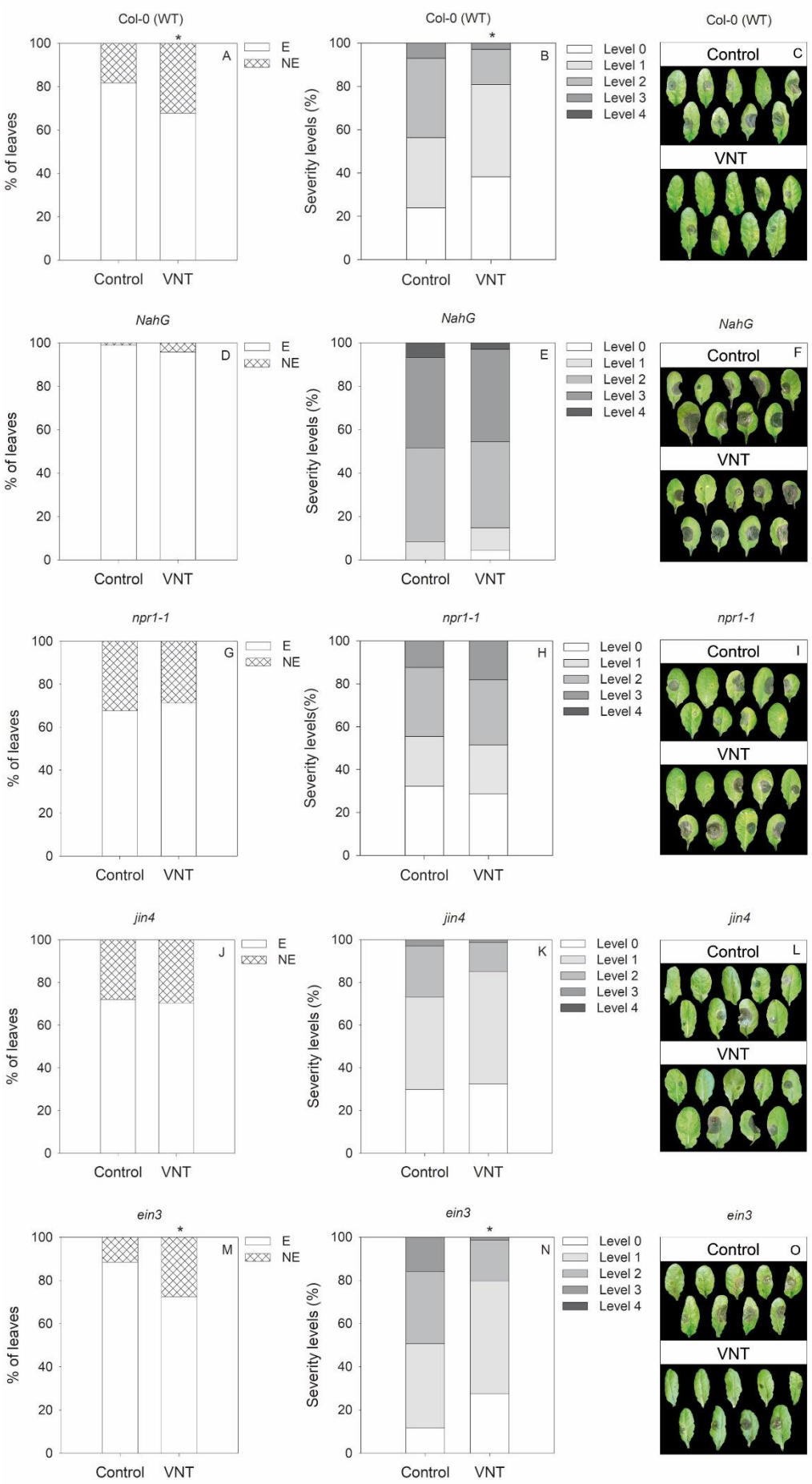
Col-0 (WT)
A
B
C
NahG
D
E
F
npr1-1
G
H
I
jin4
J
K
L
ein3
M
N
O
% of leaves
Severity levels (%)
Severity levels(%)
E
NE
Level 0
Level 1
Level 2
Level 3
Level 4
Control
VNT
*
0
20
40
60
80
100

Figure 5.6. (see previous page) Effect of vanillyl nonanoate (VNT) on Col-0 and four Arabidopsis mutants defective in main defensive hormones signalling pathways. (A, D, G, J, M) Incidence of lesions. E: expanding lesions; NE: non-expanding lesions. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in a Chi-squared test. (B, E, H, K, N) Severity of symptoms using a severity levels scale that goes from level 0 (no symptoms) to level 4 (the lesion affects to whole leaf). To see the scale check the section 5.2.2, figure 5.1. Data are the mean ± SE. The asterisk (*) means significant differences ($p<0.05$) in a Chi-square test. (C, F, I, L, O) Aspect of leaves 3 days after infection. At least three independent experiments with 7 plants per group, as minimum, were performed.

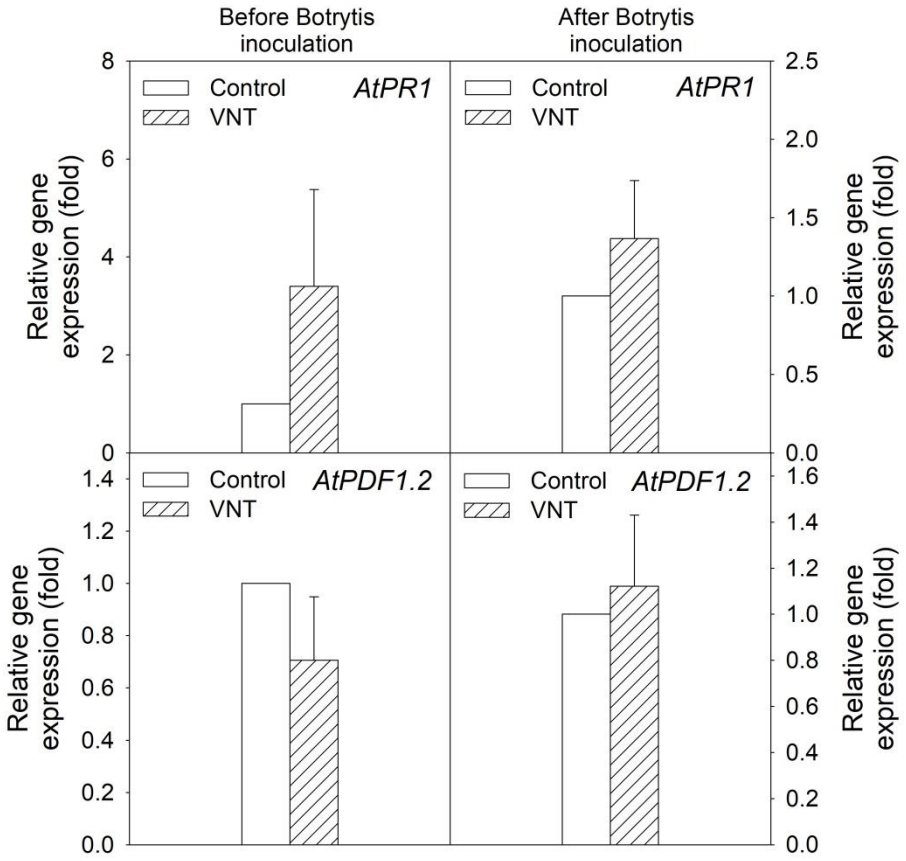

Figure 5.7. Effect of vanillyl nonanoate (VNT) on marker genes *AtPR1* (first row) and *AtPDF1.2* (second row) before and after (8 h) *B. cinerea* inoculation. Data are the mean ± SE. Asterisk (*) means significant differences ($p<0.05$) in a Kruskall-Wallis test. Six independent experiments were carried out for *AtPR1* and four independent experiments were performed for *AtPDF1.2*.

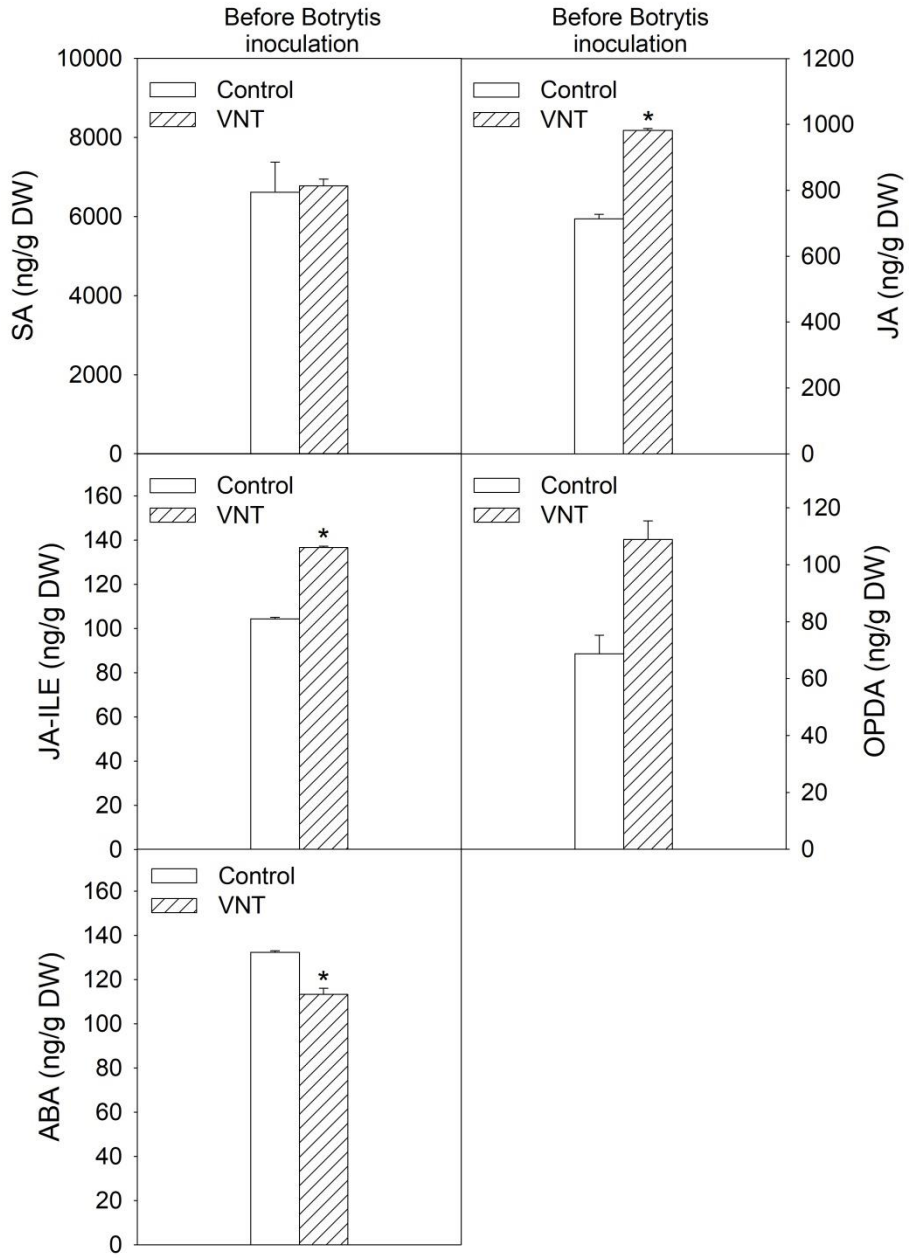

Figure 5.8. Effect of vanillyl nonanoate (VNT) on hormones in leaves of Arabidopsis 24 h after treatment. Two independent experiments were performed per hormone. The data are the mean ± SE. Asterisk (*) indicates significant differences ($p < 0.05$) in a t-Student test.

5.4. Discussion

Capsaicinoids are compounds that induce resistance against pathogens but their use is limited because they are irritant (García *et al*., 2018). In previous chapters of this thesis, capsinoids were tested as an alternative to capsaicinoids. This project was performed in pepper but do capsaicinoids and capsinoids induce resistance in other plants? In 2013, Song *et al*. published an article in which Arabidopsis and tobacco

plants were treated with capsaicin and inoculated with necrotrophic bacteria *Pectobacterium carotovorum* supsp. *carotovorum* SCC1 and biotrophic bacteria *Pseudomonas syringae* pv. tomato DC3000 (Song *et al*., 2013). Song *et al*. (2013) applied the capsaicin by the roots and inoculated the leaves, that is, they tested the systemic resistance. In Arabidopsis, they observed a reduction of symptoms (4.6-fold less) caused by capsaicin (Song *et al*., 2013). On the other hand, they observed a reduction of 4.5-fold in tobacco plants infected with *P. carotovorum*. These results suggest that the protective effect of capsaicinoids is not only restricted to pepper. Then capsaicinoids can be used as inducers of resistance in other plants.

In this chapter we studied if VNT, in the same way to capsaicin in the work of Song *et al*. (2013), is able to induce resistance in Arabidopsis against *B. cinerea* as capsaicin. We found that VNT is able to reduce the disease symptoms, that is, VNT also induced systemic resistance in Arabidopsis.

One of the consequences of induction of resistance is the strengthening of cell wall by the deposition of lignin. This is the first physical barrier that Botrytis has to overcome and it has a critical role in the plant defence (Lloyd *et al*., 2011). In this study higher lignin level was observed in VNT-treated plants but only after inoculation. Several studies have shown the important role of cell wall in plant defence in Arabidopsis. The Arabidopsis mutant *comt1* is more susceptible to *B. cinerea* than the wild type. This is because the enzyme caffeate O-methyltransferase, involved in lignin biosynthesis, is unactive in this mutant (Quentin *et al*., 2009). Also other mutants with impaired lignin biosynthesis such as *ref3-2*, which has reduced sinapate esters and guaiacyl and syringyl residues, or *fah1-2*, which has reduced sinapoyl malate, syringyl lignin and sinapoyl choline, showed an increase of susceptibility after inoculation with *B. cinerea* (Lloyd *et al*., 2011).

Besides lignin deposition, phenylpropanoid pathway and peroxidases also participate in the strengthening of the cell wall. Phenylpropanoid pathway is responsible for, among others, supplying the substrates to be polymerized by peroxidase to form the lignin. It is difficult to obtain mutants affected in peroxidases because there are a lot of isoforms and the mutation in one of them can be alleviated by an increase in the expression of other isoform. In spite of that 44 of 73 genes, that form class III of peroxidases in *A. thaliana*, have been identified to have a putative function (Cosio & Dunand, 2009). These enzymes are related to plant defence against pathogens. In fact, it

was observed that the overexpression of peroxidases increases the plant resistance (Chassot *et al*., 2007). In addition they correlate to alterations in the cell wall as it could be observed in the present study.

Peroxidase activity increased as response to VNT before the inoculation but at that time there was no effect in phenolics or lignin. However, a reduction in soluble phenolics was appreciated after inoculation. This, together with lignin, draws a situation in which VNT induces the enzyme that starting to consume phenols. In the beginning the consumption of phenols is not too drastic. Thus there are no differences with the control, although it can be observed a certain trend in VNT-treated plants. The use of phenols becomes higher when the plants are inoculated with *B. cinerea* and this is reflected in increment of lignin in cell walls. However, after inoculation there is no correlation of lignin with peroxidase activity. Therefore increase of lignin is due to the increase in enzymatic activity before inoculation.

When we measured the expression of different genes encoding peroxidases, we observed a coincidence in time between the enzyme and one of the tested genes. This gene was *PRX17*. The PRX17 protein is located in the cell wall and it is involved in lignification during development and flowering (Cosio *et al*., 2017). There are no studies about the relation between this enzyme and the response to a pathogen except the microarray data stored in AtGenexpress that showed no induction to Phytophthora. However, Tran & Plaxton (2008) observed accumulation of this enzyme when Arabidopsis cell culture was deprived of phosphorous. This increase was related to response to oxidative stress (Tran & Plaxton, 2008). Moreover, PRX17 was found among first proteins secreted after treatment with SA (Cheng *et al*., 2008). We observed an increase on gene expression of PRX17 after treatment. Considering the function and location describe by Cosio *et al*. (2017), we could assume that strengthening of the cell wall is conducted at least in part by this enzyme. In addition, the results suggest that the activation of mechanisms took place in those 24 h after VNT treatment and the reinforcement of cell wall happened after Botrytis inoculation.

During the pathogen attack, there is also an induction of PR proteins that work together with physical barriers to resist the attack. Among these proteins there are two proteins, β-1,3-glucanase and chitinase, that cooperate degrading the fungal cell wall. The first enzyme degrades β-1,3-glucan and the second enzyme degrades chitin. The action of both enzymes implies on one side the lysis of fungal cell and on the other side

the production of DAMPs that will activate the plant defences (Balasubramanian *et al*., 2012). VNT induced the activity of both enzymes before inoculation. Also the treatment with a capsaicinoid induced the activity of chitinase as well as the expression of genes that code for β-1,3-glucanase and chitinase (Veloso *et al*., 2014). It has been demonstrated that there is a correlation between an induction of those enzymes and resistance against *B. cinerea* (Magnin-Robert *et al*., 2007; Magnin-Robert *et al*., 2013; Wang *et al*., 2016). Therefore, the reduction in symptoms observed is, partially, due to the induction of those enzymes by VNT because the integrity of the pathogen was affected by them.

Several signals are produced after pathogen recognition by the plant. This is the case of hormones that will activate and modulate the defence genes. Generally speaking, there are two well known pathways: one is controlled by SA and the other one is controlled by JA/ET (Dodds & Rathjen, 2010). In order to know which of these pathways are mediating VNT-induced resistance, Arabidopsis mutants *NahG*, *npr1-1*, *jin4* and *ein3* were selected.

The Arabidopsis *NahG* plants are defective in SA accumulation. Therefore they are used to study the role of SA in plant-pathogen interaction or the effect of an inducer. This mutant expresses the *Pseudomanas putida* NahG gene that encodes a salicylate hydroxylase. This enzyme converts SA in catechol (van Wees & Glazebrook, 2003). These plants were treated with VNT and inoculated with *B. cinerea* as well as it was the wild type. The absence of SA abolished the resistance conferred by VNT. Similar results were obtained by Song *et al.* (2013) with Arabidopsis *NahG* tested with capsaicin and inoculated with the necrotrophic bacteria *P. carotovorum*. Both results point out that the SA is an important signal in the induced resistance by capsaicinoids and capsinoids. To corroborate the role of SA, we used the mutant *npr1-1* in which SAR cannot be triggered. The data supported that VNT-induced resistance is SA-dependent. Similar results were observed by Song *et al.* (2013) in *npr1* treated with capsaicin. However, in our case the data of gene expression (*AtPR1*) and hormone (SA) quantification showed no differences between treatments before inoculation. It is possible that the SA signalling would be important but the amount of SA did not change. Other possibility is that the amount of SA would change after inoculation. Hael-Conrad *et al*. (2015) observed an increase in PR1 expression in plants treated with AsES, an extracellular elicitor protein produced by *Acremonium strictum* isolate SS71,

only at 6 h after inoculation and then decreased till 48 h after inoculation. They also measured the free SA at the same times but no differences due to the treatment were observed (Hael-Conrad *et al*., 2015). The values of SA were low at all the time points except at 48 h after inoculation. Similar observations were found by Mendez-Bravo *et al.* (2011), they observed an up-regulation in PR1 and no changes in SA in plants treated with N-isobutyl decanamide, an alkamide produced by *Acmella radicans* and *Cissampelos glaberrima*. Those experiments point out that the amount of SA can be independent of the expression of PR1. To further clarify the role of SA in VNT-induced resistance more experiments should be done as quantification of SA after inoculation or use other gene markers for SA such as *PR2, PR5, PAD4* (Phytoalexin deficient 4) (Li *et al*., 2017) or *PRP6* (Pathogenesis-related protein P6) (Martínez-Medina *et al*., 2017).

On the other hand, the mutant *jin4,* which is characterized to be insensitive to JA (Berger *et al*., 1996), and the mutant *ein3,* which is ET-insensitive, were tested to know if those hormones are involved in VNT-induced resistance. In the bioassays carried out with *jin4*, the absence of JA abolished the effect of VNT. However, the plant resistance was still triggered in *ein3* mutant by VNT. Similar results were obtained by Song *et al*., (2013) applying capsaicin in mutant *jar1-1* (JA-insensitive) and *etr1-3* (ET-insensitive). In order to confirm these results, we measured the expression of *AtPDF1.2*, which is considered a marker for JA, but the result was unclear. However, JA and JA-ILE increased their levels. Taking together these results, it is clear that JA signalling pathway is involved in VNT-induced resistance against *B. cinerea*.

On the other hand we measured the content of ABA. The ABA content was less in VNT-treated plant than in the control. ABA is a negative regulator of SA and also JA/ET (Anderson *et al*., 2004; Caarls *et al*., 2015). Due to this, ABA decreases its levels to activate the defences by SA and JA.

Generally speaking there is a pattern in which plant hormones should act in a pathogen challenge. SA is effective against biotrophic pathogen and JA is effective against necrotophic pathogens. Moreover, both pathways are antagonistic (Pieterse *et al*., 2012). Then, according with this the hormone involved in defence against *B. cinerea* should be JA. However, Ferrari *et al*. (2003) and Chassot *et al.* (2007) observed induction in *PR1,* marker for SA, and *PDF1.2,* marker for JA, at the same time. Moreover, this cross talk between these hormones has been observed during other interactions. The reason why in some cases SA and JA are antagonistic and in other are

synergistic is the concentration of each hormone. Mur *et al*. (2006) observed a synergetic effect when SA and JA were in low concentrations (10-100 µM) but when the concentration was high (250 µM), an antagonistic effect appeared.

To sum up, the VNT treatment is able to protect Arabidopsis plants against *B. cinerea* by plant induced resistance. The mechanism involved is the reinforcement of cell wall by deposition of lignin with induction of peroxidase activity and consumption of soluble phenolics. In addition, the enzymes β-1.3-glucanase and chitinase were also induced. Regarding to the signalling, the hormones SA and JA were involved in VNT-induced resistance. However, more work is necessary to fully understand the cross-talk of SA and JA after infection.

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GENERAL DISCUSSION

6.1. Capsinoids in Galician pepper and their utility as phytosanitary products

One characteristic of some pepper fruits is the hot flavour. This is a consequence of their content in secondary metabolites called capsaicinoids (Díaz *et al*., 2004). However, the analysis of different species and ecotypes of pepper showed the presence of similar compounds but the fruits were non-pungent. These compounds are the capsinoids and the capsiconinoids (Sutoh *et al.*, 2006; Lourdes-Reyes-Escogido *et al.*, 2011). The most studied between them are the capsinoids. In fact, they share pharmacological properties with the capsaicinoids as analgesic activity, antitumor activity, antioxidant activity or weight reduction (Luo *et al*., 2011). Then, their presence in pepper fruit would be a nutritional extra among the qualities the pepper fruits already have. For this reason, we analysed the content in capsinoids in the main Galician cultivars (Padrón, Couto, Arnoia, Branco Rosal, Oímbra, Mougán, Piñeira, Punxín, Vilanova and Couto Grande). We searched for capsiate, vanillyl nonanoate and dihydrocapsiate but none of them were found in any cultivar. As it was discussed in chapter 1, it is probably that in those cultivars the non-pungent analogues were capsiconinoids or simply they only have pungent compounds, but more research should be done to test it.

A role described for capsaicinoids in wild peppers was seed protection against the pathogen Fusarium (Tewksbury *et al*., 2008). This antimicrobial property was used for some human cultures to avoid the diseases caused for microbes (Cichewicz, & Thorpe, 1996). Moreover, the alteration of capsaicinoid accumulation pattern under stress conditions (Estrada *et al*., 1999; Medina-Lara *et al*., 2008; Phimchan *et al*., 2012) as well as the transport of capsaicinoids from fruits to the rest of plant (Estrada *et al*., 2002) convert these compounds in excellent candidates to use them in plant disease management. In fact, there are two articles, Song *et al*. (2013) and Veloso *et al*. (2014), in which the plant treatment with capsaicin and vanillylnonamide decreased the plant susceptibility. However, their irritant properties limit their use by farmers. Then, in this thesis we have proposed to use the capsinoids instead of capsaicinoids based on their similar chemical structure and similar pharmacological properties.

To develop this idea we have chosen vanillyl nonanoate (VNT), a synthetic analogue of capsiate. We used VNT instead capsiate because the synthesis of capsiate is

more complex than VNT due to the differences of the alkyl side chain (Reddy *et al*., 2011) and capsiate is more expensive.

We studied, on one side, the direct effect of VNT on spores of *Phytophthora capsici*, *Botrytis cinerea* and, on the other, the effect of VNT on pepper plants ecotype Padrón infected with *Phytophthora capsici*, *Botrytis cinerea* and *Verticillium dahliae*. VNT had antimicrobial activity on zoospores of *P. capsici* but not on *Botrytis* spores. However, their application to the plant reduced locally the severity of symptoms with evidence of induced resistance, except in the case of *V. dahliae*.

Because we observed an induced resistance with VNT we explored several defensive mechanisms and also components of signalling to elucidate the putative mechanism of action triggered by VNT treatment.

6.2. Effect of VNT in the interaction pepper-*Phytophthora capsici*

P. capsici is one of the most destructive pathogen that affects pepper production (Lamour *et al.*, 2012). It can cause from 30-40% of losses in less severe years till completely devastation of the crop (Zhou *et al*., 2017). Their control with fungicides is difficult due to the increase of resistant isolates. Then it is necessary to find new strategies of control.

VNT induced systemic resistance to *P. capsici* in the roots, reducing both symptoms and pathogen colonization. Therefore, we studied how VNT affect the cell wall lignification, some biochemical defences (PR1 gene, capsidiol biosynthesis gene, β-1,3-glucanase activity and chitinase activity) and the biosynthesis of the main hormones involved in defence in first hours of interaction pepper-pathogen.

24 h after VNT treatment, the plant showed a strengthen of cell wall (lignin deposition), increase of capsidiol biosynthesis gene and β-1,3-glucanase activity as well as an increase in the expression of ET biosynthesis genes. These results point out the VNT increase the plant defenses. In fact, these results would explain why VNT-treated plants had less pathogen biomass than control. However, these changes were not enough to avoid the disease because plants still showed some symptoms. Then, we studied what happened in pepper roots after Phytophthora inoculation.

The first barrier that pathogen has to defeat is the cell wall. Cell wall has two roles in a defensive process: a physical barrier and a source of signal (Bacete *et al*., 2018). As we mentioned previously, the cell wall was reinforced with lignin mediated by peroxidases after VNT treatment. After Phytophthora inoculation, VNT-treated plants also showed higher lignin. These results suggested that the most active deposition of lignin started after VNT treatment. Normally, deposition of lignin use to be linked to pathogen attack, but in some cases the application of elicitors in absence of pathogen can have the same effect. For example, the application of different extracts obtained from *Fusarium oxysporum* f. sp. *lycopersici* and *Trichoderma viride* were able to induce the cell wall reinforcement in root of tomato (Mandal & Mitra, 2007). Therefore, the lignin would make more difficult the penetration of *P. capsici* when it arrives.

Once *P. capsici* arrives, it starts to degrade the cell wall to enter in the cell. As a consequence, DAMPs are formed and sense by the cell generating signals as ROS (Hématy *et al*., 2009). We have measured the levels of hydrogen peroxide in the eight first hours after inoculation and we observed a peak at 4 h. This peak was higher in VNT-treated plants. This could be the moment that plant recognizes the presence of *P. capsici* and then, it activates the defences. However, the functions of H_2O_2 are not only acting as a signal but also H_2O_2 is used by peroxidase to produce lignin or acts as toxic molecule for the pathogen (Lehmann *et al*., 2015).

After recognition the plant accumulated around the penetration point capsidiol and PR1 proteins which have demonstrated to have importance in defence against *P. capsici* (Egea *et al*., 1996; Dunn & Smart, 2015; Gamir *et al*., 2017)

Also we could observe that roots started to synthetize the hormones SA, ET and JA after inoculation. These results suggested that the three hormones were modulating the response induced by VNT. Serrano Criollo de Morelos 334 (SCM334) is a pepper cultivar resistant to *P. capsici*. This cultivar showed an initial increase of JA with low levels of SA followed by an increase of SA with low levels of JA (Ueeda *et al*., 2006). It was suggested that initial increase of JA would be needed to induce the H_2O_2 production and, then, a HR (Ueeda *et al*., 2006). On the other hand, the reduction of the amount of SA abolished the resistance against this oomycete (Wang *et al*., 2018). Jin *et al*. (2016) observed an increased in the expression of *CaPTI1*, an ethylene-responsive factor, after *P. capsici* infection. Moreover, this factor could be modulating

the signal because it response to SA, MeJA, ethephon and ROS and also it has been described as an integrative factor of SA, ET and JA in Arabidopsis.

In figure 6.1 are summarised all the components that we have found to be induced a response due to VNT treatment and Phytophthora inoculation.

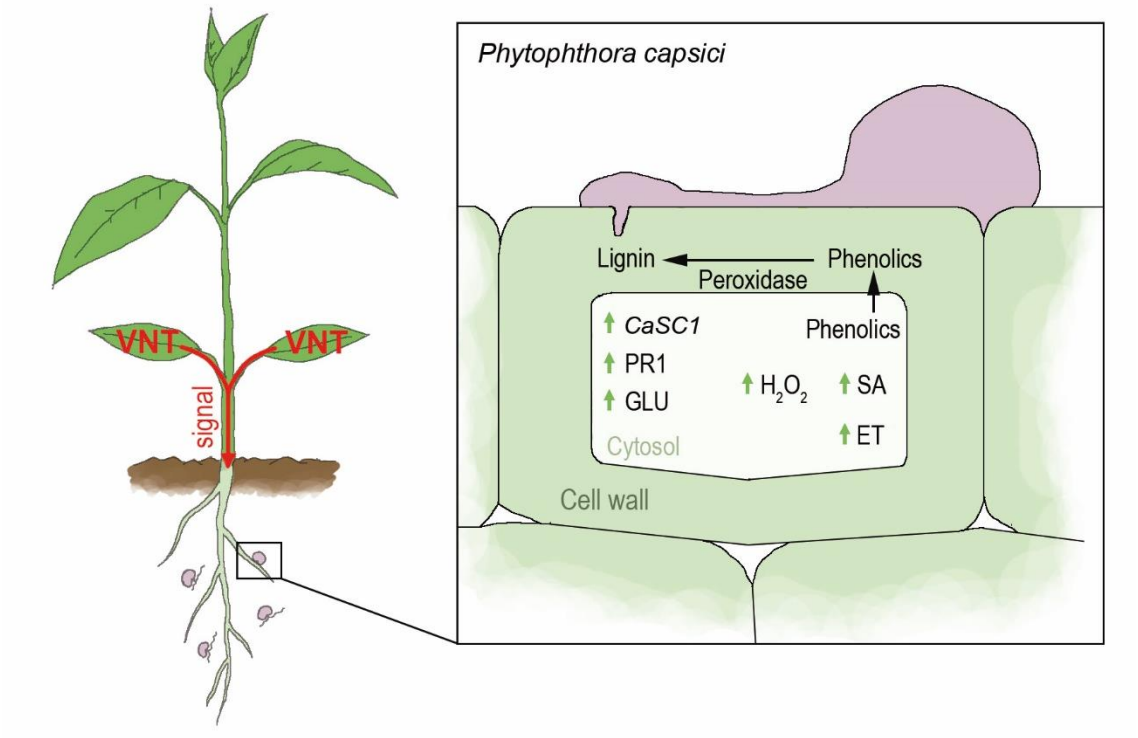

Figure 6.1. Proposed model about the effect of vanillyl nonanoate (VNT) on the interaction of *Capsicum annun-Phytophthora capsici*. ROS: reactive oxygen species, SA: salycilic acid; ET: ethylene; JA: jasmonate acid; PR1: pathogenesis-related proteins 1; GLU: β-1,3-glucanase. Green arrows mean that this parameter showed high levels in VNT-induced resistance.

6.3. Effect of VNT in the interaction pepper-*Botrytis cinerea*

Botrytis cinerea was selected to study the effect of VNT in leaves and, also, if this compound is able to protect systemically the leaves against pathogens.

As it was mentioned above, VNT had no antimicrobial activity against spores of *B. cinerea*. However, when it was applied in the plant by cotyledon infiltration and inoculation of the leaves, we could see a reduction of lesion correlated with less plant colonization by pathogen. These results indicated that VNT was able to induce resistance in other plant organs and against other pathogens than *P. capsici*, which is a hemibiotroph pathogen and *B. cinerea* is necrotroph.

Therefore, our next step was to study the mechanism that could be involved in VNT-induced resistance. Following the same scheme as in roots, we studied the lignification of cell wall, some biochemical defences and finally some signals.

To study the lignification we measured the activity of the enzymes peroxidases, beacuse they are involved in lignin polimerization (Almagro *et al*., 2009), and soluble phenolics as a source of monomers to form lignin. The results suggest that VNT also induce the deposition of lignin in cell wall there being more differences after inoculation. The data pointed out the peroxidase were responsible of that lignification, particularly *CaPO1* and *CaPO2*. *CaPO1* is related with lignification (Chmielowska *et al*., 2010) and *CaPO2* is related with H_2O_2 production (Choi *et al*., 2007). In fact, VNT-treated plants showed high levels of H_2O_2. Overall, the data suggest that this H_2O_2 could be produced, partially, by *CaPO2*. Then this H_2O_2 could be used by *CaPO1*, among other peroxidases, to increase the lignin content in cell wall.

The lignification study in both roots and leaves of pepper indicated that the reinforcement of cell wall is a common response to VNT treatment independently of plant organ explaining that in both roots and leaves we observed less pathogen colonization.

As we mentioned previously, ROS are formed in the moment that plant sense the stress (Lehmann *et al*., 2015). In the same way than pepper roots, the leaves had high levels of H_2O_2 but the accumulation pattern is different. In roots the accumulation was concentrated between 4 h and 6 h but in leaves the accumulation starting at 2 h creating an oxidative environment that can abolish the growth of Botrytis during its short biotrophic stage. Also in that moment, some changes are triggered inside the plant, and, as a consequence, PR1 proteins and β-1,3-glucanase started to expressed and some hormones are synthesized.

To study the role of hormones in leaves, we measured the expression of genes involved hormone biosynthesis, we used inhibitors for those hormones and we quantified the hormones too. After these analyses we concluded that, in the same way as in roots, hormones SA, ET and JA were involved in VNT-induced resistance. In addition ABA and 4-HBA would be regulators of SA and JA. We observed that pepper plants have 4-HBA, a compound that was related with the biosynthesis of cell wall components (Okrent *et al*., 2009) as well as it was found it during a abiotic and a biotic

stress (Smith-Becker *et al*., 1998; Tan *et al*., 2004; Horváth et al., 2007). Moreover, in this thesis we observed a corelation between 4-HBA and SA amounts. This is, when SA increased its levels, 4-HBA decreased it and viceversa.

In figure 6.2 are summarised all the components that we detected to be induced or repressed due to VNT treatment and Botrytis inoculation.

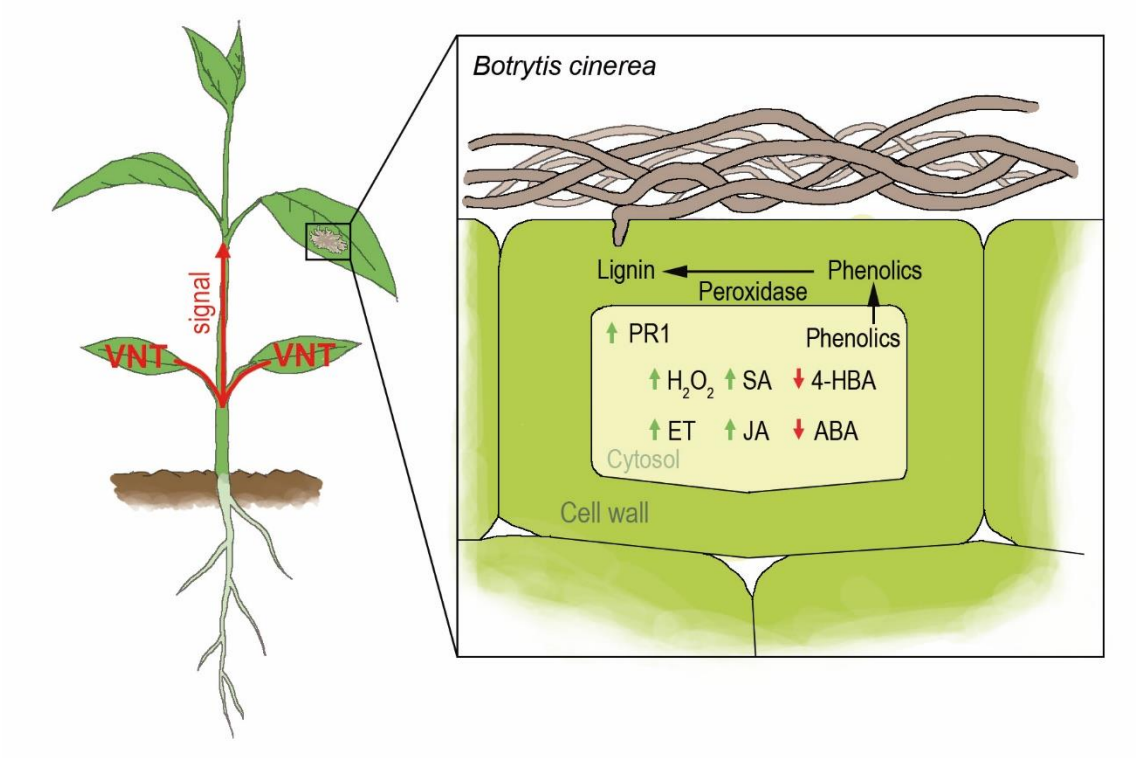

Figure 6.2. Proposed model about the effect of vanillyl nonanoate (VNT) on the interaction of *Capsicum annun-Botrytis cinerea*. PR1: pathogenesis-related proteins 1; ROS: reactive oxygen species, SA: salycilic acid; ET: ethylene; JA: jasmonate acid; 4-HBA: 4 hydroxybezoic acid; ABA: abscisic acid. Green arrows mean that this parameter showed high levels in VNT-induced resistance. Red arrows mean that this parameter decresed its amount during the process.

6.4. Effect of VNT in Arabidopsis thaliana against *Botrytis cinerea*

A. thaliana was selected as a host of *B. cinerea* to study if VNT was able to induce resistance in other species and if the mechanism could be similar to pepper.

Therefore the first step was to treat the plants with VNT and to inoculate them systemically with *B. cinerea* following the same temporal scheme used in pepper. The results showed a reduction in symptoms with systemic induction of resistance. This result together with the results of Song *et al*., (2013), in which tobacco and Arabidopsis

plants treated with capsaicin showed induction of resistance, suggest that both capsaicinoids and capsinoids are able to induce resistance in plants that do not produce those compounds.

Based on the results obtained with pepper and *B. cinerea* as well as with *P. capsici*, we studied the reinforcement of cell wall measuring peroxidase activity, phenolic, lignin and some peroxidases genes. In the same way than in pepper, cell wall was strengthened with lignin in a process mediated by peroxidases, especially PRX17, with consumption of phenolics. These similitudes of response to VNT point to that the cell wall is a common target independently of the plant species or even the organ. In fact the cell wall is a critical point in defence because it is the first barrier that pathogens have to defeat. Moreover, alterations in cell wall, including lignin content, modify plant resistance (Egea *et al*., 2001; Lloyd *et al*., 2011; Khan *et al*., 2018).

Together with the cell wall strengthening, the Arabidopsis plants treated with VNT also produced the enzymes β-1,3-glucanase and chitinase. Both enzimes are involved in fungal cell wall degradation and they have an important role intensifying the plant defence (Mauch *et al*., 1988; Hwang *et al*., 1997; Magnin-Robert *et al*., 2007).

Finally, we studied the hormones involved in the VNT induced resistance In Arabidopsis against *B. cinerea*. We observed that the hormones involved were SA and JA modulated by ABA. When we compared the players that modulates the VNT response were a bit different between pepper and Arabidopsis. In the last host, the ET was not relevant as it was observed with *ein3*, but the expression of ET biosynthesis genes as well as the assays with MCP in pepper demonstrated the ET was involved. Another difference that we observed between the response of host was 4-HBA. This compound is associated to defensive process and cell wall (Smith-Becker *et al*., 1998; Tan *et al*., 2004; Okrent *et al*., 2009) and we observed a correlation with SA. However, 4-HBA was absent in Arabidopsis leaves suggesting that this compound is only present in pepper under the studied conditions.

In figure 6.3 are summarised all the components that we have found to be induced on response due to VNT treatment and Botrytis inoculation in Arabidopsis plants.

This study points out that capsinoids as well as capsaicinoids protects plants from other families what spread their use as phytosanitary compound. In addition, the

mechanisms of action are similar, a fact that could facilitate to obtain a deeper sight of VNT-induced resistance.

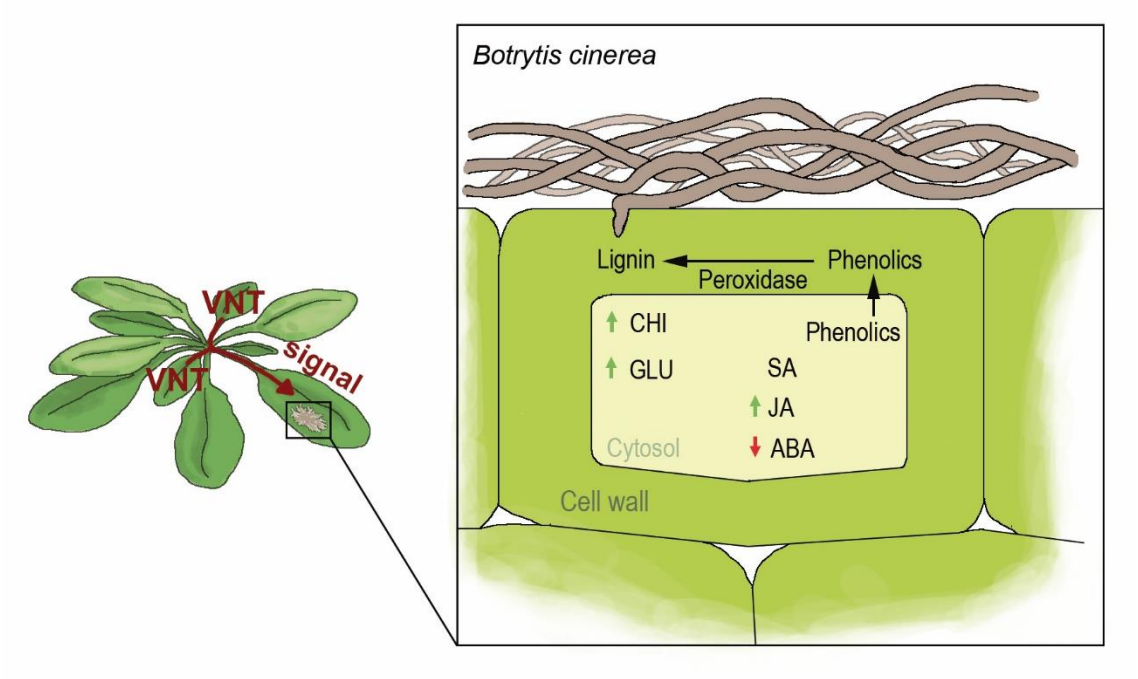

Figure 6.3. Proposed model about the effect of vanillyl nonanoate (VNT) on the interaction of *Arabidopsis thaliana-Botrytis cinerea*. CHI: chitinase; GLU: β-1,3-glucanase; SA: salycilic acid; JA: jasmonate acid; ABA: abscisic acid. Green arrows mean that this parameter showed high levels in VNT-induced resistance. Red arrows mean that this parameter reduced its amount during the process.

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CONCLUSIONS

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

1. The fruits from the Galician pepper cultivars (Padrón, Couto, Arnoia, Branco Rosal, Oímbra, Mougán, Piñeira, Punxín, Vilanova and Couto Grande) did not have the capsinoids capsiate, dihydrocapsiate and vanillyl nonanoate.

2. The synthetic capsinoid vanillyl nonanoate, but not its precursor vanillyl alcohol and nonanoic acid, has an antimicrobial effect on *Phytophthora capsici*. However, *Botrytis cinerea* is not affected by this compound.

3. Vanillyl nonanoate induces local resistance on leaves of pepper against *Phytophthora capsici* and *Botrytis cinerea*. The reduction of symptoms was correlated by an increase of the expression of genes as *CaBGLU1*, *CaSC1* and *CaPAL1*.

4. The application of vanillyl nonanoate to cotyledons of pepper plants induces systemic resistance to *Phytophthora capsici* in the roots and to *Botrytis cinerea* in the true leaves. However vanillyl nonanoate does not induce resistance to *Verticillium dahliae*.

5. The systemic resistance induced by vanillyl nonanoate in pepper is due to the deposition of lignin in the cell wall driven by peroxidases, as well as to the expression of biochemical defences as a capsidiol biosynthesis gene (*CaSC1*), a PR1 gene and β-1,3-glucanase.

6. The systemic resistance induced by vanillyl nonanoate in pepper is driven by the increase of several signals: Hydrogen peroxide, salicylic acid and ethylene in both roots and leaves, and jasmonic acid only in the case of roots. Interestingly, in the leaves 4-hydroxybenzoic acid decreased when salicylic acid increased, pointing to a cross-regulation of both. Abscisic acid is downregulated by vanillyl nonanoate in the leaves.

7. The treatment of *Arabidopsis thaliana* with vanillyl nonanoate also induces systemic resistance to *Botrytis cinerea* in the leaves. The resistance observed is

correlated with strengthening of cell wall with deposition of lignin mediated by the action of peroxidases with consumption of phenolics. Vanillyl nonanoate also induces the activity of PR enzymes as β-1,3-glucanase and chitinase. The resistance process is mediated by salicylic acid and jasmonic acid, whereas abscisic acid is downregulated.

8. The reinforcement of cell wall by lignification is a common feature of resistance induced by vanillyl nonanoate, regardless of the plant and the pathogen assayed. However, other features as biochemical defences or signalling are a bit different among the three combinations of plant and pathogen that were studied in this thesis. This fact points to the necessity of studying plant pathogen interactions other than those based in the model plant Arabidopsis.

ANNEX I

Los pimientos son un conjunto de especies pertenecientes a la familia de las Solanáceas. Su origen se encuentra en las regiones de América Central y las Indias Occidentales, siendo introducidos en Europa tras los viajes de Colón. Su capacidad para adaptarse a diferentes regiones climáticas ha permitido que su cultivo se extienda a todo el mundo, y hoy en día sus frutos son ampliamente utilizados en la industria alimentaria por sus propiedades nutricionales y organolépticas.

La presencia de este cultivo en Galicia está representada por varios ecotipos pertenecientes a la especie *Capsicum annuum*. Parte de estos ecotipos han sido designados como Denominación de Origen Protegida o como Indicación Geográfica Protegida.

Las enfermedades del pimiento causan graves pérdidas económicas entre ellas se estudiaran en este trabajo la tristeza o seca del pimiento y la podredumbre gris. La tristeza o seca del pimiento está causada por dos hongos, *Phytophthora capsici* o *Verticillium dahliae*. *Phytophthora capsici* es altamente dinámico y muy destructivo. No solo afecta al pimiento, sino también a un buen número de otras especies de interés agrícola, incluyendo berenjena, tomate, melón, calabaza o judía. Puede afectar a cualquier parte de la planta en cualquier estado de desarrollo. Los síntomas más característicos son la podredumbre del cuello de la planta y la marchitez. *Veticillium dahliae* vive en el suelo y suele atacar las raíces. Los síntomas que causa son podredumbre, pérdida total o parcial de turgencia, manchas marrones, abscisión de las hojas, clorosis, necrosis y enanismo.

Botrytis cinerea es un patógeno necrotrofo que ataca a unas 1000 especies de monocotiledóneas y dicotiledóneas, entre ellas el pimento. Este hongo, considerado como un hongo necrotrofo modelo, es responsable de la podredumbre gris. Los síntomas varían en función del tipo de tejido y del órgano afectado.

Las defensas de las plantas frente al ataque de patógenos pueden ser divididos en dos tipos de barreras. Barreras pre-existentes, se sintetizan de manera constitutiva y se encuentran presentes antes del ataque del patógeno, Barreras inducidas, son activadas una vez que la planta reconoce al patógeno. El reconocimiento del patógeno tiene lugar mediante receptores que perciben moléculas presentes en el patógeno. Estas moléculas se pueden ser PAMPs o MAMPs (Patrones Moleculares Asociados a Patógenos o Microorganismos, respectivamente), las cuales están altamente conservadas. La planta

también puede reconocer la presencia de un patógeno mediante moléculas derivadas del daño que causan en el huésped los patógenos y se denominan DAMPs (Patrones Moleculares Asociados al Daño). Estos patrones moleculares activan la PTI (Inmunidad desencadenada por PAMPs), considerada la primera línea defensiva inducida por el patógeno. La PTI puede ser suprimida por los patógenos mediante la secreción de moléculas denominadas efectores provocando que la planta sea susceptible al ataque del patógeno. Este estado se conoce como Susceptibilidad Desencadenada por Efectores (ETS). Sin embargo, los efectores pueden ser reconocidos desencadenándose la ETI (Inmunidad desencadenada por efectores).

Algunas veces la PTI y la ETI son capaces de activar las defensas en tejidos sistémicos que no han sido atacados por un patógeno. Este fenómeno se conoce como Resistencia Sistémica Adquirida (SAR). Las plantas también pueden adquirir este estado de resistencia mediante la exposición a organismos beneficios, denominándose este proceso ISR (Resistencia Sistémica Inducida).

La SAR se caracteriza por proporcionar una resistencia de larga duración así como por la expresión de proteínas PR o la respuesta hipersensible. Diversos estudios han demostrado que varias señales son necesarias para el establecimiento de la SAR, siendo la más característica el ácido salicílico (SA). En general, la SAR es efectiva contra un amplio rango de patógenos, aunque se ha observado que es más efectiva contra biotrofos y hemibiotrofos.

A diferencia de lo que ocurre con la SAR, en la señalización que establece la ISR depende del ácido jasmónico (JA) y del etileno (ET). Sin embargo, la acumulación de estas hormonas y la consecuente activación de los genes de defensa regulados por ellas, no tiene lugar hasta que la planta es atacada por un patógeno.

En general, las hormonas SA, JA y ET son consideradas como las principales reguladores de la defensa, pero se ha observado que el ácido abscisico, las citoquininas, los brasinoesteroides y las auxinas también participan modulando la respuesta defensiva.

Tanto la SAR como la ISR son formas de respuesta inmediatas al estrés, aunque también pueden desencadenar un estado de *priming*. El *priming* es un estado de “alerta” en el que la planta responde de manera más intensa y rápida a un segundo ataque. Por este motivo el *priming* es considerado como un tipo de memoria y podría participar en

la adaptación de las plantas a condiciones de estrés. De hecho, se ha observado que el *priming* es trasmitido de generación en generación en aquellas plantas expuestas de forma prolongada a un estrés. Este fenómeno se denomina *priming* transgeneracional.

Se ha observado que la aplicación de compuestos denominados inductores es capaz de activar las defensas de la plantas. Los inductores son todos aquellos compuestos que se forman durante la interacción planta-patógeno (lipopolisacaridos de la pared de los patógenos, oligómeros de quitina y glucano o exudados bacterianos entre otros), metabolitos secundarios (fitoanticipinas y fitoalexinas) así como compuestos similares al SA (ácido 2,6-dicloroisonicotinico, INA, o benzotiadiazol, BTH). También se consideran inductores aquellos organismos vivos usados en el control biológico como la rizobacteria *Pseudomonas fluorescens* y algunos hongos como por ejemplo *Trichoderma* sp.

La utilización de estos compuestos en la agricultura para el control de las enfermedades ha ido ganando popularidad en los últimos años. Su éxito se debe principalmente a que cumplen todos los requisitos para su aplicación segura tanto en invernadero como en campo incluyendo: no son tóxicos para las plantas o los animales, no tienen efectos negativos en el crecimiento, el desarrollo o en la producción, tienen un amplio espectro de acción, se usan en bajas concentraciones, confieren protección duradera y reducen el gasto económico de los granjeros.

En el pimiento, unos de los metabolitos que han suscitado un mayor interés son los capsicinoides. Estos compuestos se encuentran principalmente en el fruto, y son los responsables de la pungencia o picor de algunos cultivares de pimiento, al acumularse en la placenta del fruto durante su maduración. Sin embargo, el análisis de los frutos de diferentes especies y ecotipos de pimiento ha demostrado la existencia de varios compuestos similares a los capsicinoides, pero que carecen de pungencia. Estos compuestos son los capsinoides y los capsiconinoides. Los capsicinoides, capsinoides y capsiconinoides tienen una estructura molecular similar en la que destacan un anillo aromático y una larga cadena alifática. La única diferencia estructural entre ellos es el tipo de enlace que une ambas estructuras: un enlace amida, en el caso de los capsicinoides, y un enlace éster, en el caso de capsinoides y capsiconinoides. Los metabolitos más estudiados son los capsicinoides seguidos por los capsinoides. El interés de los capsinoides radica en que comparten ciertas propiedades farmacológicas con los capsicinoides, como son el efecto analgésico, la actividad antitumoral, la

actividad antioxidante o la reducción de peso, pero sin efecto pungente. Por lo tanto, la presencia de estos compuestos en los frutos del pimiento tiene un valor añadido y su cuantificación resulta de gran interés. Por estos motivos, analizamos el contenido de capsicinoides y capsinoides en los principales cultivares de pimiento gallegos (Padrón, Couto, Arnoia, Branco Rosal, Oímbra, Mougán, Piñeira, Punxín, Vilanova y Couto Grande). Sin embargo, no se encontraron capsinoides en ningún cultivar, aunque en los ecotipos Padrón y Mougan se pudo cuantificar la presencia de capsicina y dihidrocapsicina.

Además de las propiedades farmacológicas, se ha observado que los capsicinoides también tienen actividad antimicrobiana contra hongos, bacterias y levaduras. También se ha observado que la aplicación en planta de estos compuestos es capaz de inducir resistencia en plantas. A pesar de estos beneficios, el carácter irritante de los capsicinoides limitaría su uso por parte de los agricultores. En base a las similitudes entre los capsicinoides y los capsinoides, hemos propuesto que los capsinoides podrían ser sustitutos de los capsicinoides. Para abordar esta idea, elegimos el vanillilnonanoato (VNT) que es un capsinoide sintético similar al capsiato. Primero probamos si el VNT tiene propiedades antimicrobianas contra los patógenos del pimiento *Phytophthora capsici* y *Botrytis cinerea*. Para ello, verificamos la germinación y la longitud del tubo germinativo de las esporas expuestas a diferentes concentraciones del VNT y también de sus precursores, el vanillil alcohol (VOH) y el ácido nonanoico (NNA). Solo las esporas de *P. capsici* se vieron afectadas por la presencia de VNT. Del mismo modo, los precursores VOH y NNA no presentaron efecto antimicrobiano excepto VOH, que fue capaz de reducir la longitud del tubo germinativo de las esporas de *B. cinerea*.

Por otro lado, evaluamos el efecto del VNT contra esos mismos patógenos, aplicando el compuesto mediante pulverización a las plantas del pimiento de Padrón e inoculando las hojas con los patógenos. Las plantas tratadas con dicho compuesto mostraron una reducción en los síntomas causados por estos hongos lo que podría indicar una resistencia inducida. Para verificar esta hipótesis aplicamos el VNT, a la misma concentración, en los cotiledones mediante infiltración con ayuda de una jeringuilla. En este caso la inoculación se realizó en las raíces con *P. capsici* y en las hojas con *B. cinerea*. Al igual que en los ensayos por pulverización, las plantas infectadas mostraron una reducción en los síntomas así como una menor biomasa

patogénica en los órganos inoculados. Esto nos confirmó que el VNT es capaz de inducir resistencia sistémica en pimiento contra *P. capsici* y *B. cinerea*.

El siguiente paso fue estudiar qué respuestas defensivas fueron inducidas por VNT tanto en las raíces como en las hojas. Estudiamos defensas físicas y bioquímicas. Dentro de las defensas físicas exploramos la lignificación de la pared celular. Para ello medimos la actividad peroxidasa, enzima implicada en la formación de lignina, el contenido en compuestos fenólicos solubles, como fuente de sustratos necesarios para el refuerzo de la pared, y la lignina, producto final de la reacción de las enzimas anteriormente mencionadas. Tanto las raíces como las hojas mostraron, después del tratamiento con VNT, un aumento de lignina en el cual participaron las peroxidasas, observándose consumo de fenoles solo en las hojas.

También estudiamos las defensas bioquímicas midiendo la expresión de los genes *CaSC1* (una sesquiterpeno ciclasa implicada en la biosíntesis de capsidiol), *CaBPR1* (una proteína PR1), *CaBGLU1* (una β-1,3-glucanasa), y medimos la actividad β-1,3-glucanasa y quitinasa, ambas enzimas implicadas en la degradación de la pared celular de los patógenos. En las raíces observamos que el tratamiento con el VNT inducía la expresión de los genes *CaSC1*, *CaBPR1* y la actividad enzimática de la β-1,3-glucanasa. Sin embargo, en las hojas observamos un aumento de la expresión de los genes *CaBPR1* y *CaBGLU1*.

Además de las barreras físicas y bioquímicas, estudiamos el papel de las especies activas de oxígeno (ROS) y las principales hormonas implicadas en defensa, SA, ET y JA, en la inducción de resistencia mediante la aplicación de VNT. Se determinaron los niveles de una ROS (el peróxido de hidrógeno) durante las primeras ocho horas después de la inoculación. En las raíces, el tratamiento con VNT produjo una alta acumulación de peróxido de hidrógeno. Este aumento podría ser debido a que la planta reconoció la presencia de *P. capsici*. En las hojas, los niveles de peróxido de hidrogeno se mantuvieron altos la mayor parte del tiempo. Los ensayos con DTT (ditiotreitol), un secuestrador de peróxido de hidrógeno, mostraron que tanto en las raíces como en las hojas son necesarios altos niveles de peróxido de hidrógeno para desencadenar la resistencia inducida por VNT.

Para comprender el papel de las hormonas en la resistencia inducida por VNT, medimos la expresión de genes implicados en su biosíntesis, ya que en pimiento no hay

genes marcadores que respondan al efecto de una sola hormona. Los genes seleccionados fueron: *CaPAL1* (una fenilalanina amonio liasa) para el SA, *CaAOS* (una óxido de aleno sintasa) para el JA y *CaACS* (1-aminociclopropano-1-carboxilato (ACC) sintasa), y *CaACO*, (ACC oxidasa) para el ET. Los datos de expresión fueron confirmados con ensayos con inhibidores de síntesis o de percepción de las hormonas y cuantificando los niveles hormonales en hojas con espectrometría de masas. Los resultados mostraron que las hormonas SA y ET, en raíces, y además el JA en hojas, participan en la resistencia inducida por VNT en pimiento. También se detectaron cambios en los niveles de 4-HBA (ácido 4-hidroxibenzoico, un compuesto estructuralmente parecido al SA) en respuesta al VNT.

En la última parte de la tesis se abordó la cuestión de si el VNT podía inducir resistencia en otras especies de plantas y no solo en el pimiento. Para ello elegimos la planta *Arabidopsis thaliana* porque es un organismo modelo y, además, hay mucha información sobre sus procesos defensivos contra *B. cinerea*. En este caso, el VNT también fue capaz de inducir la resistencia contra *B. cinerea*. Basándonos en los resultados obtenidos con pimiento, estudiamos el efecto del VNT en la lignificación de las hojas de Arabidopsis midiendo la actividad peroxidasa, el contenido en fenoles solubles, la cantidad de lignina y la expresión de algunos genes que codifican para peroxidasas. Al igual que en pimiento, la pared celular fue reforzada con lignina en un proceso mediado por las peroxidasas, especialmente la enzima PRX17, con consumo de fenoles. Estas similitudes apuntan a que la pared celular es un elemento común en la respuesta inducida por el tratamiento con VNT independientemente de la especie huésped empleada.

En la interacción Arabidopsis-*Botrytis cinerea* también se determinaron las actividades de β-1,3-glucanasa y quitinasa. Al igual que en el pimiento ambas enzimas se vieron inducidas por el tratamiento con VNT.

Finalmente se estudió que hormonas regulan la resistencia inducida por el VNT en Arabidopsis contra *B. cinerea*. Para abordar este punto se realizaron ensayos con mutantes afectados en las tres principales hormonas implicadas en defensa, es decir, *NahG*, el cual tiene una salicilato hidroxilasa que degrada el SA, *npr1-1*, mutante incapaz de desencadenar la SAR, *jin4*, afectado en la ruta del JA, y *ein3*, afectado en la ruta del ET. A mayores se midió la expresión de los genes marcadores *AtPR1*, para SA, y *AtPDF1.2*, para JA y ET y se cuantificaron los niveles hormonales mediante

espectrometría de masas. Los resultados de estos análisis mostraron que las hormonas SA y JA están implicadas en la regulación de la resistencia inducida por VNT. A diferencia de lo que pasa en pimiento, en este caso el ET no sería necesario para la respuesta al VNT. El 4-HBA está presente en pimiento y ausente en Arabidopsis.

En resumen, el VNT fue capaz de inducir resistencia contra *P. capsici* y *B. cinerea* en pimiento y contra *B. cinerea* en Arabidopsis, independientemente de la ausencia de capsinoides y la maquinaria necesaria para sintetizarlos, en el último patosistema. Además, VNT indujo el refuerzo de la pared celular mediante el depósito de lignina mediado por las enzimas peroxidasas, así como varias defensas bioquímicas moduladas por el peróxido de hidrógeno, el SA, el ET y el JA, en el caso del pimiento, y el SA y el JA, en el caso de Arabidopsis.

Estos resultados apuntan a que tanto los capsinoides como los capsicinoides son capaces de ejercer un efecto protector sobre plantas de diferentes familias, lo que amplía su uso como potenciales compuestos fitosanitarios. Además, el hecho de que los mecanismos de acción sean similares podría ser de ayuda para obtener un conocimiento más profundo de la resistencia inducida por el VNT.

Basándonos en los resultados anteriormente comentados, podemos concluir los siguientes puntos:

1. Los frutos de los cultivares gallegos de pimiento (Padrón, Couto, Arnoia, Branco Rosal, Oímbra, Mougán, Piñeira, Punxín, Vilanova y Couto Grande) no tenían los capsinoides capsiato, dihidrocapsiato y vanillilnonanoato.

2. El capsinoide sintético, vanillilnonanoato, pero no sus precursores el vallil alcohol o el ácido nonanoico, tiene efecto antimicrobiano sobre *Phytophthora capsici*. Sin embargo, *Botrytis cinerea* no se ve afectado por este compuesto.

3. El vanillilnonanoato induce resistencia local en las hojas de pimiento contra los patógenos *Phytophthora capsici* y *Botrytis cinerea*. La reducción de los síntomas se correlacionó con un aumento de la expresión de genes como *CaBGLU1*, *CaSC1* y *CaPAL1*.

4. La aplicación de vanillilnonanoato en los cotiledones de plantas de pimiento induce resistencia sistémica frente a *Phytophthora capsici* en las raíces y a Botrytis

cinerea en las hojas verdaderas. Sin embargo, el vanillilnonanoato no induce resistencia frente a *Verticillium dahliae*.

5. La resistencia sistémica inducida por vanililnonanoato en pimiento se debe al depósito de lignina en la pared celular llevada a cabo por las peroxidasas, así como a la expresión de defensas bioquímicas como un gen de biosíntesis de capsidiol (*CaSC1*), un gen PR1 y β- 1,3-glucanasa.

6. La resistencia sistémica inducida por el vanillilnonanoato en el pimiento está regulada por el aumento de varias señales: el peróxido de hidrógeno, el ácido salicílico y el etileno en las raíces y en las hojas, y el ácido jasmónico solo en el caso de las raíces. En las hojas el ácido 4-hidroxibenzoico disminuyó cuando aumentó el ácido salicílico, lo que indica una regulación cruzada de ambos. El ácido abscísico está regulado negativamente por el vanillilnonanoato en las hojas.

7. El tratamiento de *Arabidopsis thaliana* con el vanillilnonanoato también induce resistencia sistémica frente a *Botrytis cinerea* en las hojas. La resistencia observada se correlaciona con el fortalecimiento de la pared celular con la deposición de lignina mediada por la acción de las peroxidasas y el consumo de compuesto fenólicos. El vanillilnonanoato también induce la actividad de proteinas PR como la β-1,3-glucanasa y la quitinasa. El proceso de resistencia está mediado por ácido salicílico y ácido jasmónico, mientras que el ácido abscísico está regulado negativamente.

8. El refuerzo de la pared celular mediante lignificación es una característica común de la resistencia inducida por el vanillilnonanoato, independientemente de la planta y del patógeno analizado. Sin embargo, otras características como las defensas bioquímicas o la señalización son un poco diferentes entre las tres combinaciones de plantas y patógenos que se estudiaron en esta tesis. Este hecho apunta a la necesidad de estudiar otras interacciones planta-patógenos distintas a las basadas en la planta modelo Arabidopsis.