MOLECULAR CHARACTERIZATION OF THE INTERACTION BETWEEN *HELIANTHUS ANNUUS* AND *VERTICILLIUM DAHLIAE*

BY

ZHEN YAO

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Zhen Yao, The University of Manitoba, September, 2009. **Molecular Characterization of the interaction between *Helianthus annuus* and *Verticillium dahliae*.**
Advisors: F. Daayf and K.Y. Rashid.

The soil-borne fungus *Verticillium dahliae* Klebahn is the primary causal agent of *Verticillium* wilt in sunflower. The long viability of the resting structures, which can survive in the soil for more than 10-15 years, makes it more difficult to control this disease. To date, only limited information is available regarding the interaction between *V. dahliae* and sunflower, although such information is critical for a better understanding of plant defense mechanisms against this pathogen and for developing an effective strategy to manage *Verticillium* wilt in sunflower. In this study, two highly aggressive (Vd1396-9 and Vd1398-21) and two weakly aggressive (Vs06-07 and Vs06-14) isolates of *V. dahliae* were used to inoculate susceptible (IS8048) and moderately resistant (IS6111) sunflower hybrids. A fusion protein of *VdNEP* (*V. dahliae* necrosis and ethylene-inducing protein) gene was produced, purified, and used as another treatment. The disease severity and symptoms caused by four *Verticillium* isolates and VdNEP were compared and induced plant defense responses were investigated in order to study the function of VdNEP involved in the sunflower-*V. dahliae* interaction. Our results indicated that VdNEP caused typical symptoms of *Verticillium* wilt in sunflower, including chlorosis, necrosis and vascular discoloration. The disease severity caused by VdNEP was similar to that caused by *Verticillium* isolate Vs06-14, which indicated that VdNEP not only acted as an elicitor but also as a pathogenicity factor involved in the

disease development of *Verticillium* wilt. Furthermore, VdNEP induced the hyper-sensitive response, oxidative burst and the accumulation of fluorescent compounds in sunflower. Pathogenesis-related genes (*Ha-PR-3,* and *Ha-PR-5*), two defensin genes (*Ha-PDF* and *Ha-CUA1*) and genes encoding *Ha-ACO*, *Ha-CHOX*, *Ha-GST* and *Ha-SCO* were up-regulated by VdNEP, which indicated that multiple signaling pathways were involved. Two genes (*Ha-PAL* and *Ha-NML1*) were slightly inhibited after infiltration with VdNEP, which suggested that sunflower resistance against *Verticillium* wilt might be SA-independent. Interestingly, VdNEP enhanced the root fresh and dry weight in the moderately resistant sunflower hybrid but not in the susceptible one, indicating that it may play other roles in the interaction between sunflower and *V. dahilae* such as promoting plant growth.

FOREWORD

This thesis is written in manuscript style. A general introduction and review of the literatures precedes a manuscript that comprised the main part of the thesis. The manuscript consists of an Abstract, Introduction, Materials and Methods, Results and Discussion. A General Discussion and a list of References Cited follow the manuscript.

1.0 INTRODUCTION

Sunflower (*Helianthus annuus* L.), a member of the Asteraceae family, is an economically important oilseed crop worldwide (FAS, 2005). South Manitoba is the major production area for sunflower in Canada, which constitutes approximately 85% of Canadian sunflower seed production, followed by Saskatchewan, and Alberta (AAFC, 2005).

Verticillium wilt, primarily caused by the soil-borne fungus *Verticillium dahliae*, is a disastrous disease causing significant yield losses of sunflower production (Sackston, 1957). *V. dahliae* has a broad host range, including more than 200 dicotyledonous plant species, such as potato, cotton and sunflower (Agrios, 2005). The fungus attacks sunflower roots through wounding and spreads to the aerial parts of plant by systemic invasion, causing leaf vein browning, chlorosis and necrosis, stunting, wilting, leaf defoliation, vascular discoloration and death in the later stage of the disease (Sackston, 1957). *V. dahliae* can survive in the soil for nearly 10-15 years in the form of microsclerotia (Agrios, 2005).

Verticillium wilt of sunflower was firstly observed in Manitoba in the early 1970s and the incidence of this disease has increased in recent years (Hoes, 1972; Gulya, 2003). *Verticillium* was recorded by the National Sunflower Association (NSA, 2008) surveyors in 19% and 13% of U.S. sunflower fields in 2002 and 2003, respectively. In 2005, the presence of *Verticillium* wilt in Manitoba reached 57% of 68 fields surveyed, with incidence ranging from trace to 30% (Rashid et al., 2006).

Several strategies to control *Verticillium* wilt in sunflower have been recommended, such as crop rotation, breeding resistant varieties and soil fumigation. Unfortunately, they

are not very effective to manage this disease because of the great variability of *Verticillium* isolates, their broad host range, striking viability of the resting structures, inability of fungicides and the lack of resistant cultivars (Koike et al., 1994).

In sunflower, information about resistance to *Verticillium* and other soil-borne pathogens is very limited, for example, the pathogenesis of *V*. *dahliae* causing wilting symptoms remains ambiguous and unknown. Better understanding of the sunflower-*V*. *dahliae* interaction is important towards controlling this disease. Pathogen-derived elicitors that activate plant defense responses is a key component and a prerequisite for accurately deciphering the interplay of host-pathogen interactions (Radman et al., 2003).

VdNEP is a *V*. *dahliae* necrosis and ethylene-inducing protein. It caused wilting symptoms on cotton and triggered a hypersensitive response-like cell death in many plant species (Wang et al., 2004). However, the role of VdNEP in the sunflower-*V*. *dahliae* interaction is not clear. Several questions have to be answered, for example, how VdNEP contributes to the development of *Verticillium* wilt symptoms and how the plant defense mechanisms work when *V*. *dahliae* attempts to infect the plant.

The objectives of this study were to: (i) analyze the expression of *VdNEP* in *V*. *dahliae* at gene and protein levels; (ii) express and purify VdNEP in pET-32a (+)/BL21 *E*. *coli* strain; (iii) compare disease severity and symptoms in sunflower caused by *V*. *dahliae* and VdNEP; and (iv) determine the defense responses induced by VdNEP.

2.0 LITERATURE REVIEW

2.1 Sunflower (*Helianthus annuus* L.)

2.1.1 Family and species

Helianthus annuus L. known as sunflower, is an annual cultivated plant native to North America. The genus *Helianthus* comprises 67 species and several subspecies in the Asteraceae family, with a typical composite flower (Heiser, 1978). The term *Helianthus* comes from two Greek words, *helios* and *anthos,* meaning sun and flower (Fletcher and Taylor, 1939). There are other species related to sunflower, such as Jerusalem artichoke (*Helianthus tuberosa*) and Mexican sunflower (*Tithonia rotundifolia*), grown as ornamentals or weeds (Paniego et al., 2007).

2.1.2 Sunflower history

Sunflower is originated from North America and it is now the national flower of Russia and Peru, the state flower of Kansas, USA, and the city flower of Kitakyushu (Wikipedia, 2009). As most crops grown in the earlier time, sunflower had many alternative uses. It was grown by indigenous people for food, dye and medicinal purposes before colonization of the New World (Putt, 1978). The early Spanish explored sunflower seeds from North America, and sunflower was widely adopted as an ornamental garden flower in Spain by 1850. It was spread along the trade route to Europe, Asia, Africa and the Middle East by English and French explorers (Putt, 1978; Stevens et al., 1993). Sunflower developed as a major source of edible vegetable oil in Russia and has been accepted as a source of high quality, edible oil throughout Europe. Since the 1990s, sunflower has become an economically important crop worldwide due to the industrial value of its oily seeds and nutritional value as forage (Stevens et al., 1993).

2.1.3 Economic importance

Sunflower is the fifth most important source of vegetable oil worldwide, ranked after soybean, rapeseed, cotton, and peanut (FAS, 2005). The total annual yield of sunflower seeds is approximately 28 million metric tons, (Table 2.1). And the major portion of sunflower production, estimated about 107 million tons, is devoted to oil extraction, providing 8.2% of total world volume (Dorrell and Vick, 1997). There are three types of sunflowers classified based on their breeding objectives and end use: (1) Oilseed varieties with small black seeds and up to 50% oil content, (2) Confectionary varieties with large striped seeds, used for snack food as well as for birds food, and (3) Ornamental varieties. Based on 2007 FAO statistical data, Argentina, Europe, China, Russia, United States, India and Turkey are major sunflower producers and they provide about 88% of the world's production of sunflower. Sunflower oil is valued for its light taste, frying appearance and health benefit. It contains proteins, vitamins, especially vitamin E and substantial amounts of essential minerals, such as magnesium, iron, copper and zinc. There are three types of sunflower oils based on the oil composition, i.e., NuSun, linoleic, and high oleic oil. Linoleic sunflower oil is specially suited for cooking due to its clean taste, high smoke point and low level of saturated fat (NSA, 2008).

Table 2.1. Sunflower seed production (in 1,000 metric tons)

Sunflower Seed Production						
Item	**2002/03**	**2003/04**	**2004/05**	**2005/06**	**2006/07**	**2007/08**
Area Harvested (1,000 HA)	19,941	22,820	21,254	22,944	23,912	23,374
Yield (MT/HEC)	1.2	1.18	1.23	1.32	1.26	1.24
Seed Production						
Canada	157	150	52	89	157	125
Argentina	3,340	2,980	3,730	3,840	3,350	4,400
Other Europe	2,019	2,670	2,250	682	764	295
European Union	3,718	4,070	4,069	5,717	6,388	4,932
China (Peoples Republic of)	1,946	1,820	1,700	1,830	1,850	1,800
Russia/Ukraine	7,194	9,348	8,001	11,390	11,650	10,250
United States	1,112	1,209	930	1,720	997	1,309
India	1,060	1,160	1,300	1,490	1,380	1,400
Turkey	830	560	640	780	820	670
Other	2,738	3,069	3,555	2,852	2,854	3,889
TOTAL	**23,957**	**26,886**	**26,175**	**30,301**	**30,053**	**28,945**

(Source http://www.sunflowernsa.com/stats/table.asp?contentID=109&htmlID=74&submit170=View&submit.x=59&submit.y=13)

2.1.4 Sunflower in Manitoba, Canada

According to the report of Agriculture and Agri-Food Canada (AAFC) in 2005, Canada is the thirteenth largest exporter and twenty-fifth largest producer of sunflower seeds in the world. The United States is by far the largest importer of Canadian sunflower seeds which constitutes 80% of total Canadian exports. Manitoba, Saskatchewan, and Alberta account for the majority of Canadian sunflower seed production. Over 85% of sunflowers are grown in South Manitoba known as the Canada's "Sunflower Belt" and all varieties grown in Canada are hybrids (AAFC, 2005).

2.2 *Verticillium* Wilt

2.2.1 Introduction of *Verticillium* wilt

Verticillium wilt, primarily caused by two species of *Verticillium*: *V. dahliae* and *V. albo-atrum*, is a serious vascular disease with a wide host range, including over 400 plant species in more than 200 families (Pegg and Brady, 2002; Pegg, 1974). It is a major problem in many crop plants and trees, such as tomato, potato, alfalfa, cherry, strawberry and maple tree (Pegg and Brady, 2002).

The genus name *Verticillium* derives from the morphological structure about the verticillate arrangement of conidiophores (Fig2.1 A). The conidiospores are ovoid or ellipsoid and usually single celled, borne on long phialides which are specialized hyphae, produced in a whorl around each conidiospore (Fig2.1 B). Normally, the vegetative mycelium of *Verticillium* spp. is hyaline, septate and multinucleate and mostly haploid (Tolmsoff, 1973). *V. dahliae* can survive in the soil for nearly 15 years in the form of microsclerotia (Agrios, 2005) which are clusters of thick-walled heavily melanized cells separated as discrete bodies from the parent mycelium (Fig2.1 C).

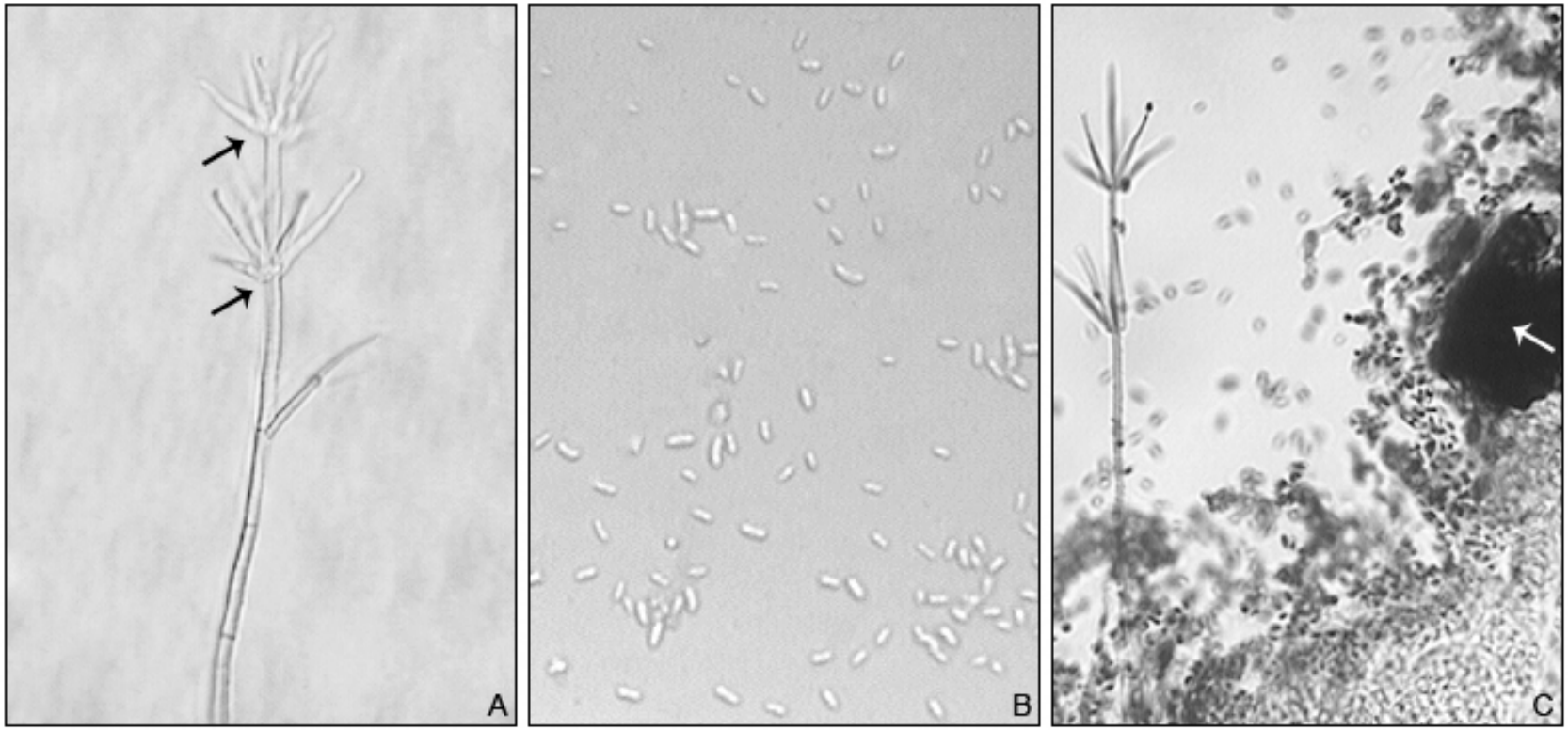

Figure 2.1. Microscopical structure of *V*. *dahliae*. (A) Verticillate arrangement of *V*. *dahliae* conidiospores. (B) Microscopical observation of *V*. *dahliae* conidiospores. (C) Microscopical observation of *V*. *dahliae*, especially microsclerotia.

2.2.2 Verticillium species

Verticillium spp. are among the most widely distributed and destructive plant pathogens in the temperate, tropical and subtropical regions of the world (Pegg and Brady, 2002). Six major *Verticillium* species have been well studied based on their molecular characteristics and the diseases caused, including two hemi-biotrophic pathogens *V. dahliae* Klebahn and *V. albo-atrum* Reinke & Berthold which infect over 200 dicotyledonous plant species (Agrios, 2005). Both species are soil-borne and truly plant pathogenic, but different in their resting structures. *V. dahliae* Klebahn (Klebahn, 1913) produces microsclerotia while *V. albo-atrum* Reinke & Berthold only produces dark resting mycelium. Three other species including *V. nigrescens* Pethybridge, *V. nubilum* Pethybridge and *V. tricorpus* Isaac are also wilt pathogens but in general, are weaker and less important pathogens. *V. theobromae* (Turconi) Mason & Hughes causes fruit-rot on banana and produces a pale brown resting-mycelium (Barbara and Clewes, 2003; Pegg and Brady, 2002).

2.2.3 Host range of *Verticillium* disease

Verticillium wilt is a destructive fungal disease with a wide host range including vegetables (eggplant, pepper, potato, and tomato), field crops (soybean, sunflower, cotton and flax), trees (olive, ash and maple), shrubs (rose, lilac), fruits (strawberry), and weeds (Hiemstra, 1998; Bhat and Subbarao, 1999; Ligoxigakis et al., 2002; Zeise and von Tiedeman, 2002; Pegg and Brady, 2002). Although most monocotyledonous plants are non-hosts of *Verticillium* species (Fradin and Thomma, 2006), the colonization of *V. albo-atrum* or *V. dahliae* in some monocotyledonous plants such as barley, wheat and tulips has been reported (Malic & Milton, 1980; Mathre, 1986; Mathre, 1989).

2.2.4 Symptoms of *Verticillium* wilt in sunflower

Verticillium wilt symptoms vary depending on the host and the *Verticillium* species, and are affected by environmental conditions during the infection process. Therefore, there are no unique symptoms for *Verticillium* wilt (Fradin and Thomma, 2006). *Verticillium* disease might cause sudden wilting of small branches, yellowing of foliage, stunting of growth and premature defoliation.

In sunflower, early symptoms appear on lateral branches or lower leaves and subsequently develop onto higher leaves and stems (Sackston, 1957). The infected tissues between leaf veins become yellow, then brown with a mottled appearance (Fig 2.2 A). These symptoms can easily be mistaken for natural senescence, since they look similar in appearance (Sackston, 1957). In the later stage of disease symptoms, including chlorosis, yellowing, wilting, vein browning, leaf defoliation, stunting and eventual collapse of infected plants can occur (Fradin and Thomma, 2006; Mace et al., 1981). Internal symptoms include dark-brown vascular discoloration arised from the protective plugging of the phenol-pectin mix (Beckman & Talboys, 1981). When an infected stem is cut laterally, vascular tissue appears as a dark ring in cross sections or shows brown to black discoloration in longitudinal sections (Fig 2.2 B). Severely affected sunflower plants die before seeds have developed and masses of tiny black bodies, called as microsclerotia are produced inside the stalk (Fig 2.2 C). Heads of diseased sunflower plants are much smaller and the seed yield and oil quality is highly reduced (Hoes, 1972).

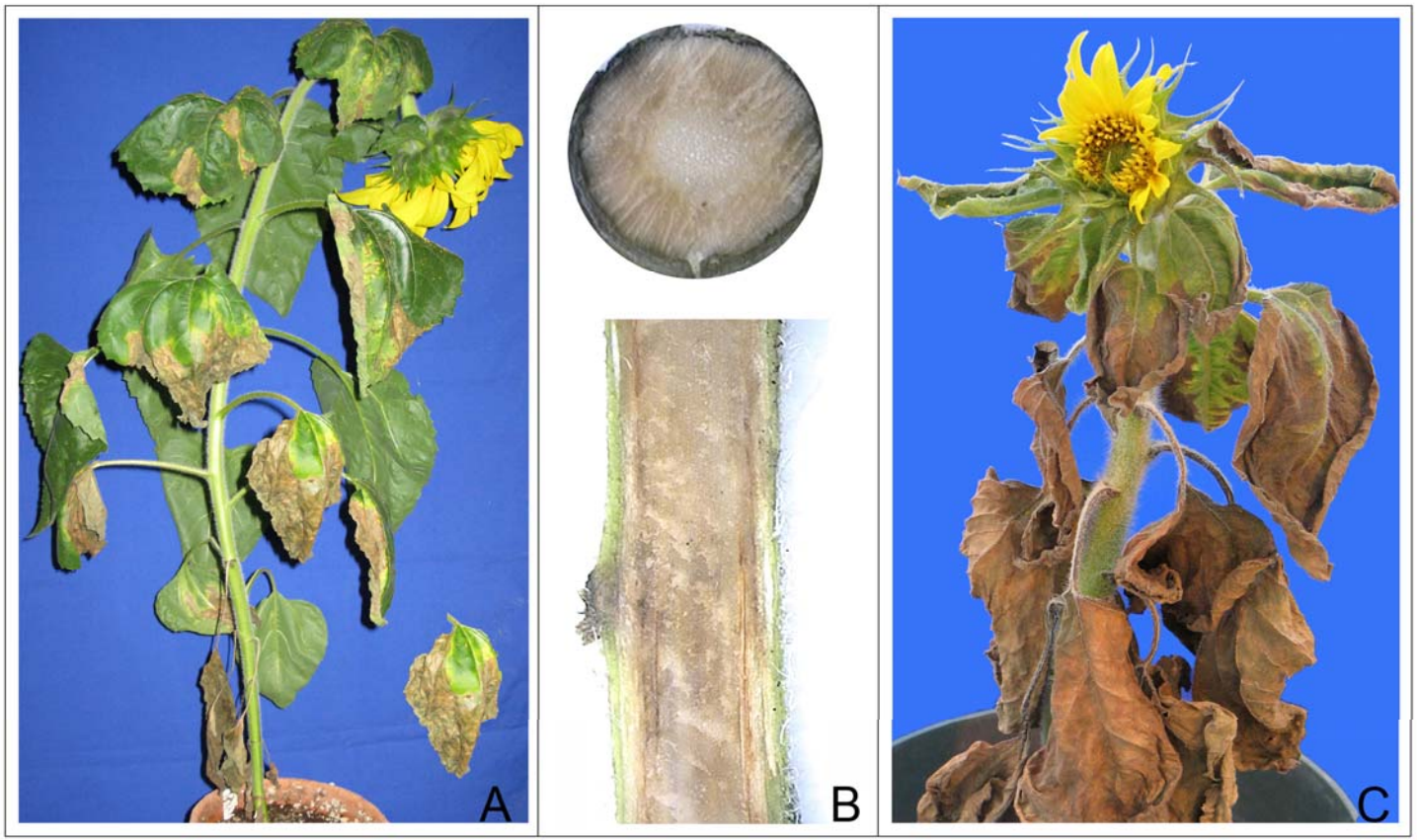

Figure 2.2. *Verticillium* wilt symptoms on infected sunflower. (A) Early symptoms on sunflower infected by *V.dahliae* showing mottled appearance. (B) Dark-brown discoloration of sunflower stem, caused by phenolic vascular plugging. (C) Later symptoms of *Verticillium* wilt in sunflower.

2.2.5 The *Verticillium* disease cycle

Verticillium dahliae causes a monocyclic disease, which can be divided into two phases. During the first phase, *V. dahliae* infects the plant vascular tissue as an endophyte. Then the semi-necrotrophic phase occurs and resting structures, such as microsclerotia, are produced. In the soil, microsclerotia germinate after they are stimulated by plant root exudates secreted from root tip and hair (Mol & Vanriessen, 1995). *V. dahliae* enters the plant either through plant roots or via wounds. Further invasion occurs by colonization of the root cortex and entry into xylem vessels, where *V. dahliae* causes chlorosis, necrosis and foliage wilting. At later stages, the pathogen colonizes senescing tissues and develops microsclerotia, which can survive for 10-15 years in soil (Wilhelm, 1955).

2.2.6 Disease management

Verticillium wilt can cause a disease incidence of up to 70% and is very difficult to control because of the great variability of *Verticillium* isolates, their broad host range and long viability of the resting structures in the soil (Koike et al., 1994). The rate of pathogen colonization is an important factor in determining the level of susceptibility of plants to this disease. Therefore, limiting the amount of inoculum in the soil and developing tools to predict future disease incidence based on the level of soil inoculum are major points of *Verticillium* wilt management. Some approaches can be used to reduce inoculum including physical, chemical and biological methods.

The thermal death point (TDP) for hyphae and conidia of *V. dahliae* is a minimum of 5 min at 47°C in water, and 10 min in soil at 50°C for microsclerotia (Nelson & Wilhelm, 1958). Heat and solarization are the most common methods used in physical control to raise soil temperature to the TDP of *Verticillium* spp. thereby eliminating

inoculum of *Verticillium* pathogens (Nelson & Wilhelm, 1958). However, they are limited to warm climate.

Chemical control methods can be directed against inoculum levels in the soil. For instance, using soil fumigants such as Methyl bromide (CH_3Br), Chloropicrin and Methylisothiocyanate (MIT) compounds is effective on a wide range of crops (Voth et al., 1973). However, detriment to the environment and public health, in addition to the huge cost restrict the use of chemicals. Moreover, at the early stage of the disease, the pathogen grows and propagates inside the vascular tissue of the plants (Schnathorst, 1981), which makes it impossible for non-systemic chemicals to kill *Verticillium* effectively without affecting the host plant.

Some biological control alternatives have been tried such as green manure with Sudan grass, rhizobacteria like *Burkholderia cepacia* and the non-pathogenic bacteria *Paenibacillus alvei* K165 (Berg, 1996; Mannanov, 2001; Tjamos *et al*., 2005). Moreover, crop rotation may keep the microsclerotia under the threshold that leads to losses (Shetty et al., 2000). However, *Verticillium* inoculum can persist for 10-15 years in the soils, therefore, although they can be useful, short rotation practices alone are not very effective. Several *Verticillium* non-host crops such as cereals, corn and onion can be used in rotation with host plants (Wilhelm, 1955).

Due to various reasons, above-mentioned strategies are often inefficient and unappealing. Currently, breeding resistant cultivars against *Verticillium* spp. is the most effective and economic means of managing *Verticillium* wilt. However it's facing many difficulties, including the great variability of *Verticillium* isolates. Cloning resistance genes is important to such a process, but only one resistance locus against *Verticillium*,

called *Ve*, has been cloned to date (Kawchuk et al., 2001). In tomato, *Ve1* and *Ve2* which are two closely linked genes, encoding a leucine-rich repeat (LRR) peptide, confer a race-specific resistance against *V. albo-atrum* (Kawchuk *et al*., 1998; 2001). The *Ve* genes express a class of cell-surface glycoproteins with receptor-mediated endocytosis-like signals and leucine zipper or Pro-Glu-Ser-Thr (PEST) sequences. Cloning has been made of *Ve*-homologues in *Solanum torvum* Swartz (Fei *et al*., 2004) and *S. licopersicoides* (Chai *et al*., 2003). The *Arabidopsis* genome contains several leucin-rich repeats (LRR) linked to plant defense, also found in the *Ve* genes, but no further homology is seen. However, a large family of *Cf2/Clavata1*-like genes that are similar to the *Ve* gene family can be found in *Arabidopsis* and are known to be involved in resistance to *Cladosporium fulvum* in tomato (Brading *et al*., 2000). Therefore, the use of major resistant genes against *Verticillium* wilt and the development of transgenic plants with increased resistance, like that achieved by *Ve1* or *Ve2* may thus be a good alternative.

2.3 Molecular aspects of host-pathogen interactions

2.3.1 Overview of the host-pathogen interaction

Molecular negotiation between plants and pathogens commence almost immediately after the first touch or initial plant surface contact (Fujita et al., 2004). On the one hand, pathogen attempts to infect the host tissue by using their various weapons, such as cell wall degrading enzymes and toxins. After perceiving and deciphering the pathogen signals, on the other hand, plants defend themselves by inducing synthesis of several antimicrobial compounds and fortifying their cell wall components to ward off the pathogens (Mengiste et al., 2003). Interplay of the plant and pathogen signals is a key

component in the development of diseases in plants (Yang et al., 2005). Resistance or susceptibility are often a result of the battle between the plant and the pathogen. If the pathogen is able to overcome plant's defense mechanisms by producing appropriate signals, the disease develops; otherwise disease resistance occurs if plants have evolved local and systemic defense response to withstand an attack by the pathogen.

How does the plant-pathogen interaction work? Generally, pathogen secretes elicitors that can trigger a number of induced defenses in plants. Plants are able to recognize pathogen-derived elicitors, resulting in several early intracellular events including rapid ion fluxes, activation of Kinase cascades, and generation of reactive oxygen species (ROS) (Radman et al., 2003). Some other defense events are induced subsequently, such as induction of a hypersensitive response (HR), programmed cell death (PCD), and phytolexins (Montesano et al., 2003). Intercellular defense signaling is mediated by a set of endogenous signaling molecules. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are three well-known molecules which are crucial in two major defense signaling pathways, one SA-dependant and the other one SA-independent but involving JA and ET (Kunkel and Brooks, 2002). Some other molecules such as ROS, nitric oxide (NO) and abscisic acid (ABA) are also involved in plant defense signaling (Montesano et al., 2003).

2.3.2 Pathogenesis of *Verticillium* species

There are several important questions about pathogenesis of *Verticillium* species that remain unanswered. For instance, it's unknown why the pathogen rarely leaves the xylem vessels until the death of the host. The cause of wilting is the crucial question of wilt pathogenesis. Over the last 50 years, two notable presumptions have persisted: vessel

occlusion and toxin activity (Pegg and Brady, 2002). Nevertheless, evidence for the important role of physical blockage of the plant's xylem and toxins in pathogenicity is ambiguous and inconsistent.

2.3.2.1 Involvement of hydrolytic enzymes

Verticillium species produce hydrolytic enzymes such as polygalacturonase (PG), pectin esterases (PE), pectinolytic and β-glucosidases that can induce symptoms of wilt when introduced into plants (Dobinson et al., 1997, 2004). Deese and Stahmann (1962) found the production of PE and PG from *V. albo-atrum* on tomato stem and wheat bran. Moreover, the enzyme activity on a susceptible tomato cultivar is 3-10 times higher than on resistant cultivars (Deese and Stahmann, 1962). However, the relationship between the enzyme-producing ability and virulence of *Verticillium* isolates has been controversial. It has been demonstrated that non-pathogenic isolates of *V. dahliae* failed to produce pectic enzymes in culture (Leal and Villaneuva, 1962). On the contrary, Talboys and Busch (1970) found that there was no correlation between enzyme production and virulence from the study of 23 isolates of *V. albo-altrum*, 16 of *V. dahliae*, two of *V. tricorpus*, four of *V. nigrescens* and one of *V. nubilum*.

2.3.2.2 Involvement of toxins

As an alternative to vascular occlusion, the toxin basis of symptom induction of *Verticillium* wilt has been widely reported over 50 years. There are two kinds of toxins, low molecular weight toxins which are lower than 1,000 Dalton and macromolecular weight compounds that are usually polymers of polysaccharides or proteins. Studies about low molecular weight toxins in *Verticillium* wilt are limited. In 1957, the existence of a low molecular weight toxin responsible for wilt was found in hop infected by *V.*

albo-atrum (Talboys, 1957). Similar low molecular weight substances including pigments, pentaketides and neutral lipids were found from cotton strains of *V. dahliae* (Ten et al., 1977, 1981). However, there is no further evidence to support a small molecular toxin basis for *Verticillium* symptoms.

More studies are available on macromolecular toxins, for example, Green (1954) precipitated a thermolabile protein-polysaccharide mixture from *V. dahliae* in 90% ethanol which can induce wilting in tomato shoots. Interestingly, the wilting shoots can get recovery after a section of the stem base was removed which indicated wilting was due to vascular occlusion rather than toxins. Malysheva and Zeltser (1968) isolated a protein-lipopolysaccharide (PLP) causing chlorosis and necrosis in cotton shoots and young plants. Similarly, PLP compounds from 3-day cultures of *V. dahliae* were toxic on cotton plants, had a molecular weight of 3,000kD and consisted of 75% polysaccharide and 15% protein and lipid (Keen and Long, 1972). Up to date, the work on PLP in cotton provides the best evidence for a toxin basis for Verticillium pathogenesis. However, evidence for the role of toxins in *Verticillium* wilt disease is often contradictory and with many inconsistencies.

2.3.2.3 Elicitors

2.3.2.3.1 Introduction

The term "elicitor" derives from the molecules with an ability to elicit plant defense responses (Keen and Long, 1972; Hahn, 1996). There are two groups of elicitors, general and race-specific elicitors. General elicitors, just as the name implies, are able to trigger defense responses both in host and non-host plants. Race-specific elicitors induce defense responses only in specific host cultivars following the gene-for-gene model. In this case,

the elicitor encoded by an avirulence (avr) gene in a particular race of the pathogen will elicit defense responses leading to disease resistance only in specific host lines carrying the corresponding resistance (R) gene (Kruger et al., 2003; Vidhyasekaran, 2004).

Elicitors do not have any common structures and they belong to a wide range of chemical classes including oligosaccharides, proteins, peptides, glycoproteins and lipids (Montesano et al., 2003). The terms of elicitors and toxins are often overlapping, however, elicitors act as signal compounds to trigger plant defenses at low concentration, while toxins may damage the plant only at higher concentration without inducing plant defense mechanisms (Boller, 1995).

2.3.2.3.2 Elicitors from *Verticillium dahliae* (VdNEP)

Verticillium dahliae is also reported to produce some elicitors inducing plant defense responses (Davis et al., 1990; Wang et al., 2004). A 65-kDa glycoprotein was isolated from culture fluids of *V. dahliae* and is responsible for the elicitor activity inducing the production of phytoalexin in cotton cell suspension cultures (Davis et al., 1990).

More recently, another elicitor named **VdNEP** has also been isolated from *V. dahliae* eliciting a hypersensitive response-like cell death in various plant species (Wang et al., 2004). VdNEP is a member of NLPs (Nep1-like proteins; Pemberton and Salmond, 2004), named after the first member Nep1 (Necrosis- and ethylene-inducing protein-1) that was isolated from culture filtrates of *Fusarium oxysporum* inducing necrosis and ethylene production in leaves of many dicot plant species (Bailey, 1995). Further study shows Nep1 induces accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), also activates the production of ROS and cell death in tobacco leaves (Jennings et al., 2001).

2.3.3 Plant defense responses

2.3.3.1 Cell wall fortification

The plant cell wall is the first barrier for the invasion of fungal pathogens (Thordal-Christensen, 2003) and consists of a highly complex array of polysaccharides, lignin, suberin, waxes, proteins, enzymes and certain inorganic compounds (Showalter, 1993). When the pathogen attempts to penetrate the host cell by producing a serial of cell-wall-degrading enzymes, such as pectinases, cellulases, xylanases and polygalacturonases (Herbert et al., 2004; Yang et al., 2005), the host plant defends itself by fortifying the cell wall with papilla (Gjetting et al., 2004), callose (Soylu et al., 2004; He and Wolyn, 2005), lignin (Zheng et al., 2005), wall-bound phenolics (De Ascensao and Dubery, 2003), suberin (Chen et al., 2004), and minerals (Ghanmi et al., 2004).

2.3.3.2 Cell death program during host-pathogen interaction

In response to fungal infections, scientists have named various types of cell death, including programmed cell death (PCD) (Wang et al., 2005; Zuppini et al., 2005), hypersensitive cell death (HCR or HR) (Dangl et al., 1996; Sasabe et al., 2000), spontaneous cell death (Dong, 2004) and susceptibility-related cell death (Eckardt, 2005).

Programmed cell death is a functional term used to describe cell death which is a normal part of the life of multicellular organisms (Martin et al., 1994). It is the cellular suicide, consisting of condensation of the cytoplasm and nucleus and nuclear DNA fragmentation (Wang et al., 1996; De Jong et al., 2000) to eliminate the harmful, unwanted cells and plays a crucial role in plant development and morphogenesis (Sanmartin et al., 2005). Programmed cell death is also involved in defense mechanisms against pathogen infections in plants (Greenberg et al., 1994; Zuppini et al., 2005).

The term of hypersensitive response describes the localized and rapid cell death in and around the initial infection sites, characterized by a rapid loss of membrane integrity in the infected host cells and the accumulation of phenolic compounds in response to attempted invasion by pathogens (Morel and Dangl, 1997). The hypersensitive response is induced by plant signals and hence it's also called host-induced cell death (Dickman et al., 2001). Moreover, in most plant-pathogen interactions, it's coordinately expressed with other plant defense responses to restrict the pathogen and protect the plant (Lam et al., 2001).

Spontaneous cell death is induced in some mutant and transgenic plants which is similar to hypersensitive cell death except that it's induced in the absence of a pathogen attack (Dong, 2004; Park et al., 2004; Xiao et al., 2003). For instance, some mutants called lesion mimics show cell death spontaneously without a pathogen attack (Morel and Dangl, 1999).

2.3.3.3 Induction of pathogenesis-related proteins

When a pathogen invades a plant, a group of proteins known as pathogenesis-related or PR proteins are induced and accumulated locally and often systemically in the infected plant tissues (Vidhyasekaran, 2004). Based on the protein structure and sequence similarity, PR proteins are classified into 17 groups given designation PR-1 through PR-17 (Van Loon et al., 1994). PR proteins are induced in the hypersensitive response and also during systemic acquired resistance (SAR) (Faize et al., 2004). For example, PR-2 (β-1, 3-glucanase) and PR-3 (chitinase) have antifungal activity *in vitro* (Cutt and Klessig, 1992). Moreover, transgenic plants over-expressing some of the PR proteins show enhanced disease resistance (Chen et al., 1999; Datta et al., 1999; Gao et al., 2000).

2.3.3.4 Reactive oxygen species in signal transduction

The rapid production of reactive oxygen species, such as hydrogen peroxide (H_2O_2) superoxide (O_2^-) and hydroxyl radical (-OH) , known as the oxidative burst, is one of the earliest events of plant defense responses that are associated with hypersensitive response (HR) in many plant-pathogen interactions (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). For example, H_2O_2 is produced within 5 min of *Verticillium dahliae* elicitor addition in cultured soybean cells (Apostol et al., 1989). ROS have been shown to play an important role in plant defense response for the following three reasons. Firstly, the ROS have a direct antimicrobial effect on the pathogen. Secondly, they are involved in the oxidative cross-linking of cell walls around the site of infection (Bradley et al., 1992). Thirdly, these ROS have been implicated as inducers of defence genes and have a role in the development of cell death during the HR (Alvarez et al., 1998; Grant and Loake, 2000; Orozco-Cardenas et al., 2001).

2.3.3.5 Nitric Oxide in signal transduction

Among activated oxygen species, nitric oxide (NO) is a molecule recently recognized to be involved in defence signaling as a cellular mediator in plants (Gould et al., 2003). For instance, NO content dramatically increased in wheat after 24 hour inoculation with *Puccinia striiformis* race CY22-2 (Xu et al., 2004). Foissner et al. (2000) found an elicitor named cryptogein from *Phytophthora cryptogea*, triggers a NO burst within minutes in epidermal sections from tobacco leaves (*N. tabacum* cv. Xanthi), with maximum increase within 30 min (Lamotte et al., 2004). Nitric oxide is also used by plants as a signaling molecule in transduction pathways (Wendehenne et al., 2002; Lamotte et al., 2004).

2.3.3.6 Hormone signaling pathways in plants

2.3.3.6.1 Salicylic acid (SA) signaling pathway

2.3.3.6.1.1 SA signaling in plant defense responses

Salicylic acid (SA) has been reported as one of the important signal molecules accumulated in infected plants (Nicholson and Hammerschmidt, 1992; Meuwly et al., 1995). White (1979) found that exogenous SA induced resistance in tobacco to Cucumber mosaic virus (CMV), which showed the first hint that SA may be involved in the regulation of plant resistance. The importance of SA in induction of plant defense was further studied in tobacco or cucumber plants transformed with the *NahG* gene that codes for the enzyme salicylate hydroxylase as a mean of reducing the amount of SA by converting it into catechol (Gaffney et al., 1993). *NahG* transgenic plants are unable to accumulate SA or develop SAR in response to TMV, suggesting that SA might be a natural signal for SAR in plants (Gaffney et al., 1993; Delaney et al., 1994). In addition, SA has been proposed as a candidate for the translocated signal involved in SAR (Dean and Kuć 1986), which means SA must be produced in the infected plant tissue and then translocated throughout the whole plant. Rasmussen et al. (1991) found that there is an increased SA in the phloem of the cucumber leaf above the inoculated leaf by *Pseudomonas syringae* pv. *dyringae*, indicating that SA might be moving from one leave to another. The role of SA as the translocated signal was further studied by grafting wide type tobacco and *NahG* rootstocks (Vernooij et al., 1994). The authors found that TMV-inoculated leaves on the *NahG* rootstocks were able to induce resistance and SA accumulation in the wild type tobacco. Therefore, they concluded that SA was not the primary translocated signal, but was the secondary signal that was induced by primary,

translocated signal generated at the infection site. SA accumulation induces disease resistance and the expression of defense-related genes, and this has been well characterized in *Arabidopsis*, tobacco and cucumber (Dorey et al., 1997). However high endogneous SA levels have been found in plant species, such as potato and rice (Raskin et al., 1990; Dempsey et al., 1999), which makes plant cells less responsive to exogenous SA signal. Up to date, little is known about the involvement of SA in disease resistance in these species.

Although SA signaling is important in plant defense, SA is not to be involved in host defense responses in many plant-pathogen interactions (Hückelhoven et al., 1999). For instance, SA application didn't enhance resistance in *Arabidopsis* against *Hyaloperonospora parasitica* (McDowell et al., 2000), *Botrytis cinerea* (Govrin and Levine, 2002), or *Alternaria alternate* (Ryals et al., 1996), which indicates that besides SA, other signaling pathways may play a crucial role in triggering plant defense responses.

2.3.3.6.1.2 Signal perception of SA

The first 650 kDa protein shown to reversibly bind SA was a catalase (CAT) from tobacco, originally termed SABP (SA-binding protein) (Chen and Heath, 1993). In addition to catalase, a second SA-binding protein (SABP2) has been identified in tobacco cytoplasma which has a low molecular weight of approximately 25 kDa and reversibly binds SA, showing 150-fold higher affinity than that between SA and catalase (Du and Klessig, 1997). Although silencing of the *SABP2* gene suppressed expression of the PR-1 induced by SA (Kumar and Klessig, 2003), the biochemical function of SABP2 in inducing SA signaling is unclear.

2.3.3.6.1.3 Upstream of SA signaling

In the SA signaling, H_2O_2 has been well established to be important for SA biosynthesis and accumulation (Leon et al., 1995; Summermatter et al., 1995). In transgenic tobacco plants, when catalase inducing the accumulation of H_2O_2 was suppressed, most plants lost the ability to induce the expression of constitutive *PR* genes (Takahashi et al., 1997). Moreover, in *NahG* plants, H_2O_2 was unable to induce *PR* gene expression, which suggested that SA may act downstream of H_2O_2 in inducing PR genes (Bi et al., 1995).

2.3.3.6.1.4 Downstream of SA signaling

In *Arabidopsis*, *NPR1* (non-expressor of *PR1*) / *NIM1* (non-inducible immunity 1) acts as a very important regulator of *PR* gene expression, downstream of SA signaling (Shah et al., 1997; Zhang et al., 2003). In non-induced cells, oxidized *NPR1* forms inactive oligomers that remain in the cytosol. After stimulation by SA, The *NPR1* inactive oligomeric complex turns into an active monomeric state and translocates to the nucleus where it interacts with TGA transcription factors that bind the SA-responsive promoter elements (TGACG), resulting in the activation of PR genes (Kinkema et al., 2000; Mou et al., 2003).

SA induces the expression of several *PR* genes to defend plants against pathogens, including *PR-1* (involved in plant cell wall thickening), *PR-2* (β-1, 3,-Glucanase) and *PR-5* (thaumatin-like proteins) (Malamy et al., 1990). Several *Arabidopsis* mutants with high endogenous SA level showed high constitutive expression of *PR* genes (Dangl et al., 1996; Ryals et al., 1996). On the contrary, the SA-intensive (*sai1*) mutant plants are unable to express *PR* genes even after treatment with SA (Cao et al., 1997).

2.3.3.6.2 Jasmonate (JA) signaling pathway

2.3.3.6.2.1 JA signaling in plant defense responses

Jasmonates (JAs) including jasmonic acid and its cyclic precursors and derivatives were first detected as essential oils (Demole et al., 1962). The importance of JA in plant defense responses was first found by Farmer and Ryan (1990), that is the methyl ester of jasmonic acid (MeJA), volatilized from sagebrush could trigger defense gene expression in adjacent tomato plants. The enhanced resistance against a bacterial pathogen *Pseudomonas syringae* was found in *Arabidopsis* mutant with endogenous high level of JA (Ellis et al., 2002). On the contrary, JA-deficient mutants of *Arabidopsis* were unable to induce defense responses and were highly susceptible to *Pythium mastophorum* (Thomma et al., 1998). The application of exogenous JA enhanced the resistance level close to that of wild-type controls (Thomma et al., 1998; Li et al., 2002). JA deficiency in tomato mutants increased the susceptibility of the plants to *Phytophthora infestans* and some soil-borne pathogens, such as *Fusarium oxysporum* and *Verticillium dahliae* (Thaler et al., 2004). However, no increased susceptibility was found to three other fungi, *Septoria lycopersici*, *Cladosporium fulvum* and *Oidium neolycopersici* (Thaler et al., 2004) suggesting that JA signaling affects different pathogens in varied manners (Thomma et al., 2001).

JA induces the accumulation of several proteins called JIPS (JA-induced proteins). Little information is available about the functions of those proteins, but some of them may have antimicrobial activity (Reinbothe et al., 1994).

2.3.3.6.2.2 Signal perception of JA

The JA signal is probably transformed by the activation of receptors that bind the JA

molecule. However, there are no receptors indentified, like SABP2 as SA binding protein (Turner et al., 2002). In *Arabidopsis*, two genes are involved in the perception of JA signaling, *JAR1* (JA-resistant 1) and *COI1* (coronatine-insensitive 1). JAR1 is similar to auxin-induced GH3 gene product from Soybean and COI1 is a 66 kDa protein containing an F-box motif and a LRR domain (Xie et al., 1998). Both of them show no homology to plant receptor proteins reported previously (Gilroy and Trewavas, 2001), but they may regulate the defense responses induced by JA (Turner et al., 2002).

2.3.3.6.2.3 Upstream of JA signaling

The accumulation of JA is activated by mechanical wounding and water stress (Creelman and Mullet, 1997), herbivory (McCloud and Baldwin, 1997) and microbial elicitors (Blechert et al., 1995). AOS (Active Oxygen Species) are important in the synthesis of JA. The potato plants over-expressing the flax AOS gene accumulated high levels of JA (Harms et al., 1995). The JA-dependant pathway is up-regulated by protein phosphatase 2A, but is down-regulated by a protein kinase (Rojo et al., 1998).

2.3.3.6.2.4 Downstream of JA signaling

Jasmonates (JAs) induce many defense genes (Sasaki et al., 2001), including phenylalanine ammonia lyase (PAL) (Gundlach et al., 1992), proline-rich cell wall protein (Creelman et al., 1992), thionin (*Thi2.1*) (Andresen et al., 1992), plant defense gene (*PDF1.2*) (Manners et al., 1998), proteinase inhibitors (Farmer and Ryan, 1990), chalcone synthase (Creelman and Mullet, 1995) and several secondary metabolites, including various alkaloids, flavonoids, terpenoids and anthraquinones (Gundlach et al., 1992).

2.3.3.6.3 Ethylene (ET) dependent signaling pathway

2.3.3.6.3.1 ET signaling in plant defense responses

The rapid accumulation of ethylene (ET) is one of the earliest events occurring during the plant-pathogen interaction (Geraats et al., 2003). However, the involvement of ethylene signaling seems complex. ET stimulates defense mechanisms in various plants (Boller, 1991). For example, enhanced resistance against *Botrytis cinerea* was found in ET-pretreated tomato (Díaz et al., 2002). On the contrary, ET also induces susceptibility to this pathogen in tomato, pepper and other crops when it is applied exogenously (Boller, 1991). The ET-insensitive mutant of tomato showed reduced disease severity when inoculated by *Fusarium oxysporum* (Lund et al., 1998) and *Arabidopsis* mutant *ein2-1* (ET-insensitive 2-1) showed enhanced resistance to *Pseudomonas syringae* and *Xanthomonas campestris* (Benta et al., 1992). By contrast, *ein2-1* showed increased susceptibility to *B. cinerea* and *Pythium* spp. (Geraats et al., 2002), which indicated ET may regulated the disease reaction depending on the pathogen.

2.3.3.6.3.2 Signal perception of ET

Although the role of ET in plant-pathogen interaction is unclear, the biosynthesis pathway of ET has been well studied (Kende, 1993; Wang et al., 2002). S-adenosylmethionine (S-AdoMet) and 1-Aminocyclopropane-1-carboxylic acid (ACC) are the precursors of ET. ACC synthase (ACS) and ACC oxidase (ACO) are the most important enzymes involved in ET synthesis to convert S-AdoMet to ACC and ACC to ET (Wang et al., 2002).

After ET synthesis, it is perceived by a family of membrane-localized receptors (Pirrung, 1999). Five ET receptors exist in *Arabidopsis*: ETR1 (ethylene response 1),

ETR2, EIN4 (ethylene-insensitive 4), ERS1 (ethylene response sensor) and ERS2 (Wang et al., 2002; Klee, 2004). Based on the structure similarities, the receptor family can be divided into two subfamilies: ETR1- and ETR2-like subfamilies. ETR1-like subfamily, including ETR1 and ERS1, contains an ET-binding site with three transmembrane (TM) domains at the N-terminal region and a conserved histidine kinase domain at the C-terminal region (Hall et al., 2000). The ETR2-like subfamily includes ETR2, EIN4, and ERS2, which contains an ET-binding site with four transmembrane (TM) domains and a degenerate histidine kinase domain (Hall et al., 2000). Six ET receptors have been reported in tomato showing a similar structure to those in *Arabidopsis* (LeETR1-6) (Klee, 2004).

2.3.3.6.3.3 Upstream of ET signaling

Rapid activation of SIPK (salicylic acid-induced protein kinase), a mitogen-activated kinase (MAPK), highly induced the ET production and ACS activity in tobacco (Kim et al., 2003) indicating SIPK may be an important factor in the process of ET accumulation.

2.3.3.6.3.4 Downstream of ET signaling

In *Arabidopsis*, CTR1 belonging to Raf family of Serine/threonine protein kinases, is a negative regulator in the downstream ethylene signaling pathway through an MAPK cascade (Wang et al., 2002). According to genetic epistasis analysis, EIN2 (ET-insensitive 2) is a downstream target of CTR1. EIN2 encodes a protein with 12 putative membrane-spanning domains, suggesting the possible function as a transporter (Alonso et al., 1999). The Null mutations of EIN2 cause the loss of ET- responsiveness completely, which indicates that EIN2 acts as a positive regulator in ET signaling

pathway (Alonso et al., 1999). EIN3, a novel nuclear-localized protein, binds to the promoter of ERF1 gene and activates downstream ET responses (Chao et al., 1997). ERF1 is a member of ET-response-element binding proteins (EREBPS), which are able to bind to the GCC box, a DNA motif associated with ethylene- and pathogen-induced gene expression (Solano and Ecker, 1998). EIN 3 is necessary for *ERF1* expression, indicating ERF1 may regulate the ET signaling pathway downstream of EIN3. In tomato, a transcription factor Pti4 is homologous with EREBPS and specifically binds to the GCC box in the promoter of *PR* genes. ET induces the expression of *Pti4* and several *PR* genes in tomato leaves (Gu et al., 2000). ET also induces the expression of PAL, HRGPS and osmotin-like proteins (Boller, 1991).

2.3.3.6.4 Abscisic acid (ABA) signaling pathway

Abscisic acid (ABA) is not only an important signaling molecule in seed maturation and germination, but is also involved in the adaption of plants to abiotic stress (Leung et al., 1997). The finding that ABA accumulates in infected tissues during fungal invasion (Bothe et al., 1994) suggests that this hormone may also be involved in plant disease resistance. In rice plants, ABA induces the expression of PR-1 gene (Agrawal et al., 2001). It causes the rapid induction of proteinase inhibitor genes in tomato as well (Peňa-Cortés et al., 1991). ABA signaling may act upstream of the octadecanoid pathway which is a well-characterized biosynthetic pathway for the production of JA (Bostock, 1999).

On the other hand, ABA may enhance disease susceptibility in various plant species, including soybean (*Glycine max*) to *Phytophthora soja*e (Mcdonald and Cahill, 1999), tomato to *Botrytis cinerea* (Audenaert et al., 2002), rice to *Magnaporthe grisea* (Koga et

al., 2004) and *Arabidopsis thaliana* to *Peronospora parasitica* (Mohr and Cahill, 2003). ABA may suppress SA-dependant defense signaling pathway in tomato and induce susceptibility to *Botrytis cinerea* (Audenaert et al., 2002). It's widely reported that SA highly induces the expression of PAL gene (Audenaert et al., 2002). However, ABA suppresses the PAL activity in tomato and soybean (Ward et al., 1989) suggesting that there might be a negative crosstalk between SA and ABA signaling. ABA also inhibits ET and JA signaling pathways (Anderson et al., 2004). *PDF1.2* is an important defensin gene induced by ET and JA. Exogenous treatment of ABA significantly depresses the expression of *PDF1.2* in *Arabidopsis* (Anderson, 2004).

2.3.3.6.5 Other signaling pathways

In *Arabidopsis*, some transcription factors are not responsive to SA, JA, ET and ABA (Chen et al., 2002), which indicates that there are some other signaling pathways involved in plant defense responses. However, what other molecules are involved in plant defense is unknown.

2.3.3.6.6 Network and interplay of signaling pathways

2.3.3.6.6.1 Coordinated regulation among SA, JA and ET signaling

The hormone molecules SA, JA and ET are important signals in plant primary resistance that do not act independently, but coordinately, to fine-tune plant defense responses (Nandi et al., 2003). As mentioned above, the expression of the defensin gene *PDF1.2* and the *PR* genes are required for all these three signaling pathways in *Arabidopsis* (Nandi et al., 2003). In some cases, JA/ET signaling pathways are required for SA (Shah et al., 1999). In *Arabidopsis* mutant *cpr5* (constitutive expression of *PR5*),

the expression of defense genes are interdependent of JA, SA and ET signaling pathways (Clarke et al., 2001).

2.3.3.6.6.2 Coordinated regulation of JA and ET signaling

JA and ET signaling pathways usually work together to induce expression of defense or signaling genes involved in plant defense responses (Anderson et al., 2004). For example, PR proteins were not only induced by JA but also by ET in potato (Xu et al., 1994). Induced systemic resistance (ISR), which is triggered by selected strains of non-pathogenic rhizobacteria, requires both JA and ET (Van Loon et al., 1998). In *Arabidopsis*, ISR is not dependent on SA or PR gene activation, but is dependent on JA/ET, because JA/ET deficient mutants are unable to induce ISR (Ton et al., 1999). Although pathogen-induced SAR and rhizobacteria-mediated ISR take part in distinctly different signaling pathways, both of them require *NPR1* gene in *Arabidopsis*. The *npr1* mutant plants fail to express PR protein accumulation and ISR signaling (Pieterse et al., 1998), suggesting NPR1 acts downstream of SA, JA and ET signaling pathways, as a common intermediate between SAR and ISR.

ET may act downstream of the JA signaling pathway. Application of MeJA to Douglas fir (*Pseudotsuga menziesii*) stems highly induces the expression of ACO (ACC oxidase) which is involved in the last step of ET synthesis. On the contrary, pretreatment with an ET synthesis inhibitor makes Douglas fir plants lose their sensitivity to MeJA (Hudgins and Franceschi, 2004). ET and JA signaling pathways may converge in the expression of *ERF1* gene, which encodes an ET-response-element binding protein (EREBP) interacting with the GCC box to regulate the expression of pathogen-induced genes (Solano and Ecker, 1998). Neither JA- nor ET-deficient *Arabidopsis* mutants are

able to induce the expression of *ERF1* (Lorenzo et al., 2003). On the contrary, over expression of *ERF1* induces the accumulation of *PR* genes and some other defense responses in both *coi1* (defective in JA-dependent pathway) and *ein2* (ethylene-insensitive 2) *Arabidopsis* mutants (Lorenzo et al., 2003), suggesting that *EFR1* operates downstream of the intersection between JA and ET signaling pathways.

2.3.3.6.6.3 Cross talk between SA, JA and ET signaling

Cross talk between SA, JA and ET signaling pathways is involved in fine-tuning the plant defense responses, and has been broadly reported (Xu et al., 1994; Chen et al., 2002; Devoto and Turner, 2003). In most plant-pathogen interactions, SA and JA/ET antagonize each other (Felton et al., 1999; Takahashi et al., 2004). SA suppresses the expression of JA-dependent defense genes and *vice versa* (Bowling et al., 1997; Van Wees et al., 1999). For instance, TMV-infected tobacco plants expressing SAR are unable to develop JA-mediated wound responses (Preston et al., 1999). In *Arabidopsis* mutants *ssi* (suppressor of SA insensitivity) and *fab2* (allelic with ssi2), which accumulate elevated level of SA, the expression of *PDF1.2*, which is a marker gene in JA/ET-dependant signaling pathways, is suppressed (Kachroo et al., 2003). On the other hand, JA and ET may inhibit SA synthesis or signaling. For example, pretreatment of tomato plants with JA suppresses the accumulation of PR proteins, which are induced by SA (Bostock, 1999). Accordingly, eight genes induced by SA are down-regulated by MeJA in *Arabidopsis* (Schenk et al., 2000). Over expression of *ERF1*, which is a key element in the integration of both JA and ET signaling pathways for the regulation of plant defense responses, makes *Arabidopsis* plants less responsive to SA and more

susceptible to bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Berrocal-Lobo et al., 2002).

2.3.3.6.6.4 Cross talk between ABA, JA and ET signaling

ABA may antagonize JA/ET signaling pathways (Anderson et al., 2004). Exogenous application of ABA suppresses the expression of JA/ET-mediated defense genes, such as *PDF1.2* (Anderson et al., 2004). Upregulation of JA/ET-dependent defense genes was found in *Arabidopsis* ABA-deficient mutant plants (Anderson et al., 2004). In reverse, ET-intensive mutants show enhanced sensitivity to ABA (Anderson et al., 2004), indicating that the antagonistic interaction between ABA and JA/ET signaling pathways is involved in fine-tuning plant defense responses.

3.0 MANUSCRIPT

VdNEP is both a pathogenicity factor and an elicitor in the interaction between *Verticillium dahliae* and *Helianthus annuus*

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3.1 Abstract

Verticillium wilt, caused by the soil-borne *Verticillium dahliae* Klebahn is a serious problem in the production of sunflower worldwide. To date, information on sunflower resistance to *Verticillium* spp. is very scarce, although it is critical for an effective management of this pathogen. In this study, two highly aggressive (Vd1396-9 and Vd1398-21) and two weakly aggressive *V. dahliae* isolates (Vs06-07 and Vs06-14) were used to inoculate moderately resistant (IS6111) and susceptible (IS8048) sunflower hybrids. VdNEP (*V. dahliae* necrosis and ethylene-inducing protein), an elicitor from *V. dahliae*, was also used to infiltrate sunflower plants. Our results indicate that VdNEP has a dual role in the interaction between sunflower and *V. dahliae*. VdNEP acted not only as a pathogenicity factor on sunflower by inducing wilting symptoms such as chlorosis, necrosis and vascular discoloration, but also as an elicitor triggering defense responses of the host. VdNEP induced the hypersensitive cell death in *Nicotiana benthamiana* leaves and sunflower cotyledons. Moreover, VdNEP activated the production of reactive oxygen species and the accumulation of fluorescent compounds in sunflower leaves. Pathogenesis-related genes (*Ha-PR-3,* and *Ha-PR-5*), two defensin genes (*Ha-PDF* and *Ha-CUA1*) and genes encoding *Ha-ACO*, *Ha-CHOX*, *Ha-GST* and *Ha-SCO* were up-regulated by VdNEP, suggesting that multiple signaling pathways are involved in this interaction. Two SA-related genes (*Ha-PAL* and *Ha-NML1*) were slightly suppressed after infiltration with VdNEP, suggesting a possible involvement of VdNEP in affecting sunflower defenses.

Keywords

Verticillium wilt, Sunflower, VdNEP, Pathogenicity, Elicitor, Plant defense response

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AOS	Active oxygen species
CAA	Casamino acid
d.a.i.	Days after inoculation
ET	Ethylene
h.a.i.	Hours after inoculation
H_2O_2	Hydrogen peroxide
His-VdNEP	His tagged fusion VdNEP protein
HR	Hypersensitive response
JA	Jasmonic acid
NEP	Necrosis- and ethylene-inducing protein-1
NLP	NEP1-like protein
NO	Nitric oxide
O_2^-	Superoxide
-OH	Hydroxyl radical
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDB	Potato dextrose broth
PE	Pectin esterases
PG	Polygalacturonase
PLP	Protein-lipopolysaccharide
PR	Pathogenesis related proteins
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid
SABP2	SA-binding protein
S-AdoMet	S-adenosylmethionine
SAR	Systemic acquired resistance
SDW	Sterile distilled water
w.a.i.	Weeks after inoculation

3.2 Introduction

Sunflower (*Helianthus annuus* L.) is an economically important crop and a major source of vegetable oil and snack food worldwide (Putt, 1978). *Verticillium* wilt, caused by the soil-borne fungus *Verticillium* spp., is a serious problem in sunflower production worldwide, causing up to 50% yield loss and dramatically reducing sunflower oil quality (Hoes, 1972). The main causal agent, *V. dahliae* Klebahn, infects sunflower roots and subsequently spreads to aerial parts of the plant through the vascular system (Sackston, 1957). Like in many economic crops, this wilt in sunflower involves chlorosis, necrosis, wilting, vein browning, leaf defoliation and stunting (Fradin & Thomma, 2006; Mace *et al*., 1981). Dark-brown vascular discoloration, mostly due to the plugging of the vessels by a phenols-pectin mix, also occurs (Beckman & Talboys, 1981).

Several strategies, developed for *Verticillium* wilt management in sunflower and other crops, are expensive and not very effective, due to the variability and host range of *Verticillium* spp., as well as the striking viability of their resting structures (Koike *et al*., 1994). Heat and solarization, which raise soil temperature to the thermal death point (TDP) of *Verticillium* spp., are energy-inefficient and limited to warm climates. Crop rotation, as currently used, is not effective due to the long-term survival of *Verticillium* resting structures (Shetty *et al*., 2000). Chemical fumigants can effectively reduce the level of *Verticillium* inoculum in soil (Voth *et al*., 1973) but are expensive and detrimental to the environment. Biological control alternatives have also been investigated in several crops (Mannanov, 2001; Uppal *et al*., 2007) but have not been consistent and their efficacy often limited to controlled conditions. Finally, breeding of resistant cultivars would be a promising and potentially efficient approach to manage

Verticillium wilt, but only one resistance locus against *Verticillium* has been cloned so far (Kawchuk *et al*., 2001) and no sunflower cultivar resistant to this pathogen is currently available (Pegg & Brady, 2002). In sunflower in particular, information about resistance to *Verticillium* is very limited. Therefore, better understanding of the sunflower-*Verticillium* spp. interaction is essential for further development of an effective strategy against this pathogen. In particular, understanding how key components such as pathogen-derived compounds regulate the interplay of the host-pathogen interaction is critical (Radman *et al*., 2003).

Phytotoxic factors or elicitor-like substances from *V. dahliae* including glycoproteins, protein-lipopolysaccharide (PLP) complex, cell-wall-degrading enzymes and small peptides have gained much attention as *Verticillium* wilt inducers. For example, a PLP complex purified from cultures of *V. dahliae* caused chlorosis and necrosis in potato shoots and young potato plants (Nachmias *et al*., 1982). However, culture filtrates of *V. dahliae*, containing complex phytotoxic proteins and carbohydrates (Low & Heinstein, 1986), also stimulated cell wall lignification in cotton hypocotyls (Smit & Dubety, 1997). Subsequently, a 26-kDa glycoprotein purified from culture fluids of *V. dahliae* induced both leaf wilting and phytoalexin production in cotton leaves (Chu *et al*., 1999). Therefore, the role of these effector molecules in disease *versus* resistance has been unclear.

VdNEP (*V. dahliae* necrosis and ethylene-inducing protein) is a *V. dahliae* protein from the NLPs family (NEP1-like proteins; Pemberton & Salmond, 2004), named after the NEP1 (Necrosis- and ethylene-inducing protein-1) isolated from *Fuasrium oxysporum* culture filtrates (Bailey, 1995). Homologs to *VdNEP* were previously identified in

Fusarium oxysporum (AAC97382) (Bailey, 1995), *Pythium monospermum* (AAQ89593), *Pythium aphanidermatum* (AAD53944) (Veit *et al*., 2001) and *Phytophthora sojae* (AAM48171) (Qutob, *et al*., 2002). VdNEP was reported to induce both necrosis in *Nicotiana benthamiana* leaves and accumulation of pathogenesis-related proteins (AtPR1, AtPDF1.2) in *Arabidopsis* (Wang *et al*., 2004). It also induced the production of phytoalexins and programmed cell death in cotton cell suspensions and wilting of cotton leaves and cotyledons after infiltration (Wang *et al*., 2004). It was thus proposed that VdNEP played an important role in the interaction between *V. dahliae* and its hosts. Nevertheless, evidence whether *VdNEP* is a common *V. dahliae* effector, to what extent it is related to *V. dahliae* pathogenicity and symptom development in its hosts, or in what proportions it contributes to triggering defense mechanisms *in planta* remained very limited. So far, the extent to which VdNEP is responsible for *V. dahliae* wilting symptoms has not been investigated. Therefore, it is important to assess the relative contribution of VdNEP and other effectors to both disease and defense, which will be highly valuable for deciphering the mechanisms of this interaction between *V. dahliae* and its hosts.

The first objective of this study was to compare the expression of VdNEP at the gene and protein levels in both highly and weakly aggressive isolates of *V. dahliae*. In order to assess to what extent VdNEP contributes to *V. dahliae* pathogenesis on sunflower, the second objective was to produce a VdNEP fusion protein *in vitro* and compare the disease severity and symptoms it causes in sunflower with those caused by weakly and highly aggressive isolates of *V. dahliae*. Finally, in order to determine the responses induced by VdNEP in sunflower, the third objective was to assess the defense

reactions induced in sunflower leaves in response to infiltration with VdNEP, including the hypersensitive reaction, production of reactive oxygen species and activation of defense-related genes.

3.3 Materials and methods

3.3.1 Sunflower plants and *Verticillium* isolates

To analyze the presence of *VdNEP* in the genome of *V. dahliae*, ten single-spore cultures of *V. dahliae* isolates from our lab collection were used, including six sunflower isolates (Vs06-01, Vs06-02, Vs06-03, Vs06-06, Vs06-07 and Vs06-14) and four potato isolates (Vd1396-9, Vd1398-21, Vs04-09 and Vs04-47) (Uppal *et al*., 2007; Alkher *et al*., 2009). Four were selected to assess the expression level of *VdNEP* and their pathogenicity on sunflower: Vd1396-9 and Vd1398-21, previously determined to be highly aggressive on sunflower, and Vs06-07 and Vs06-14, which were weakly aggressive (Uppal *et al*., 2007; Alkher *et al*., 2009). Two sunflower cultivars, IS6111 (moderately resistant) and IS8048 (highly susceptible) were used to compare the disease severity and symptoms caused by *V. dahliae* isolates and purified His-VdNEP protein.

Sunflower seeds were germinated and grown as previously described (Alkher *et al*., 2009). The conidia were harvested from *V. dahliae* grown on potato dextrose agar (PDA) for two weeks at 20°C (Fisher Scientific incubator Model 146E). The final concentration of the conidial suspension from each *Verticillium* isolate was adjusted to 1×10^6 conidia/ml. Two-week old sunflower seedlings were inoculated by root dipping (Daayf *et al*., 1998) with purified His-VdNEP protein at 20 μg/ml (Wang *et al*., 2004) and four *V.*

dahliae isolates: Vd1396-9, Vd1398-21, Vs06-07 and Vs06-14. Sunflower seedlings treated with sterile distilled water (SDW) were used as controls.

3.3.2 DNA and RNA isolation of *V. dahliae*

Total genomic DNA was extracted from *V. dahliae* as described previously (Alkher *et al*., 2009). The *VdNEP* gene was amplified using primers, VdNEP-F (5'-AAACAGCCATCTGCACCT-3') and VdNEP-R (5'-TATGCTAGCACTCGACT-3') in a 50µl reaction volume. The PCR amplification mixture consisted of 0.5 µl of genomic DNA from *V. dahliae*, 1×amplification buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 2U *Taq* DNA polymerase, 200 µM each of dNTP and 0.5 µM of each primer. The amplification profile in a Thermal Cycler (Techne, Princeton, N.J.) consisted of the initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 60s, followed by a final extension at 72°C for 10 min. Master mix with no template DNA was used as a negative control. Amplified PCR products were analyzed on a 1% agarose gel stained with ethidium bromide in TBE buffer (0.089 mM Trizma base, 0.089 mM Boric acid, 2.0mM EDTA pH 8.0) and 1Kb Plus DNA Ladder (Invitrogen). The electrophoresis was run at 150 volts for 20-30 minutes. The stained gel was photographed by a UV transilluminator (Alpha Innotech, San Leandro, U.S.A.). All PCR products were sequenced by Macrogen (Rockville, Maryland, U.S.A.).

Total RNA from mycelium of *V. dahliae* was extracted at 3, 7, 10, 14, 21 and 40 days after media inoculation using TRIzol reagent (Invitrogen), as described by Triant and Whitehead (2009) then treated with amplification grade DNAse I (Invitrogen) to

remove genomic DNA contaminants. For reverse transcription-PCR (RT-RCR), the first strand of cDNA was synthesized with 2 μg of total RNA in a 20 μl mixture consisting of 200U of M-MLV reverse transcriptase (Invitrogen), 1×first-strand buffer, 20 mM DTT, 500 μM each of dNTP and 20ng/μl oligo $dT_{(18)}$ at 42°C for 50min according to the manufacturers' recommendations. Following the reaction, 1 μl of the first strand cDNA was used as the template for the second strand amplification with the primers: VdNEP1-F (5'-ATGCTTCCCTCCGCAGTCTTCT-3') and VdNEP1-R (5'-CAGATGACGACGTTCTCCCAGT-3') as described above.

3.3.3 Expression of VdNEP in pET-32a (+) / BL21 *Escherichia coli* strain

The pGEM-T vector containing *VdNEP* was provided by Shanghai Institutes of Biological Science, Chinese Academy of Science. *VdNEP* was amplified using primers described previously (Wang *et al*., 2004) and constructed into the expression vector pET-32a (+) (Novagen, Madison, Wis.). Competent cells of *E. coli* BL21 (DE3) were transformed with pET-32a (+) vector harboring *VdNEP* gene to express His-tagged VdNEP fusion protein (His-VdNEP). *E. coli* cells were grown in a shaker flask at 37ºC in LB broth medium containing 50 μg/ml ampicillin until OD=0.6. Then 1 mM of isopropyl-l-thio-β-D-galactopyranoside (IPTG) was added to the medium and the expression of His-VdNEP was induced for 12 hours at 20 °C on a shaker rotated at 120 rpm (C2 Platform Shaker, Edison, NJ, U.S.A.). *E. coli* cells were harvested by centrifugation at 3,200 ×g for 10 min at 4°C. His-VdNEP was purified using Ni-NTA column (Qiagene) according to the manufacturer's instructions and dialyzed against Tris·HCl buffer (20 mM, pH 8.0) at 4°C overnight. Proteins were examined using sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 1-mm-thick 12% running gel according to the method described previously (Laemmli, 1976). The protein concentrations were determined with the protein assay kit (Bio-Rad) based on the manufacturer's protocols.

3.3.4 Antibody of VdNEP and Western blot analysis

The polyclonal antibody against purified His-VdNEP from this study was commercially produced by GenScript, U.S.A.. *V*. *dahliae* isolate, Vd1396-9 was cultured in 50 ml of Czapek-Dox broth liquid medium for 14 days in the dark at room temperature. The cell paste and liquid culture were collected to assess the native VdNEP proteins secreted from *V*. *dahliae*. For Western blot analysis, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes in 25 mM Tris-glycine buffer. The native VdNEP proteins were detected with One-Step Western Basic Kit (GenScript, U.S.A.) according to the manufacturer's instructions.

3.3.5 Assessment of disease severity and growth parameters

The disease severity was assessed at 2, 3, 4, and 5 weeks after inoculation (w.a.i.) using the disease rating scales described by Uppal *et al*. (2007) and Alkher *et al*. (2009). Vascular discoloration was evaluated at 5 w.a.i. with a scale ranging from 0 to 5 (Alkher *et al*., 2009). Plants were taken from pots and washed free of clay, then spread on paper for determination of plant height at the end of the experiment (5 w.a.i.). Plants were further cut into roots and shoots, and fresh weight measured. Plant shoots and roots were dried for 48 h at 38°C and dry weight recorded. There were 10 replicates in each

treatment.

3.3.6 Assay of necrosis-inducing activities

The bacterially-expressed fusion proteins of His-VdNEP in PBS buffer were infiltrated into 4-week-old *N. benthamiana* leaves or 5-day-old sunflower cotyledons. Sterile distilled water (SDW) and His-tag proteins from empty pET-32a (+) vector were used as negative controls. A 20 µl of protein solution was used in each infiltration at the concentrations noted in Fig. 3.7, which was performed by using 1 ml plastic syringes without needles. Hypersensitive cell death or necrosis symptoms were viewed and photographed 24 or 12 hour after penetration.

3.3.7 Examination of the production of fluorescent compounds and active oxygen species, and DNA laddering in sunflower leaves

To examine the accumulation of fluorescent compounds, sunflower leaves were stained with Trypan blue in lactophenol as previously described (Wang *et al*., 2004). The leaves were then mounted on a slide with chloral hydrate and photographed under a Leica DMRB microscope equipped with a Leica Fibre Optic light source and PL FLUOTAR 10´ and 40´ phase contrast lens. Images were recorded with a Leica DFC290 digital camera and processed with Leica image application suiteâ 2007 (Leica) provided by microscope manufacturer.

To view the production of reactive oxygen species (AOS), sunflower leaves were stained for 12 h with a 3,3-diaminobenzidine (DAB) solution (1 mg/ml, pH 3.8) and then decolorized in 96% ethanol. Samples were mounted on a slide with 60% glycerol and

photographed under a light microscope. AOS were detected as reddish brown coloration.

Genomic DNA of sunflower was isolated using CTAB (Doyle & Doyle, 1990). DNA was analyzed on a 2% agarose gel stained with ethidium bromide in TBE buffer. DNA laddering in the genomic DNA was observed and photographed under UV light.

3.3.8 RT-PCR analysis of sunflower defense and signaling gene expression

Total RNA from sunflower was isolated from sunflower leaves using TRIzol reagent (Invitrogen) and treated with amplification grade DNAse I (Invitrogen). The expression of genes related to defense responses and signaling in sunflower were determined using RT-RCR as described previously. Gene-specific primers were designed using the software OLIGO 6.6 (Table 3.1). *EF-1α* gene exhibiting a constitutive expression in sunflower was used as the internal control to ensure a linear relationship between the amount of RNA used and the amount of cDNA fragment amplified and to check the quality of both extracted RNA and RT-PCR reactions.

3.3.9 Data analysis

Sunflower height, shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), disease severity and vascular discoloration were analyzed using generalized linear models in SAS system (Statistical Analysis Systems Institute Inc., Cary, N.C, and U.S.A.). The percentage of infected leaf area (chlorosis and necrosis) was estimated using the image analysis software Assess (Lamari, 2002). All experiments were repeated at least once.

Gene	Primer sequence	Tm (ºC)	No. of cycles	Accession no.
Ha-PR-5	For 5'-ACTATACCAATGCAGCCGTGTT-3' Rev 5'-TTGATCAAGGGCAGAATATAACGTC-3'	55	28	AF364864
Ha-Chi	For 5'-GCCAGCAGAGCGATGATACTGAA-3' Rev 5'-CGTACTTGGGAGAACTCTTGATTGA-3'	55	30	EF431955
Ha-PDF	For 5'-GAGCTTGAGCTTAGTTCAGTAACTT-3' Rev 5'-AGCAACTGATTCCAATTTCC-3'	54	30	AF364865
Ha-GST	For 5'-CTCAGGATGCTTACGAGAAGGCTC-3' Rev 5'-CAACCAGGTTGATGTTCTCACCTCC-3'	55	28	AY667502
Ha-SCO	For 5'-TGTTCCTCTTAGTTCTCGCT-3' Rev 5'-ATAGCCCTCATGTTGACGAG-3'	50	28	AF364866
Ha-PAL	For 5'-TGGTGAAGTTGGGTGGCGAGACT-3' Rev 5'-GACCTTCTTTGGGCTGTAACTCGAA-3'	55	30	Y12461
Ha-NML1	For 5'-TAAGCTTCACCTCATCTTCACAC-3' Rev 5'-GCTTACTGTCCTTCTCTACACAA-3'	50	31	AY640383
Ha-ACC	For 5'-ACCATGGGATTCCTCATGATTTA-3' Rev 5'-CTCCAACCCAAGATTCTCACATA-3'	54	30	L29405
Ha-CHOX	For 5'-ACAACGTTGGAGTCGAAGTTCTT-3' Rev 5'-GGAGAACCTCAAGTCTGAGTTTC-3'	55	29	AF472609
Ha-CUA1	For 5'-CTGAGCCATAGTTTCAAGGG-3' Rev 5'-ACTGGTTCGATCTAGCAA TG-3'	50	30	AF178635
Ha-EF-1α	For 5'- TCTTCTTGCTTTCACCCTCGG-3' Rev 5'- GATCACACCAGTCTCAACACGTCCC-3'	55	25	AY094064

Table 3.1. Primer sequences, Tm (ºC), and number of cycles used for polymerase chain reaction. For: Forward primer; and Rev: Reverse primer.

3.4 Results

3.4.1 Detection of *VdNEP* in genomic DNA using PCR

VdNEP was amplified using specific primers in ten *V. dahliae* isolates, six from sunflower and four from potato (Fig. 3.1). All 10 isolates were positive for *VdNEP* amplification, showing a band with the size of 834 bp and no corresponding band was amplified in the negative control. The amplification products from all 10 *V. dahliae* isolates were sequenced. High sequence similarities (~98%) were found among *VdNEPs* amplified from the different *V. dahliae* isolates.

3.4.2 *VdNEP* over time expression in *V. dahliae*

The expression of *VdNEP* was compared in two highly and two weakly aggressive isolates of *V. dahliae* using RT-PCR (Fig. 3.2). In the highly aggressive isolate 1396-9, *VdNEP* was expressed in cultures since 3 days after media inoculation (d.a.i.) but the highest level was found after 40 days. In the other highly aggressive isolate 1398-21, *VdNEP* was significantly expressed at 3, 7 and 40 d.a.i. In comparison, the expression of *VdNEP* in the two weakly aggressive isolates, Vs06-07 and Vs06-14, was visibly lower than in the highly aggressive counterparts (Fig. 3.2).

3.4.3 *VdNEP* Expression in *Escherichia coli*

The insertion of *VdNEP* in the pET-32a (+) vector was confirmed using enzymatic digestion. Fragments corresponding to the *VdNEP* insert (648 bp) and the empty pET-32a

(+) vector (5.9 Kb) were observed after the construct was digested with *EcoR* I and *NOT* I, indicating that *VdNEP* was successfully inserted into pET-32a (+) (Fig. 3.3a). The expression of His-VdNEP was induced with 1 mM IPTG for 12 hours at 20°C and the protein extract was analyzed using SDS-PAGE. His-VdNEP of 383 amino acid residues appeared as a sharp band with an estimated molecular mass of 42 kDa in the electrophoresis (Fig. 3.3b). The concentration of His-VdNEP was estimated to be 2,276 µg/ml (Fig. 3.3c).

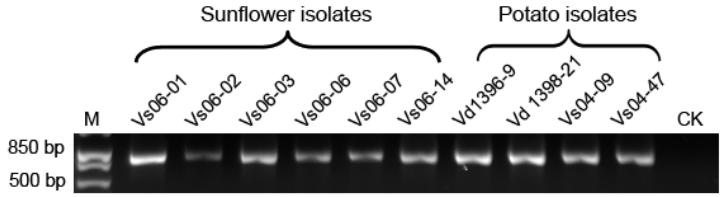

Figure 3.1. PCR amplification of *VdNEP* from 10 *V. dahliae* isolates. Lane M represents DNA ladder and Lane CK is the negative control with master mixture and no template DNA.

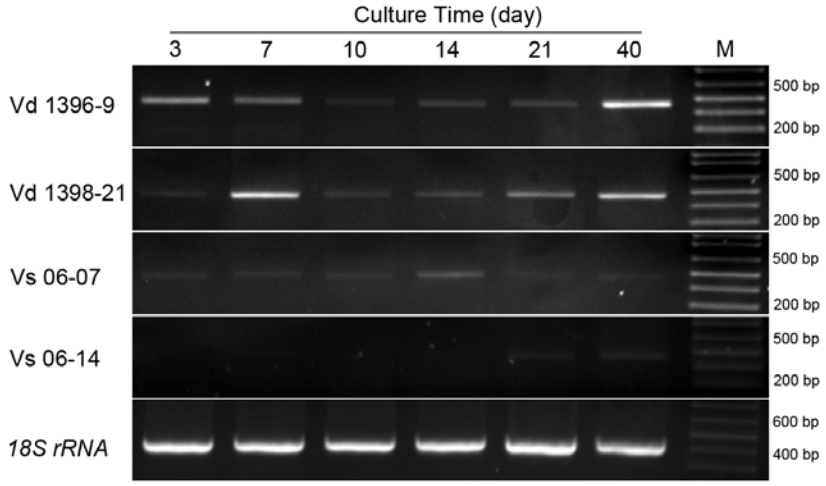

Figure 3.2. RT-PCR analysis about expressions of *VdNEP* in *V. dahliae* cultured from 3 to 40 days. *V. dahliae* Vd1396-9 and Vd1398-21 are highly aggressive on sunflower whereas Vs06-07 and Vs06-14 are weakly aggressive isolates. Lane M represents DNA ladder. Transcripts of *18S of V. dahliae* were amplified by 26 cycles of PCR and used an internal control. *VdNEP* was amplified by 30 cycles.

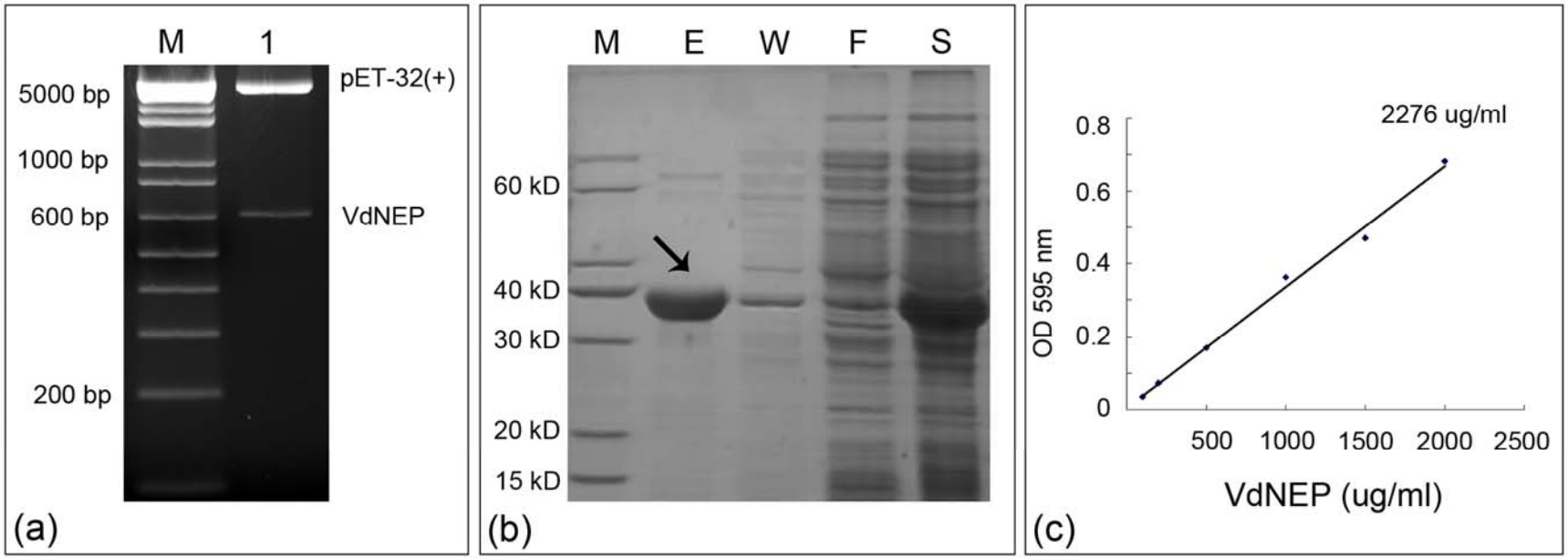

Figure 3.3. Double digestion of pET-32a (+) with *VdNEP* insert by restriction enzymes *EcoR* I and *NOT* I (a); SDS-PAGE of VdNEP protein expressed in pET-32a (+) / BL21 *E. coli* strain (b); Lane M represents the protein size standard in kDa; lane S shows total protein extracts from supernatant of BL21 after sonification; Lane F shows protein extracts flowing through from His Bond Resin; Lane W represents protein extracts washed by 20 mM imidazole and Lane E shows purified His-VdNEP protein eluted by 250 mM imidazole; The concentration of His-VdNEP is 2276 ug/ml (c).

3.4.4 Analysis of native VdNEP protein in *V. dahliae* by Western blot

The expression of native VdNEP protein in the highly aggressive isolate Vd1396-9 was analyzed by western blot using a VdNEP-specific polyclonal antibody. VdNEP was expressed both in the mycelium and the liquid media after 14 days (Fig. 3.4), indicating that it is secreted and present both in fungal cells and their extracellular space.

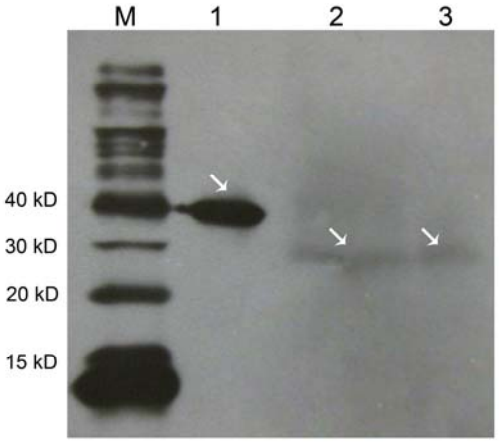

Figure 3.4. Western blot about the secretion of VdNEP in highly aggressive isolate Vd 1396-9 cultured for 14 days. Lane M shows the size standards in kDa; Lane 1 represents purified His-VdNEP (100 ng) used as the positive control; Lane 2 and 3 are VdNEP proteins extracted from liquid culture and mycelium of Vd 1396-9, respectively.

3.4.5 Comparison of *V. dahliae* and VdNEP effects on sunflower growth

Growth parameters, i.e., plant height, shoot fresh/dry weight and root fresh/dry weight were measured at 5 w.a.i.. Compared to the control and His-VdNEP-treated sunflower plants, significant reductions in plant height (Fig. 3.5a) and shoot fresh/dry weight (Fig. 3.5b-c), and high disease severity (Fig. 3.5g-h) were recorded in both cultivars IS6111 and IS8048 inoculated with each *V. dahliae* isolate. Interestingly, VdNEP increased both root fresh and dry weight in moderately resistant sunflower cultivar IS6111 but not in the susceptible IS8048. As a result, 1.5-fold increase in dry root weight was recorded in VdNEP-treated IS6111 plants compared to the controls (Fig. 3.5d-e). Among the four *V. dahliae* isolates, Vd1396-9 was the most aggressive and

reduced plant height, shoot fresh/dry weight and root fresh/dry weight by 37.5%, 38.4/57.0%, 63.5/69.0%, respectively, in moderately resistant cultivar IS6111.

3.4.6 Comparison of disease severity and vascular discoloration caused by *V. dahliae* and VdNEP

Disease severity in sunflower cultivars IS6111 and IS8048 inoculated with each of the four *V. dahliae* isolates or His-VdNEP were measured from 2 to 5 w.a.i.. V-shaped or mottled lesions appeared on lower leaves, and were first observed 2 w.a.i.. Disease severity was highest 5 w.a.i., with symptoms including chlorosis, necrosis, leaf defoliation and plant stunting. Disease was more severe on susceptible cultivar IS8048 than on moderately resistant IS6111. Among the four *V. dahliae* isolates assessed, Vd1396-9 caused the highest disease severity in both cultivars whereas Vs06-14 only caused slight damage in susceptible cultivar IS8048. VdNEP caused chlorosis and necrosis lesions at the edge of sunflower leaves. The margins of VdNEP-treated leaves turned yellow and curled. Disease severity and symptoms caused by VdNEP were similar to those caused by the weakly aggressive *V. dahliae* isolate Vs06-14 (Fig. 3.5g-h).

Vascular discoloration, one of the most important criteria to assess disease severity of *Verticillium* wilt, was also evaluated, 5 w.a.i, in lower stem sections of plants inoculated with *V. dahliae* or infiltrated with VdNEP. Vascular discoloration was observed in most infected plants but was greatest with Vd1396-9 (Fig. 3.5f). In comparison, the degree of vascular discoloration in sunflower inoculated with VdNEP was similar to that caused by the two weakly aggressive *V. dahliae* isolates Vs06-07 and Vs06-14 (Fig. 3.5f).

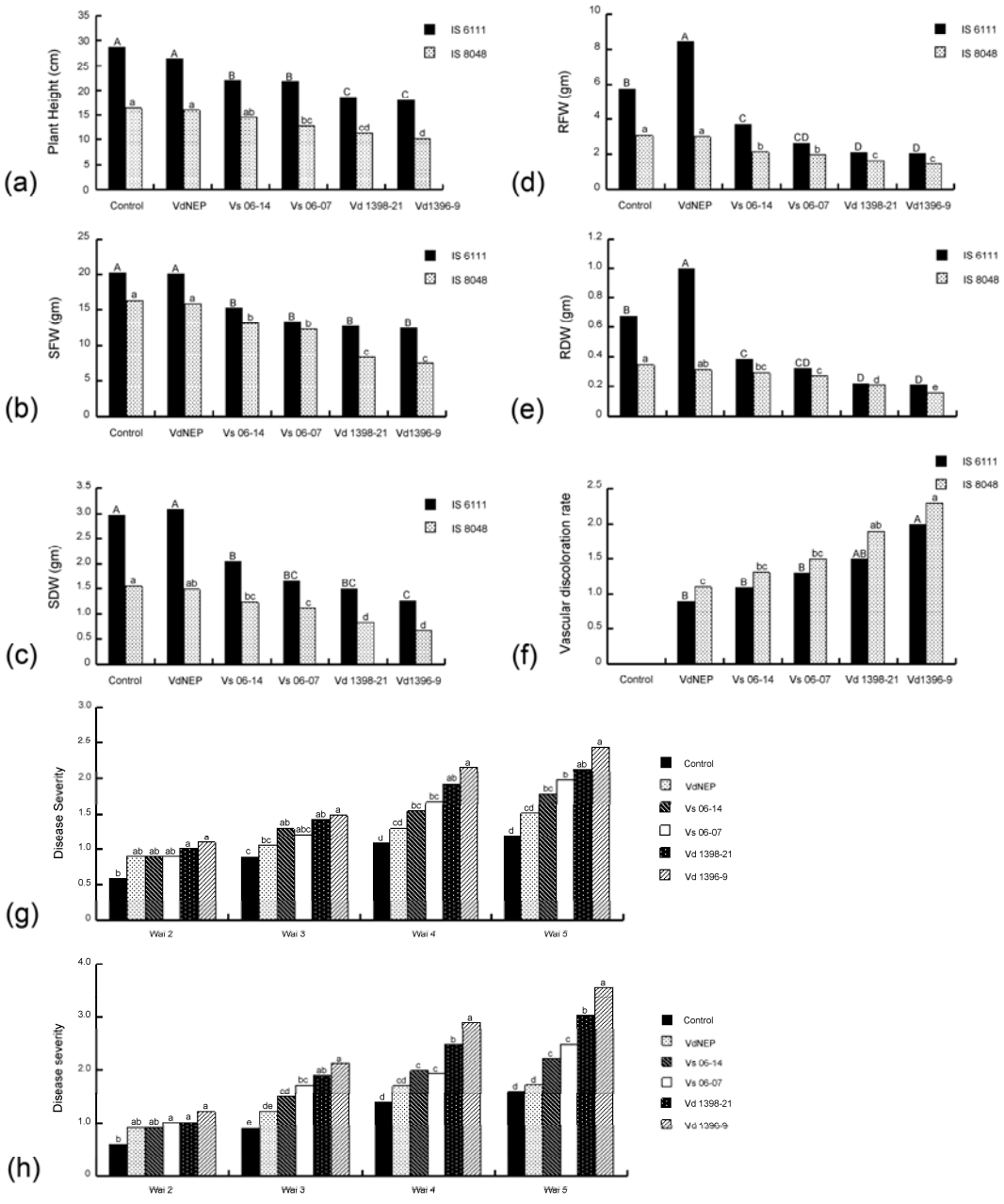

Figure 3.5. The effect of *V. dahliae* and His-VdNEP on the height of sunflower (a), shoot fresh weight (b), shoot dry weight (c), root fresh weight (d) and root dry weight (e); Vascular discoloration caused by *V. dahliae* and His-VdNEP (f); Disease severity caused by *V. dahliae* and His-VdNEP in moderately resistant sunflower IS6111 (g) and susceptible sunflower IS8048 (h). w.a.i. represents weeks after inoculation.

We have also estimated the percentage of infected leaf area (chlorosis and necrosis) in sunflower plants inoculated with VdNEP and *V. dahliae* isolates using the image analysis software Assess. VdNEP caused 8.08 and 9.13 % infected leaf area in IS6111 and IS8048, respectively, at 3 w.a.i.. This was similar to those caused by Vs06-14 at 3 w.a.i., estimated at 8.49 and 10.52 % leaf chlorosis and necrosis in IS6111 and IS8048, respectively (Fig. 3.6a-b)

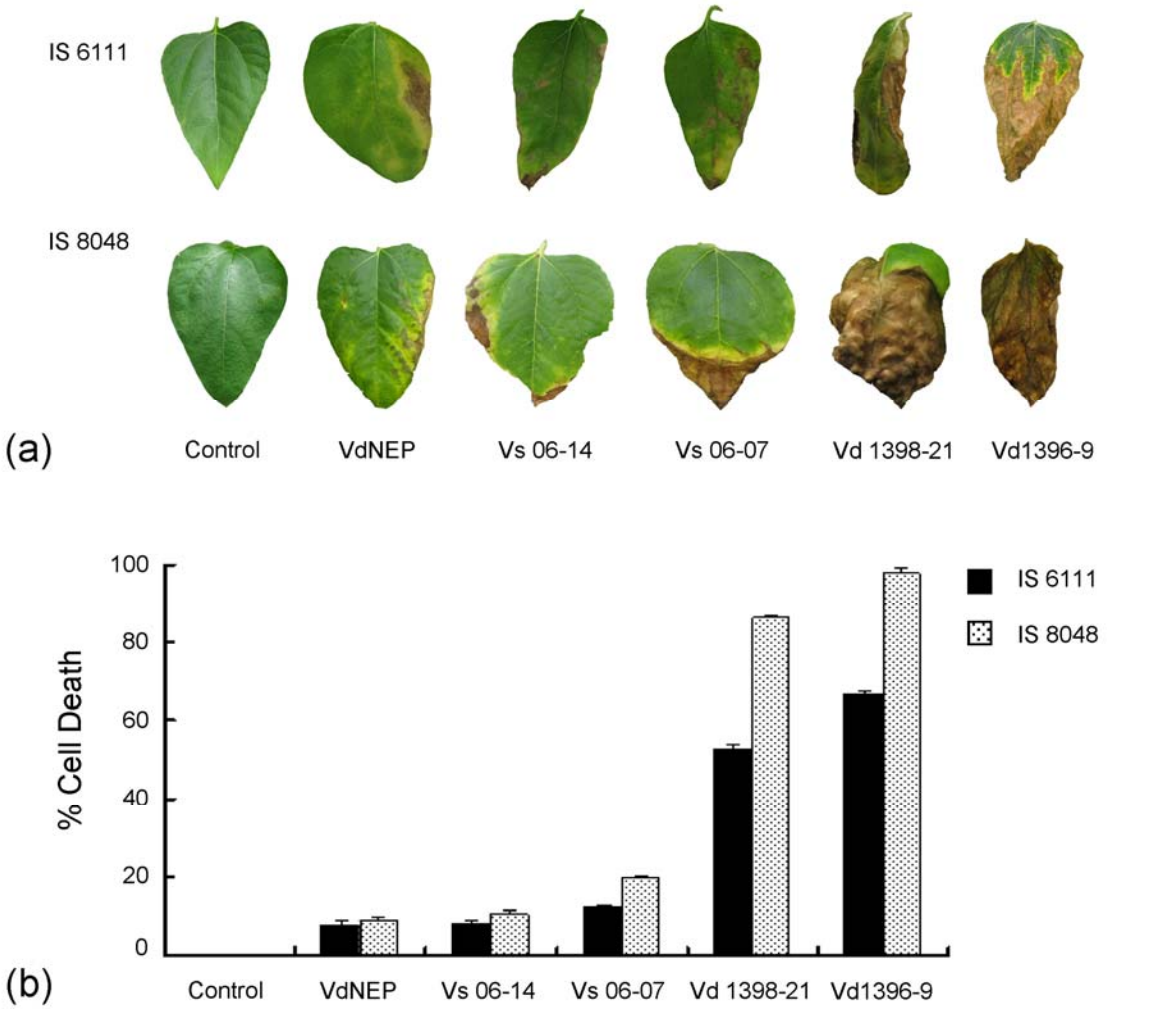

Figure 3.6. Chlorosis and necrosis in sunflower cultivars IS6111 and IS8048 induced by His-VdNEP (20 µg/ml) and *V. dahliae* isolates 3 weeks after the inoculation (w.a.i.) by root dipping (a); Percentage of cell death in sunflower leaves treated with *V. dahliae* and VdNEP (b).

3.4.7 VdNEP induced HR, DNA laddering and oxidative burst

Hypersensitive response (HR) was observed in *N. benthamiana* leaves 24 hours after infiltration with His-VdNEP at concentrations from 10 to 2000 μg/ml (Fig. 3.7a). Similarly, HR also occurred in sunflower cotyledons 12 hours post-infiltration with His-VdNEP (20 μg/ml) (Fig. 3.7b). DNA laddering, which is a characteristic feature of programmed cell death, was also observed in DNA from sunflower leaves infiltrated with His-VdNEP (Fig. 3.7c). In comparison, His tag alone or sterile water did not induce HR or DNA laddering in sunflower plants. These results indicated the occurrence of programmed cell death in sunflower leaves treated with His-VdNEP.

In addition to the hypersensitive response, the accumulation of fluorescent compounds was also observed in sunflower leaves infiltrated with His-VdNEP 12 hours after infiltration (h.a.i.), which was not observed in control leaves (Fig. 3.7d). Furthermore, His-VdNEP also induced the production of reactive oxygen species (ROS) in sunflower leaves, which showed reddish coloration after staining with 3, 3-diaminobenzidine (DAB) solution (Fig. 3.7e-f). No accumulation of fluorescent compounds or production of ROS was observed in control plants treated with His-tag protein (20 μg/ml).

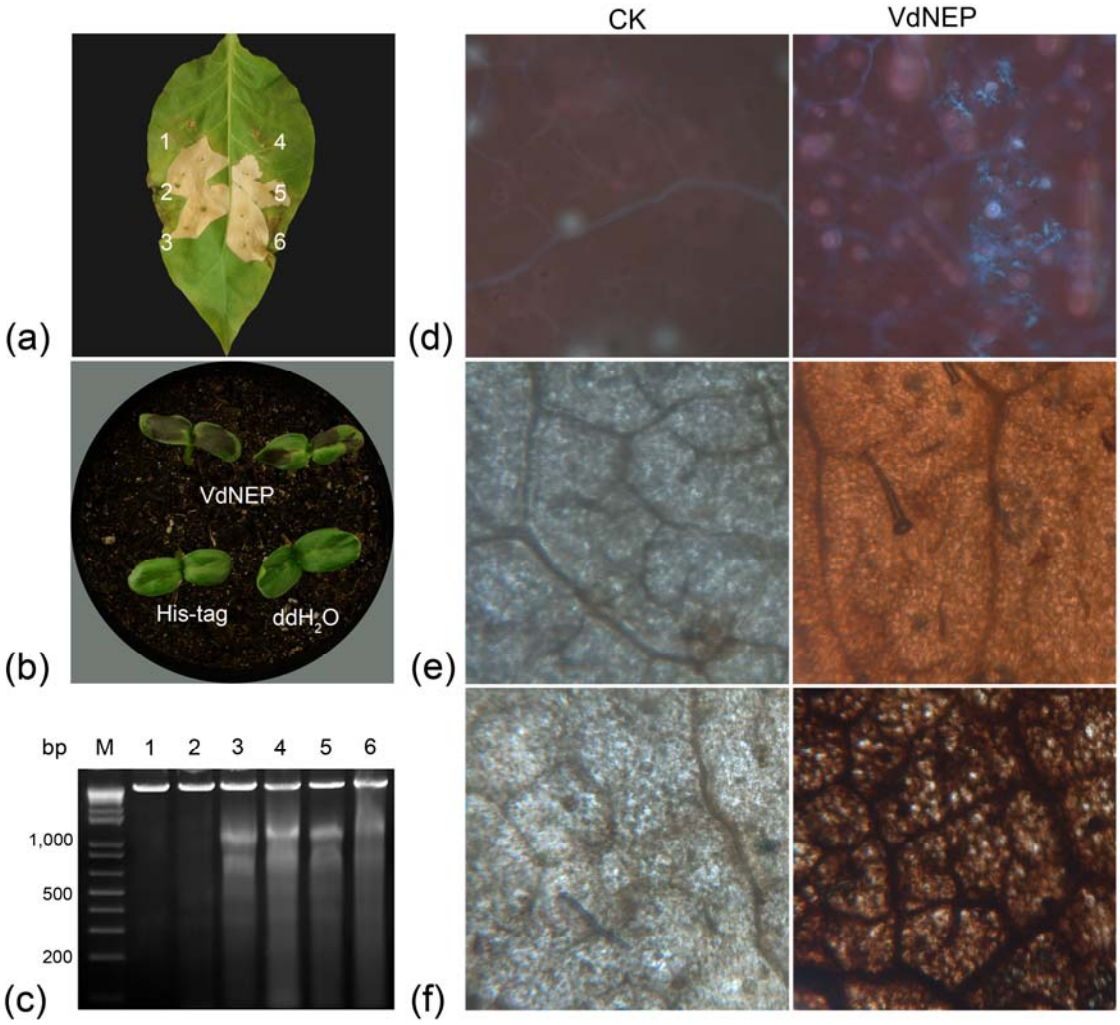

Figure 3.7. Hypersensitive response in *N. benthamiana* leaves (a) and sunflower cotyledons (b) induced by His-VdNEP 24 hour after infiltration; a1 and a4 are negative controls treated with DDW or His tag protein (20 μg/ml); a2, a3, a5 or a6 are treatments with 10, 20, 200 or 2000 μg/ml His-VdNEP; DNA laddering of 180-200 bp DNA fragments in sunflower leaves inoculated with His-VdNEP (c); Lanes: M, 1 Kb Plus DNA Ladder; 1 or 2, negative control treated with DDW or His tag protein (20 μg/ml); Lane 3, 4, 5 or 6, treatments with His-VdNEP at 0.05, 0.5, 5 or 20 μg/ml, respectively; Accumulations of fluorescent compounds (d) and oxidative burst (e, f) in sunflower leaves inoculated with His-VdNEP. The production of reactive oxygen species activated by His-VdNEP (20 μg/ml) were examined by 3,3-diaminobenzidine staining 6 hours after infiltration; CK are sunflower leaves treated with His tag protein (20 μg/ml).

3.4.8 VdNEP affected the expression of defense and defense signaling genes in sunflower

Expression of *pathogenesis-related* (*PR*) genes: *Ha-Chi* (*PR3*) and *Ha-PR5* (Fig. 3.8a-b)

RT-PCR analysis showed a 2.6-fold increase of *Ha-Chi* (*PR3*) transcripts in VdNEP-treated sunflower leaves since 6 h.a.i. and kept high until 7 d.a.i.. In comparison, it reached its maximum at 3 d.a.i. in the control plants. As such, wounding of control plants caused by root-dipping in SDW also induced the accumulation of *Ha-Chi* transcripts. Early after infiltration, *Ha-PR5* transcripts accumulated in the same way as *Ha-Chi* (*PR3*). Compared to the control, the significant increase in *Ha-PR5* transcripts was observed in VdNEP-treated sunflower as early as 6 h.a.i. and reached three-fold increase at 12 h.a.i. The expression of *Ha-PR5* declined to levels similar to that found in the control from 2 to 3 d.a.i, and then increased again at 7 d.a.i.

Expression of the defensin genes *Ha-PDF* and *Ha-CUA1* (Fig. 3.8c-d)

The expression of *Ha-PDF* was relatively low and that of *Ha-CUA1* was almost undetectable in control plants. Infiltration with VdNEP resulted in a substantial up-regulation of both genes starting at 3 d.a.i. (3.1-fold and 3.8-fold increase in *Ha-PDF* and *Ha-CUA1* transcripts, respectively).

Rapid induction of *Ha-ACO* encoding ethylene synthesis enzyme (Fig. 3.8e)

ACC oxidase (ACO) is one of the most important enzymes involved in ET synthesis. Transcripts of *Ha-ACO* were virtually undetectable before treatment with VdNEP but

their accumulation was dramatically up-regulated with elicitation as early as 6 h.a.i. The accumulation of *Ha-ACO* in VdNEP-treated plants reached its maximum 12 h.a.i., showing a 3-fold increase compared to control plants, then declined to a level similar to that found in the control. At 3 d.a.i., these transcripts increased in response to both infiltration wounding and VdNEP.

Expression of *Ha-CHOX* and *Ha-GST* and *Ha-SCO* involved in AOS (Fig. 3.8f-h)

Ha-CHOX was significantly up-regulated in leaves of VdNEP-treated sunflower from 3 d.a.i. to 7 d.a.i., whereas its expression was relatively low in control sunflower leaves. The highest level of expression was detected at 3 d.a.i.. In comparison, the up-regulation of *Ha-GST* occurred 6 h.a.i., with a 1.9-fold increase that reached a maximum 3 d.a.i. Similarly, the expression of *Ha-SCO* was also up-regulated in VdNEP-treated sunflower leaves. The induced expression of *Ha-CHOX*, *Ha-GST* and *Ha-SCO* coincided with the production of AOS in VdNEP-treated sunflower leaves (Fig. 3.7e-f).

Interference with *Ha-PAL* and *Ha-NML1* (Fig. 3.8i-j)

Compared to the control plants, the expression of *Ha-PAL* and *Ha-NML1* was lower in VdNEP-treated sunflower leaves 24 and 48 h.a.i.. The levels of these transcripts kept increasing and decreasing in both treatments, but never exceeded those at time T0.

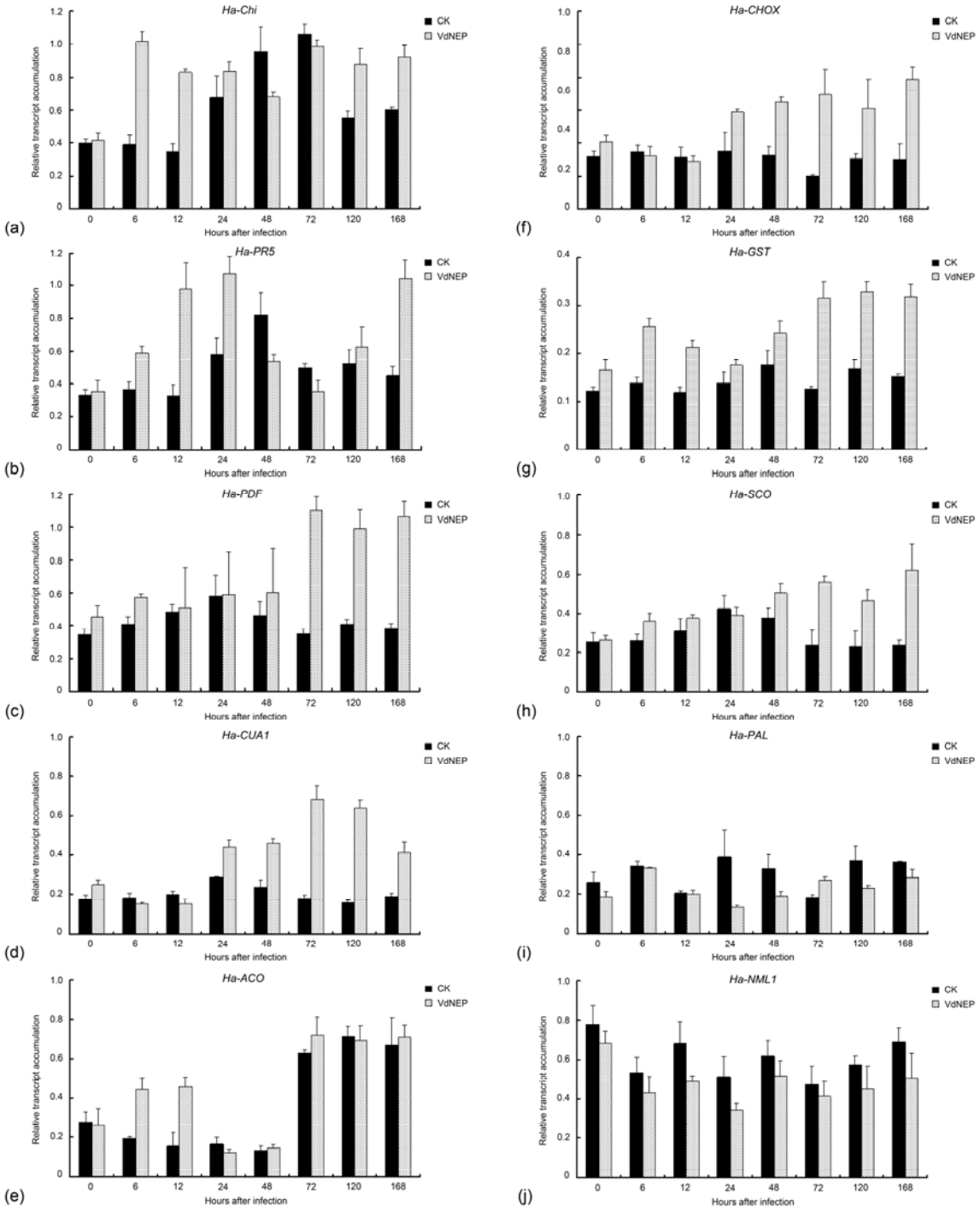

Figure 3.8. Relative expression of *Ha-Chi* (a), *Ha-PR-5* (b), *Ha-PDF* (c), *Ha-CUA1* (d), *Ha-ACO* (e), *Ha-CHOX* (f), *Ha-GST* (g), *Ha-SCO* (h), *Ha-PAL* (i) and *Ha-NML1* (j) in sunflower plants inoculated with His-VdNEP (20 µg/ml). CK represent controls inoculated with water. Leaves were collected from 0 to 168 hours after inoculation (h.a.i.).

3.5 Discussion

NLPs from diverse organisms share a high degree of sequence similarity and were reported to play important roles in pathogenesis (Pemberton & Salmond, 2004). In the present study, *VdNEP* was amplified from all ten *V. dahliae* tested isolates and sequence analysis showed that *VdNEP* was highly conserved among these isolates. In addition, *VdNEP* from *V. dahliae* showed sequence identities of 73% with *NEP1* from *Fusarium oxysporum* and 67% with *NIE* from *Pythium monospermum*.

VdNEP expression was higher in two highly aggressive isolates (Vd1396-9 and Vd1398-21) than in two weakly aggressive ones (Vs06-07 and Vs06-14). Furthermore, the native VdNEP protein was detected not only in the mycelium but also in the liquid culture. *V. dahliae* genomic sequence analysis by Wang *et al*. (2004) had predicted VdNEP to be an extracellular protein, but our study is the first one to demonstrate that VdNEP is secreted out of *V. dahliae* cells. The presence of VdNEP in the extracellular space and its correlation with *Verticillium* wilt symptoms suggested that VdNEP is closely associated to the pathogenesis of this pathogen in sunflowers.

VdNEP (233 amino acids) includes two transmembrane domains and an N-terminal signal peptide that is removed during the processing (Wang *et al*., 2004). Bailey *et al*. (1997) reported that the production of extracellular proteins, including NLPs, was dependant on growth conditions such as the media used. For example, NEP1 production by wild type *F. oxysporum* or its *NEP1*-overexpressing isolates was nil in PDB media, but it was restored by addition of 1% CAA to the medium (Bailey *et al*., 1997).

To investigate the involvement of VdNEP in *Verticillium* wilt development, four *V. dahliae* isolates and His-VdNEP were used to inoculate/infiltrate sunflower plants. Compared to the control plants, inoculation of sunflower seedlings with *V. dahliae* isolates decreased plant height, fresh and dry shoot weight in both cultivars. These results were in agreement with the finding of Karagiannidis *et al*. (2002). Although there were no significant differences in these plant growth parameters between VdNEP-treated sunflower and control plants, VdNEP significantly increased fresh and dry root weight in moderately resistant sunflower cultivar (IS6111), while such increase was not observed in the susceptible sunflower cultivar (IS8048) treated with VdNEP. This is also in line with the higher level of IS6111 resistance to *V. dahliae*, and its differential reaction to VdNEP as compared to susceptible hybrid IS8048.

It was previously proposed that chlorosis and necrosis might be related to programmed cell death in host cells (Fellbrich *et al*., 2002; Veit *et al*., 2001) and that necrosis-inducing activity of NLPs correlated with pathogenicity. For example, NEP1 and PsojNIP from plant-pathogenic *Fusarium oxysporum* and *Phytophthora sojae* induced necrosis, while NLPs from non-plant pathogenic organisms, such as *Streptomyces coelicolor* and *Bacillus halodurans* were inactive or comparatively weak in necrosis-inducing activity (Qutob *et al*., 2002). These results suggest that VdNEP is involved in *Verticillium* pathogenesis and contributes to disease development. In our study, wilting symptoms and the disease severity caused by VdNEP leveled up with those caused by the weakly aggressive isolate Vs06-14. In addition, the percentage of cell death

caused by VdNEP in sunflower IS6111 was also similar to that caused by the two weakly aggressive isolates (Vs06-14: 8.49% and Vs06-07: 12.04%). Quantifying VdNEP *in situ* during the interaction of sunflower with *V. dahliae* and comparing its activity in presence of the highly *versus* the weakly aggressive isolates will be instrumental in establishing its role in differential pathogenicity levels. In this study, we were able to detect VdNEP in culture, but attempts to detect it in sunflower roots or stems infected with one highly aggressive isolate Vd1396-9 were not successful (data not shown). This might be due to low levels of VdNEP in these tissues or the low sensitivity of western blot tests. Nevertheless, we cannot rule out the possibility that the production and processing of VdNEP were limited and/or transient in plant tissues. Similarly, NEP1 was not observed in coca stems infected with *F. oxysporum NEP1*-overexpressing isolates (Bailey *et al*., 2002).

To date, the function of VdNEP in the pathogenicity of *V. dahliae* was unclear and whether VdNEP is critical to wilt development was not known. Bailey *et al*. (2002) found that the disruption of *NEP1* using gene replacement or the overexpression of *NEP1* did not affect the pathogenicity of *F. oxysporum* toward *Erythroxylum coca*. On the contrary, Madhosingh (1995) found that virulence of *F. oxysporum* isolates positively correlated with the wilt-inducing capability of their filtrates.

Treatment with a crude extract from *V. dahliae* induced plant defense responses such as the accumulation of pathogenesis-related proteins (Dubery & Slater, 1997; Mcfadden *et al*., 2001). Further elucidating different defense responses, including signaling, induced

by VdNEP in sunflower will help to better understand the role of VdNEP in the interaction between sunflower and *V. dahliae*. In the present study, VdNEP induced the hypersensitive response both on *N. benthamiana* leaves and sunflower cotyledons. The hypersensitive response is a rapid cell death in the local region surrounding the infection which is analogous to the innate immune system found in animals (Dangl *et al*., 1996). We also observed DNA laddering and the accumulation of fluorescent compounds in VdNEP-treated sunflower leaves. DNA laddering is a typical feature of programmed cell death that results into multiple 180-185 bp genomic DNA fragments (Ryerson & Heath, 1996). Furthermore, the production of active oxygen species (AOS) also occurred in sunflower leaves inoculated with VdNEP. Associated with the hypersensitive response, active oxygen species (AOS) are produced in many incompatible plant-pathogen interactions (Levine *et al*., 1994). A rapid and transient generation of AOS, known as the oxidative burst, often results in the reinforcement of cell walls which restricts fungal growth and limits the spread of pathogens (Levine *et al*., 1994). Our results are in agreement with the findings of Wang *et al*. (2004), which suggest that VdNEP may be one of the possible signals delivered by *V. dahlia*e during infection and perceived by the plant. Similarly, NLPs members, including NPP1, elicited rapid production of active oxygen species in *Arabidopsis* via an NADPH oxidase-independent pathway (Fellbrich *et al*., 2002). In this regard, VdNEP acted similarly to one well-described protein Harpin isolated from bacterial pathogens in *Erwinia* genus. Harpin is not only related to

pathogenicity (Bauer *et al*., 1995), but is also capable of inducing hypersensitive-like cell death and the production of AOS in many plant species (Wei *et al*., 1992).

By investigating the expression of defense and signaling genes induced by VdNEP, we also aimed to search which signaling/defense genes are important in the interaction between sunflower and *V. dahliae*. In this study, VdNEP up-regulated two pathogenesis-related genes (*Ha-PR-3,* and *Ha-PR-5*), two defensin genes (*Ha-PDF* and *Ha-CUA1*) and genes encoding *Ha-ACO*, *Ha-CHOX*, *Ha-GST* and *Ha-SCO*, suggesting that multiple signaling pathways were involved in the sunflower-*V. dahliae* interactions. *Ha-PAL* and *Ha-NML1*, which are key regulatory genes in induced resistance affected by salicylic acid (SA), were slightly down-regulated in presence of VdNEP. This may indicate that the SA signaling pathway is possibly targeted by *V. dahliae* in counterdefense in this interaction.

The expression of *Ha-Chi* (*PR3*), an antifungal protein, was highly induced by VdNEP when compared to the control plant. Similarly, *Ha-PR5*, sharing high sequence similarity with published PR5 family members such as osmotin and thaumatin (Graham *et al*., 1992), was significantly up-regulated in sunflower 6 to 12h after VdNEP treatment. PR5-like proteins were demonstrated to have high antifungal activity against a variety of fungi (Liu *et al*., 1994).

Sunflower defensin genes *Ha-PDF* and *Ha-CUA1* were both up-regulated in VdNEP-treated sunflower leaves and the highest level of expression was at 72 hour after VdNEP treatment. *PDF1.2* from *Arabidopsis* is a marker gene in JA/ET-dependant

signaling pathways and its expression is SA-independent (Kachroo *et al*., 2003). Although it is still not clear whether *Ha-PDF* and *Ha-CUA1* have the same function in sunflower, the up-regulation of *Ha-PDF* in sunflower treated with VdNEP suggests that JA/ET signaling pathways are important in sunflower-*V*. *dahliae* interaction. Previous studies have implied that JA contributed to plant resistance against *V*. *dahliae*, since JA-deficient tomato and JA-insensitive *Arabidopsis* plants were much more susceptible to *Verticillium* infections (Thaler *et al*., 2004; Tjamos *et al*., 2005).

It was well established that the production of ethylene increased rapidly in plants upon the infection of *Verticillium* or the treatment with NLPs (Pegg & Cronshaw, 1976). For instance, Jennings *et al*. (2000) found that NEP1 up-regulated ACC synthase and ACC oxidase, which were two key enzymes in the biosynthesis of ethylene. In addition, Wang *et al*. (2004) found that transcripts of ACS6, a key enzyme for ethylene biosynthesis, were up-regulated in *Arabidopsis* leaves infiltrated with His-VdNEP. It is not surprising that 2.9-fold increase in *Ha-ACO* transcripts was found in VdNEP-treated sunflower leaves 6 h after treatment, which indicated that ethylene signaling pathway was involved in the interaction between sunflower and *V*. *dahliae*.

There has been a long debate regarding the role of ethylene in disease resistance. For example, ethylene-insensitive *Arabidopsis* showed more susceptibility to *Botrytis cinerea* compared to wild type (Thomma *et al*., 1999). On the other hand, it was also reported that ethylene was involved in the symptom development of *Verticillium* wilt (Robinson *et al*., 2001). Transgenic tomato expressing an ACC deaminase, which inhibited ethylene

synthesis and *Arabidopsis* ethylene-deficient mutants, displayed less symptoms upon *Verticillium* inoculation (Robinson *et al*., 2001; Tjamos *et al*., 2005).

Ha-CHOX encodes a carbohydrate oxidase in sunflower. The transgenic tobacco expressing *Ha-CHOX* displayed enhanced resistance against *Pectobacterium carotovorum* (Custers *et al*., 2004). *Ha-GST* encodes a Glutathione S-transferase which has been implicated in numerous stress responses, including those arising from pathogen attacks or oxidative stress (Marrs, 1996). *Ha-SCO,* which also encodes a carbohydrate oxidase, has high homology to an antifungal protein from *Arabidopsis* (Stuiver *et al*., 2000) and a berberine bridge enzyme involved in benzophenanthridine alkaloids pathway (Dittrich & Kutchan, 1991). A previous study found that carbohydrate oxidase or berberine bridge enzyme can produce AOS (Dittrich & Kutchan, 1991), suggesting that *Ha-SCO* may also elevate rapid production of AOS. In our study, *Ha-CHOX, Ha-GST* and *Ha-SCO* were all significantly up-regulated by VdNEP. The up-regulation of these genes was in agreement with the rapid production of AOS observed in sunflower leaves treated with VdNEP. These results suggest that the production of AOS is very important in the interaction between *V. dahliae* and sunflower. Since H_2O_2 acts as the primary signal to stimulate the transcription of GST (Tenhaken *et al*., 1995), it is possible that *Ha-GST* acts downstream of *Ha-CHOX*.

SA signaling is involved in host defense responses in many plant-pathogen interactions (Hückelhoven *et al*., 1999). Two related genes *Ha-PAL* and *Ha-NML1* were slightly down-regulated by VdNEP in sunflower. PAL (phenylalanine ammonia lyase) is

a key enzyme in phenylpropanoid pathway, inducing the production of defense related plant secondary metabolites such as SA, phytoalexins, and lignin-like polymers (Kohler *et al*., 2002). In the first step of SA synthesis, trans-Cinnamic acid is produced by L-phenylalanine, catalyzed by PAL. *NML1* (*NIM1*/*NPR1*-like 1) shares high sequence similarity with *NPR1*, which acts downstream of SA in the SAR signal transduction pathway (Cao *et al*., 1994). SA pretreatment did not enhance resistance of *Arabidopsis* against *V. dahliae* infection compared with the wild-type plants (Veronese *et al*., 2003). However, the slight down-regulation of *Ha-PAL* and *Ha-NML1* may suggest that sunflower resistance against *Verticillium* wilt might not be completely SA-independent.

In summary, the sunflower-*V. dahliae* interaction is very complex and the function of VdNEP is double-sided. VdNEP was involved in the development of *Verticillium* wilt as a pathogenicity factor, causing chlorosis, necrosis, vascular discoloration, and eventually cell death in sunflower plants. Such symptoms didn't exceed the levels induced by the weakly aggressive isolates, which may indicate that the highly aggressive isolate produce higher concentrations of VdNEP. On the other hand, VdNEP also acted as an elicitor activating a variety of defense responses against *V. dahliae*, including induction of HR, oxidative burst, and accumulation of PR proteins. Based on the results presented here, it is difficult to classify VdNEP only as a pathogenicity factor or an elicitor. However, it may be speculated that the activity of VdNEP is dose-dependent. These studies provide a foundation to further better understand the interaction between sunflower and *V. dahliae*. Further analyses of VdNEP and concurrently produced

effectors *in planta* will provide more insights into the mechanisms of host-pathogen interactions involving *V. dahliae*.

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4.0 GENERAL DISCUSSION AND CONCLUSION

4.1 VdNEP acted as a pathogenicity factor

Verticillium wilt, caused by soil-borne fungus *V*. *dahliae*, is an important vascular disease in sunflower (Hoes, 1972). Previous studies suggested that toxins or phytotoxic factors produced by *V*. *dahliae* were the primary agents of wilting symptom (Nachmias et al., 1982). In the present study, we found that *VdNEP* (*V*. *dahliae* necrosis and ethylene-inducing protein) was highly conserved in the genome of *V*. *dahliae* which indicated that *VdNEP* was unique and essential during gene evolution, which may play important roles in the pathogen (Pemberton and Salmond, 2004). The expression of *VdNEP* was higher in two highly aggressive isolates (Vd1396-9 and Vd1398-21) than in two weakly aggressive ones (Vs06-07 and Vs06-14). In addition, VdNEP caused typical *Verticillum* wilt symptoms on infiltrated sunflower plants, including leaf vein browning, chlorosis, necrosis, stunting and vascular discoloration. The disease severity and symptoms caused by VdNEP were comparable to that cause by the weakly aggressive *V*. *dahliae* isolate Vs06-14. Furthermore, VdNEP was present in both fungal cells and extracellular space through western blot. All these results suggest that VdNEP acts as a pathogenicity factor and contributed to the *Verticillium* wilt symptom development in sunflower.

4.2 VdNEP acted as an elicitor

In addition to being phytotoxic, VdNEP also functioned as an elicitor, which triggered complex defense responses in sunflower. VdNEP induced the hypersensitive response (HR) in *N. benthamiana* leave and sunflower cotyledons. HR is one of the most common defense responses associated with incompatibility, appeared as a rapid cell death in the local region surrounding the infection (Dangl et al., 1996). Associated with hypersensitive cell death, we also observed the production of active oxygen species, DNA laddering, and accumulation of fluorescent in VdNEP infiltrated sunflower. Furthermore, VdNEP up-regulated the accumulation of pathogenesis related proteins, including antifungal proteins chitinase (PR-3) and osmotin (PR-5).

4.3 Defense signalling pathways induced by VdNEP in sunflower

In the present study, VdNEP rapidly induced the expression of defense and signaling genes, including two defensin genes (*Ha-PDF* and *Ha-CUA1*) and genes involved in ethylene synthesis and active oxygen species, such as *Ha-ACO*, *Ha-CHOX*, *Ha-GST* and *Ha-SCO*. *Ha-PDF* is a marker gene in JA/ET-dependant signaling pathways and its expression is SA-independent (Kachroo et al., 2003). *Ha-ACO* encodes ACC oxidase, which is a key enzyme in the biosynthesis of ethylene (Wang et al., 2002). The up-regulation of marker genes in both JA/ET-dependant and ethylene dependant signaling pathway could suggest that both signaling pathways were activated by VdNEP. Previous studies have implied that JA signaling pathway is significant to plant resistance

against *V. dahliae*, for example, JA-deficient tomato were more susceptible to *Verticillium* infections (Thaler et al., 2004).

VdNEP treatment also induced the expression of *Ha-CHOX*, *Ha-GST and Ha-SCO*. *Ha-CHOX* and *Ha-SCO* encode the carbohydrate oxidase and *Ha-GST* encodes a Glutathione S-transferase (Custers et al., 2004; Stuiver et al., 2000; Marrs, 1996). Both enzymes are involved in the production of reactive oxygen species which is one of the earliest events of plant defense responses induced by pathogens and abiotic stress (Lamb and Dixon, 1997). These results suggest that the production of AOS is very important in the interaction between *V. dahliae* and sunflower. Since H_2O_2 acts as the primary signal to stimulate the transcription of GST (Tenhaken et al., 1995), it is possible that *Ha-GST* acts downstream of *Ha-CHOX*.

Although SA signaling has been reported to be involved in host defense responses in many plant-pathogen interactions, two genes (*Ha-PAL* and *Ha-NML1*) involved in SA signaling pathway, were slightly suppressed after infiltration with VdNEP. Similarly, Veronese et al. (2003) reported that pretreatment with SA did not enhance resistance of *Arabidopsis* against *V. dahliae* infection compared with wild-type plants. The slight down-regulation of *Ha-PAL* and *Ha-NML1* could suggest that sunflower resistance against *Verticillium* wilt might be SA-independent.

4.4. Conclusions and future perspectives

Sunflower-*V. dahliae* interaction is very complex. VdNEP acted not only as a pathogenicity factor causing disease symptom of *Verticillium* wilt but also triggered multiple defense responses against *V. dahliae* as an elicitor. Both JA/ET-dependant and ethylene dependant signaling pathways were activated by VdNEP. The defense signaling pathways induced by VdNEP seemed to be SA-independent. Our results suggest that VdNEP is very important in the interaction between sunflower and *V. dahliae*. Future studies such as site directed mutagenesis, *in situ* hybridization and genome-wide microarray analysis can further demonstrate the role of VdNEP in the pathogenesis of *V. dahliae* and in the interaction between this fungal pathogen and its hosts.

5.0 LITERATURE CITED

AAFC (Agriculture and Agri-Food Canada). (2005). http://www.agr.gc.ca/index_e.php

Agrawal, G.K., Rakwal, R., Jwa, N.S. and Agrawal, V.P. (2001). Signaling molecules and blast pathogen attack activates rice *OsPR1a* and *OsPR1b* genes, a model illustrating components participating during defense/stress responses, *Plant Physiol. Biochem*., 39:1095-1103.

Agrios, G.N. (2005). Plant Pathology. 5thed. Elsevier Academic Press, San Diego. CA. USA. pp. 526-527.

Alkher, H., El Hadrami, A., Rashid, K.Y., Adam, L.R. and Daayf, F. (2009). *Verticillium dahliae* cross pathogenicity between potato and sunflower, *Eur. J. Plant Pathol*., 124:505-519.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*, *Science*, 284:2148-2152.

Alvarez, M.E., Pennel, R.I., Meijer, P., Ishikawa, A., Dixon, R.A. and Lamb. C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity, *Cell*, 92:773-784.

Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C, Maclean, D.J., Ebert, P.R. and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signalling pathways modulates defense gene expression and disease resistance in *Arabidopsis*, *Plant Cell*, 16:3460-3479.

Andresen, I., Becker, W., Schlüter, K., Burges, J., Parthier, B. and Apel, K. (1992). The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (Hordeum vulgare), *Plant Mol. Biol*., 19:193-204.

Apostol, I., Heinstein, P.F. and Low, P.S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: Role in defense and signal transduction, *Plant Physiol*., 90:109-116.

Audenaert, K., De Meyer, G.B. and Hofte, M.M. (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms, *Plant Physiol*., 128:491-501.

Bailey, B.A. (1995). Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*, *J. Phytopathol*., 85:1250-1255.

Bailey, B.A., Apel-Birkhold, P.C. and Luster, D.G. (2002). Expression of NEP1 by *Fusarium oxysporum* f. sp. *erythroxyli* after gene replacement and overexpression using polyethylene glycol-mediated transformation, *J. Phytopathol*., 92:833-841.

Bailey, B.A., Jennings, J.C. and Anderson, J.D. (1997).The 24-kDa protein from *Fusarium oxysporum* f. sp. *erythroxyli*: occurrence in related fungi and the effect of growth medium on its production, *Can. J. Microbiol*., 43:45-55.

Barbara, D. and Clewes, E. (2003). Plant pathogenic *Verticillium* species: how many of them are there? *Mol. Plant Pathol*., 4:297-305.

Bauer, D.W., Wei, Z.M., Beer, S.V. and Collmer, A. (1995). *Erwinia chrysanthemi* HarpinEch: an elicitor of the hypersensitive response that contributes to soft-rot pathogenesis, *Mol. Plant-Microbe Interact*., 8:484-491.

Beckman, C.H. and Talboys P.W. (1981). Anatomy of resistance. In Fungal wilt diseases of plants (Mace, M.C., Bell, A.A and Beckman, C.H. eds.), Academic Press, New York. pp. 487-521.

Benta, F., Innesr, W., Ecker, J.R. and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens, *Mol. Plant-Microbe Interact.*, 5:372-378.

Berg, G. (1996). Rhizobacteria of oilseed rape antagonistic to *Verticillium dahliae* var *longisporum* Stark, *J. Plant Dis. Prot.*, 103:20-30.

Berrocal-Lobo, M., Molina, A. and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi, *Plant J.*, 29:23-32.

Bhat, T.G. and Subbarao, K.V. (1999). Host range specificity in *Verticillium dahliae*, *J. Phytopathol.*, 89:1218-1225.

Bi, Y.M., Kenton, P., Mur, L., Darby, R. and Draper, J. (1995). Hydrogen peroxide does not function down-stream of salicylic acid in the induction of PR protein expression, *Plant J.*, 8:235-245.

Blechert, S., Brodschelm, W., Hölder, S., Kammerer, L., Kutchan, T.M., Mueller, M.J., Xia, Z.Q. and Zenk, M.H. (1995). The octadecanoic pathway: Signal molecules for the regulation of secondary pathways, *Proc. Natl. Acad. Sci. U.S.A.*, 92:4099-4105.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance, *Plant Hormone Ethylene* (A.K. Mattoo and J.C. Suttle, eds), CRC Press, Boca Raton, Florida, pp. 293-314.

Boller, T. (1995). Chemoperception of microbial signals in plant cells, *Annu*. *Rev*. *Plant Physiol*. & *Plant Mol*. *Biol*., 46:189-214.

Bostock, R.M. (1999). Signal conflicts and synergies in induced resistance to multiple attackers, *Physiol*. *Mol*. *Plant Pathol*., 55:99-109.

Bothe, H., Klingner, A., Kaldorf, M., Schmitz, O., Esch, H., Hundeshagen, B. and Kernebeck, H. (1994). Biochemical approaches to the study of plant-fungal interactions in arbuscular mycorrhiza, *Experientia*, 50:919-925.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X. (1997). The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance, *Plant Cell*, 9:1573-1584.

Brading, P.A., Hammond-Kosack, K.E., Parr, A. and Jones, J.D. (2000). Salicylic acid is not required for Cf-2- and Cf-9-dependent resistance of tomato *Cladosporum fulvum*, *Plant J*., 23:305-318.

Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992). Elicitor and wound induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response, *Cell*, 70:21-30.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance, *Plant Cell*, 8:1583-1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats, *Cell*, 88:57-63.

Chai, Y., Zhao, L., Liao, Z., Sun, X., Zuo, K., Zhang, L., Wang, S. and Tang, K. (2003) Molecular cloning of a potential *Verticillium dahliae* resistance gene *SlVe1* with multisite polyadenylation from *Solanum licopersicoides*, *DNA Seq*., 14:375-384.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins, *Cell*, 89:1133-1144.

Chen, C.Y. and Heath, M.C. (1993). Inheritance of resistance to *Uromyces vignae* in cowpea and the correlation between resistance and sensitivity to a cultivar-specific elicitor of necrosis, *J. Phytopathol*., 83:8179-8183.

Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X. and Zhu, T. (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses, *Plant Cell*, 14**:**559-574.

Chen, W.P., Chen, P.D., Liu, D.J., Kynast, R., Friebe, B., Velazhahan, R., Muthukrishnan, S. and Gill, B.S. (1999). Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene, *Theor*. *Appl*. *Genet*., 99:755-760.

Chen, Z., Nunes, M.A., Silva, M.C. and Rodrigues, C.J. (2004). Appressorium turgor pressure of *Colletotrivhum kahawae* might have a role in coffee cuticle penetration, *Mycologia*, 96:1199-1208.

Chu, Z.Q., Jia, J.W., Zhou X.J. and Chen X.Y. (1999). Isolation of glycoproteins from *Verticillium dahliae* and their phytotoxicity, *Acta Bot*. *Sin*., 41:972-976.

Clarke, J.D., Volko, S.M., Ledford, H., Ausubel, F.M. and Dong, X. (2001). Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induces resistance in *Arabidopsis*, *Plant Cell*, 12:2175-2190.

Creelman, R.A. and Mullet, J.E. (1995). Jasmonic acid distribution and.action in plants: regulation during development and response to biotic and abiotic stress, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 92:4114-4119.

Creelman, R.A. and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants, *Annu*. *Rev*. *Plant Physiol*. & *Plant Mol*. *Biol*., 48:355-381.

Creelman, R.A., Tierney, M.L. and Mullet, J.E. (1992). Jasmonic acid /methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 89:4938-4941.

Custers, J.H., Harrison, S.J., Sela-Buurlage, M.B., van Deventer, E., Lageweg, W., Howe, P.W., van der Meijs, P.J., Ponstein, A.S., Simons, B.H., Melchers, L.S. and Stuiver, M.H. (2004). Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence, *Plant J*., 39:147-160.

Cutt, J.R. and Klessig, D.F. (1992). Pathogenesis-related proteins. In plant gene research (Meins, F. and Boller, T. eds.), Springer-Verlag, New York, pp. 181-216.

Daayf, F., Nicole, M., Belanger, R.R. and Geiger, J.P. (1998). Hyaline mutants from *Verticillium dahliae*, an example of selection and characterization of strains for host-parasite interaction studies, *Plant Pathol*., 47:523-529.

Dangl, J.L., Dietrich, R.A. and Richberg, M.H. (1996). Death don't have mercy: cell death program in plant-microbe interactions, *Plant cell*, 8:1793-1807.

Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G., Muthukrishnan, S. and Datta, S.K. (1999). Overexpression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease, *Theor*. *Appl*. *Genet*., 98:1138-1145.

Davis, D.A., Legendre, L., Heinstein. P. and Low, P.S. (1990). Elicitation of plant defense responses by components from *Verticillium dahliae*, *Plant Physiol*., 93:S53.

De Ascensao, A.R.F.D.C. and Dubery, I.A. (2003). Soluable and wall-bound phenolics and phenolic polymers in *Musa acuminate* roots exposed to elicitors from *Fusarium oxysporum* f. sp. *cubense*, *Phytochemistry*, 63: 679-686.

De Jong, A.J., Hoeberichts, F.A., Yakimova, E.T., Maximova, E. and Woltering, E.J. (2000). Chemical-induced apoptotic cell death in tomato cells: Involvement of caspase-like proteases, *Planta*, 211:656-662.

Dean, R.A. and Kuć, J. (1986). Induced systemic protection in cucumbers: the source of the "signal.", *Physiol*. *Plant Pathol*., 28:227-233.

Deese, D.C. and Stahmann, M.A. (1962). Formation of pectic enzymes by *Verticillium alboatrum* on susceptible and resistant tomato stem tissues and on wheat bran, *J*. *Phytopathol*., 46:53-70.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance, *Science*, 266:1247-1250.

Demole, E., Lederer, E. and Mercier, D. (1962). Isolement et determination de la structure du jasmonate de methyle, constituant odorant caracteristique de l'essence de jasmine, *Helv*. *Chim*. *Acta*, 45:675-685.

Dempsey, D., Shah, J. and Klessig, D.F. (1999). Salicylic acid and disease resistance in plants, *Crit*. *Rev*. *Plant Sci*., 18:547-575.

Devoto, A. and Turner, J.G. (2003). Regulation of Jasmonate-mediated plant responses in *Arabidopsis*, *Ann*. *Bot*., 92:1-9.

Díaz, J., ten Have, A. and van Kan, J.A.L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*, *Plant Physiol*., 129:1341-1351.

Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, F. and French, R. (2001). Abrogation of disease development in plants expression animal apoptotic genes, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 98:6957-6962.

Dittrich, H. and Kutchan, T.M. (1991). Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the responses of plants to pathogenic attack, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 88:9969-9973.

Dobinson, K.F., Grant, S.J. and Kang, S. (2004). Cloning and targeted disruption, via *Agrobacterium tumefaciens*-mediated transformation, of a trypsin protease gene from the vascular wilt fungus *Verticillium dahliae*, *Curr*. *Genet*., 45:104-110.

Dobinson, K.F., Lecomte, N. and Lazarovits, G. (1997). Production of an extracellular trypsin-like protease by the fungal plant pathogen *Verticillium dahliae*, *Can*. *J*. *Microbiol*., 43:227-233.

Dong, X. (2004). The role of membrane-bound ankyrin-repeat protein ACD6 in programmed cell death and plant defense, *Sci*. *STKE*, 221:pe6.

Dorey, S., Baillieul, F., Pierrel, M.A., Saindrenan, P., Fritig, B. and Kauffmann, S. (1997). Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor, *Mol*. *Plant-Microbe Interact*., 10:646-655.

Dorrell, D.G. and Vick, B.A. (1997). Properties and processing of oilseed sunflower in Sunflower Technology and Production (Schneiter, A.A. eds.), Amer. Soc. Agro., Crop Sci. Sco. Amer., Soil Sci. Soc. Amer. Madison, WI. 35:709-745.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue, *Focus*, 12:13-15.

Du, H. and Klessig, D.F. (1997). Identification of soluble, high-affinity salicylic acid-binding protein in tobacco, *Plant Physiol*., 113:1319-1327.

Dubery, I.A. and Slater, V. (1997). Induced defence responses in cotton leaf disks by elicitors from *Verticillium dahliae*, *Phytochemistry*, 44:1429-1434.

Eckardt, N.A. (2005). Ins and outs of programmed cell death and toxin action, *Plant Cell*, 17:2849-2851.

Ellis, C., Karafyllidis, I. and Turner, J.G. (2002). Constitutive activation of jasmonate signalling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*, *Mol*. *Plant-Microbe Interact.,* 15:1025-1030.

Faize, M., Faize, L., Ishizaka, M. and Ishii, H. (2004). Expression of potential responses of Asian and European pears to infection with *Venturia nashicola*, *Physiol*. *Mol*. *Plant Pathol*., 64:319-330.

FAO (Food and Agriculture Organization of the United Nations). (2007). Agricultural Statistics Database. http://www.fao.org

Farmer, E.E. and Ryan, C.A. (1990). Interplant communication: airborne methyl-jasmonate induces synthesis of proteinase inhibitors in plant leaves, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 87:7713-7716.

FAS (Foreign Agriculture Service) (2005). Oilseeds: world market and trades. Current World Production, Market and Trade Reports. http://www.fas.usda.gov.

Fei, J., Chai, Y., Wang, J., Lin, J., Sun, X., Sun, C., Zuo, K. and Tang, K. (2004). cDNA cloning and characterisation of the *Ve* homologue gene *StVe* from *Solanum torvum* Swartz, *DNA Seq*., 15:88-95.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Englehardt, S., Felix, G., Kemmerling, B., Krzymowska, M. and Nürnberger, T. (2002). NPP1, a Phytophthora-associated trigger of plant defense in parsley and *Arabidopsis*, *Plant J*., 32:375-390.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C. and Dixon, R.A. (1999). Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory, *Curr*. *Biol*., 9:317-320.

Fletcher, S.W. and Taylor, N. (1939). *Helianthus*. The Standard Cyclopedia of Horticulture (Bailey LH. eds.), Macmillan & Co. Ltd., London, pp. 1445-1449.

Foissner, L., Wendehenne, D., Langebartels, C. and Durner, J. (2000). In vivo imaging of an elicitor-induced nitric oxide burst in tobacco, *Plant J*., 23:817-824.

Fradin, E.F. and Thomma, B.P.H.J. (2006). Physiology and molecular aspects of *Verticillium wilt* diseases caused by *V*. *dahliae* and *V*. *albo-atrum*, *Mol*. *Plant Pathol*., 7:71-86.

Fujita, K., Suzuki, T., Kunoh, H., Carver, T.L.W., Thomas, B.J., Gurr, S. and Shiraishi, T. (2004). Induced inaccessibility in barley cells exposed to extracellular material released by non-pathogenic powdery mildew conidia, *Physiol*. *Mol*. *Plant Pathol*., 64:169-178.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J.A. (1993). Requirement of salicylic acid for the Induction of Systemic Acquired Resistance, *Science*, 261:754-756.

Gao, A.G., Hakimi, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J. and Rommens, C.M.T. (2000). Fungal pathogen protection in potato by expression of a plant defensin peptide, *Nat*. *Biotechnol*., 18:1307-1310.

Geraats, B.P.J., Bakker, P.A.H.M. and van Loon, L.C. (2002). Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*, *Mol*. *Plant-Microbe Interact*., 15:1078-1085.

Geraats, B.P.J., Bakker, P.A.H.M., Lawrence, C.B., Achuo, E.A., Höfte, M. and van Loon, L.C. (2003). Ethylene-insensitive tobacco shows differentially altered susceptibility to different pathogens, *J*. *Phytopathol*., 93:813-821.

Ghanmi, D., McNally, D.J., Benhamou, N., Menzies, J.G. and Bélanger, R.R. (2004). Powdery mildew of *Arabidopsis thaliana*: a pathosystem for exploring the role of silicon in plant-microbe interactions, *Physiol*. *Mol*. *Plant Pathol*., 64:189-199.

Gilroy, S. and Trewavas, A. (2001). Signal processing and transduction in plant cells: the end of the beginning? *Nat*. *Rev*. *Mol*. *Cell*. *Biol*., 2:307-314.

Gjetting, T., Carver, T.L.W., Skøt, L. and Lyngkjær, M.F. (2004). Differential gene expression in individual papilla-resistant and powdery mildew-infected barley epidermal cells, *Mol*. *Plant-Microbe Interact*., 17:729-738.

Gould, K.S., Lamotte, O., Klinguer, A., Pugin, A. and Wendehenne, D. (2003). Nitric oxide production in tobacco leaf cells: a generalized stress response? *Plant Cell Environ*., 26:1851-1862.

Govrin, E.M. and Levine, A. (2002). Infection of *Arabidopsis* with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic resistance (SAR), *Plant Mol*. *Biol*., 48:267-276.

Graham, J.S., Burkhart, W., Xiong, J. and Gillikin, J. (1992). Complete amino acid sequence of soybean leaf P21: similarity to the thaumatin-like polypeptides, *Plant Physiol*., 98:163-165.

Grant, J.J. and Loake, G.J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance, *Plant Physiol*., 124:21-29.

Green, R.J. (1954). A preliminary investigation of toxins produced in vitro by *Verticillium albo-atrum*, *J*. *Phytopathol*., 44:319-326.

Greenberg, J.T., Guo, A., Klessig, D.F. and Ausubel, F.M. (1994). Programmed cell death in plants: a pathogen triggered response activated coordinately with multiple defence functions, *Cell*, 77:551-63.

Gu, Y., Yang, C., Thara, Y.K., Zhou, J. and Martin, G.B. (2000). *Pti4* is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase, *Plant Cell*, 12:771-786.

Gulya, T.J. (2003). Sunflower crop survey 2002. Disease assessment across eight states. Proceedings Sunflower Research Workshop, Fargo, ND, January 16-17.

Gundlach, H., Müller, M.J., Kutchan, T. and Zenk, M.H. (1992). Jasmonic Acid is a Signal Transducer in Elicitor-Induced Plant Cell Cultures, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 89:2389-2393.

Hahn, M.G. (1996). Microbial elicitors and their receptors in plants, *Annu*. *Rev*. *Phytopathol*., 34:387-412.

Hall, A.E., Findell, J.L., Schaller, G.E., Sisler, E.C. and Bleecker, A.B. (2000). Ethylene perception by the ERS1 protein in *Arabidopsis*, *Plant Physiol*., 123:1449-1458.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996). Resistance gene-dependent plant defense responses, *Plant Cell*, 8:1773-1791.

Harms, K., Atzorn, R., Brash, A., Kuhn, H., Wasternack, C., Willmitzer, L. and Pena-Cortes, H. (1995). Expression of a Flax Allene Oxide Synthase cDNA Leads to Increased Endogenous Jasmonic Acid (JA) Levels in Transgenic Potato Plants but not to a Corresponding Activation of JA-Responding Genes, *Plant Cell*, 7:1645-1654.

He, C.Y. and Wolyn, D.J. (2005). Potential role for salicylic acid in induced resistance of asparagus roots to *Fusarium oxysporum* f. sp. *Asparagi*, *Plant Pathol*., 54:227-232.

Heiser, C.B. (1978). Taxonomy of *Helianthus* and Origin of Domesticated Sunflower. Sunflower Science and Technology (Carter, J.F. eds.), Amer. Soc. Agro., Crop Sci. Sco. Amer., Soil Sci. Soc. Amer. Madison, WI. pp. 31-52.

Herbert, C., O'Connell, R., Gaulin, E., Salesse, V., Esquerré-Tugayé, M. T. and Dumas, B. (2004). Production of a cell wall-associated endopolygalacturonase by *Colletotrichum lindemuthianum* and pectin degradation during bean infection, *Fungal Genet*. *Biol*., 41:130-147.

Hiemstra, J. (1998). Some general features of *Verticillium wilts* in trees. A compendium of *Verticillium* wilts in tree species (Hiemstra, J. and Harris, D. eds.), CPRO-DLO, Wageningen, The Neatherlands and HRI, West Malling, UK, pp. 5-11.

Hoes, J.A. (1972). Losses due to *Verticillium* wilt in sunflower, *J. Phytopathol*., 62:764.

Hückelhoven, R., Fodor, J., Preis, C. and Kogel, K.H. (1999). Hypersensitive Cell Death and Papilla Formation in Barley Attacked by the Powdery Mildew Fungus Are Associated with Hydrogen Peroxide but Not with Salicylic acid accumulation, *Plant Physiol*., 119:1251-1260.

Hudgins, J.W. and Franceschi, V.R. (2004). Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation, *Plant Physiol*., 135:2134-2149.

Jennings, J.C., Apel-Birkhold, P.C., Mock, N.M., Baker, C.J. anderson, J.D. and Bailey, B.A. (2001). Induction of defense responses in tobacco by the protein Nep1 from *Fusarium oxysporum*, *Plant Sci*., 161:891-899.

Jennings, J.C., Bailey, B.A. and Anderson, J.D. (2000). Induction of ethylene biosynthesis and necrosis in weed leaves by a *Fusarium oxysporum* protein, *Weed Sci*., 48:7-14.

Johnson, P.R. and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective, *Annu*. *Rev*. *Genet*., 32:227-254.

Kachroo, P., Kachroo, A., Lapchyk, L., Hildebrand, D. and Klessig, D.F. (2003). Restoration of defective cross talk in *ssi2* mutant: Role of salicylic acid, jasmonic acid, and fatty acids in SSI2-mediated signaling, *Mol*. *Plant-Microbe Interact*., 11:1022-1029.

Karagiannidis, N., Bletsos, F. and Stavropoulos, N. (2002). Effect of *Verticillium* wilt (*Verticillium dahliae* Kleb.) and mycorrhiza (*Glomus mosseae*) on root colonization, growth and nutrient uptake in tomato and eggplant seedlings, *Sci*. *Hort*., 94:145-156.

Kawchuk, L.M., Hachey, J. and Lynch, D.R. (1998). Development of sequence characterized DNA linked to a dominant *Vericillium* wilt resistance gene in tomato, *Genome*, 41:91-95.

Kawchuk, L.M., Hachey, J., Lynch, D.R., Kulcsar, F., van Rooijen, G., Waterer, D.R., Robertson, A., Kokko, E., Byers, R., Howard, R.J., Fischer, R. and Prufer, D. (2001). Tomato *Ve* disease resistance genes encode cell surface-like receptors, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 98:6511-6515.

Keen, N.T. and Long, M. (1972). Isolation of a phytotoxic protein–lipopolysaccharide complex from *Verticillium albo-atrum*, *Physiol*. *Plant Pathol*., 2:307-315.

Kende, H. (1993). Ethylene biosynthesis, *Annu*. *Rev*. *Plant Physiol*. & *Plant Mol*. *Biol*., 44:283-307.

Kim, C.Y., Liu, Y., Thorne, E.T., Yang, H., Fukushige, H., Gassmann, W., Hildebrand, D., Sharp, R.E. and Zhang, S. (2003). Activation of a Stress-Responsive Mitogen-Activated Protein Kinase Cascade Induces the Biosynthesis of Ethylene in Plants, *Plant Cell*, 15:2707-2718.

Kinkema, M., Fan, W. and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of *PR* Gene Expression, *Plant Cell*, 12:2339-2350.

Klebahn, H. (1913). Beiträge zur Kenntnis der Fungi Imperfecti I. Eine *Verticillium-Krankheit* auf Dahliaen, *Mykol*. *Zbl*., 3:49-66.

Klee, H.J. (2004). Ethylene signal transduction, moving beyond *Arabidopsis*, *Plant Physiol*., 135:660-667.

Koga, H., Dohi, K. and Mori, M. (2004). Abscisic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*, *Physiol*. *Mol*. *Plant Pathol*., 65:3-9.

Kohler, A., Schwindling, S. and Conrath, U. (2002). Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/ NIM1 gene in *Arabidopsis*, *Plant Physiol*., 128:1046-1056.

Koike, S., Subbarao, K., Davis, R., Gordon, T. and Hubbard, J. (1994). *Verticillium* wilt of cauliflower in California, *Plant Dis*., 78:1116-1121.

Kruger, W.M., Szabo, L.J. and Zeyen, R.J. (2003). Transcription of the defense response genes chitinase IIb, PAL, and peroxidase is induced by the barley powdery mildew fungus and is only indirectly modulated by R genes, *Physiol*. *Mol*. *Plant Pathol*., 63:167-178.

Kumar, D. and Klessig, D.F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 100:16101-16106.

Kunkel, B.N. and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense, *Curr*. *Opin*. *Plant Biol*., 5:325-331.

Laemmli, U.K. (1976). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227:680-685.

Lam, E., Kato, N. and Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response, *Nature*, 411:848-853.

Lamari, L. (2002). Assess: Image Analysis Software for Plant Disease Quantification. APS, St. Paul, MN, USA.

Lamb, C. and Dixon, R.A. (1997). The oxidative burst in plant disease resistance, *Annu*. *Rev*. *Plant Physiol*. & *Plant Mol*. *Biol*., 48:251-275.

Lamotte, O., Gould, K., Sequiera-Legrand, A., Lebrun-Garcia, A., Durner, J., Pugin, A. and Wendehenne, D. (2004). Analysis of nitric oxide signalling functions in tobacco cells challenged by the elicitor cryptogein, *Plant Physiol*., 135:516-529.

Leal, J.A. and Villanueva, J.R. (1962). Lack of pectic enzyme production by non pathogenic species of *Verticillium*, *Nature*, 195:1328-1329.

Leon, J., Lawton, M.A. and Raskin, I. (1995). Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco, *Plant Physiol*., 108:1673-1678.

Leung, J., Merlot, S. and Giraudat, J. (1997). The *Arabidopsis* abscisic acid insensitive 2 (*ABI2*) and *ABI1* genes encode redundant protein phosphatases 2C involved in abscisic acid signal transduction, *Plant Cell*, 9:759-71.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response, *Cell*, 79:583-593.

Li, L., Li, C., Lee, G.I. and Howe, G.A. (2002). Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 99:6416-6421.

Ligoxigakis, E.K., Vakalounakis, D.J. and Thanasoulopoulos, C.C. (2002). Weed hosts of *Verticillium dahliae* in Crete: Susceptibility, symptomatology and significance, *Phytoparasitica*, 30:11-518.

Liu, D., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994). Osmotin overexpression in potato delays development of disease symptoms, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 91:1888-1892.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense, *Plant Cell*, 15:165-178.

Low, P.S. and Heinstein, P.F. (1986). Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes, *Arch*. *Biochem*. *Biophys*., 249:472-479.

Lund, S.T., Stall, R.E. and Klee, H.J. (1998). Ethylene regulates the susceptible response to pathogen infection in tomato, *Plant Cell*, 10:371-382.

Mace, M.E., Bell, A.A. and Beckman, C.H. (1981). Life cycle and epidemiology of *Verticillium*, Fungal wilt disease of plants (Schnathorst, W.C. eds.), Academic Press, New York. pp. 81-111.

Madhosingh, G. (1995). Relative wilt-inducing capacity of the culture filtrates of isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the tomato crown and root rot pathogen, *J. Phytopathol*., 143:193-198.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection, *Science*, 250:1002-1004.

Malic, N. and Milton, J. (1980). Survival of *Verticillium* in monocotelydonous plants, *Trans*. *Br*. *Mycol*. *Soc*., 75:496-498.

Malysheva, K.M. and Zeltser, S.S. (1968). Protein-lipid-polysaccharide complex of culture liquid and mycelium of *Verticillium dahliae* Kleb., the causative agent of *Verticillium* wilt in cotton, *Doklady Akademii Nauk SSSR*, 179:231-234.

Mannanov, R.N. (2001). The use of natural bio-agents for the control of cotton phytopathogens, *Meded*. *Rijksuniv*. *Gent*. *Fak*. *Landbouwkd*. *Toegep*. *Biol*. *Wet*., 66:183-186.

Manners, J.M., Penninckx, I.A.M.A., Vermaere, K., Kazan, K., Brown, R.L., Morgan, A., Maclean, D.J., Curtis, M.D., Cammue, B.P.A. and Broekaert, W.F. (1998). The promoter of the plant defensin gene *PDF1.2* from *Arabidopsis* is systemically

activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid, *Plant Mol. Biol.*, 38:1071-1080.

Marrs, K.A. (1996). The functions and regulation of glutathione S-transferases in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47:127-158.

Martin S.J., Green D.R. and Cotter T.G. (1994). Dicing with death: dissecting the components of the apoptosis machinery, *Trends Biochem. Sci.*, 19:26-30.

Mathre, D. (1986). Occurence of *Verticillium dahliae* in barley, *Plant Disease*, 70:981.

Mathre, D. (1989). Pathogenicity of an isolate of *Verticillium dahliae* from barley, *Plant Dis.*, 73:164-167.

McCloud, E.S. and Baldwin, I.T. (1997). Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*, *Planta*, 203:430-435.

McDonald, K. and Cahill, D. (1999). Influence of abscisic acid and the abscisic acid biosynthesis inhibitor, norflurazon, on interactions between *Phytophthora sojae* and soybean (*Glycine max*), *Eur. J. Plant Pathol.*, 105:651-658.

McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L. and Holub, E.B. (2000). Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for *NDR1*, EDS1, *NPR1*, and salicylic acid accumulation, *Plant J.*, 22:523-531.

McFadden, H.G., Chapple, R., De Feyter, R. and Dennis, E. (2001). Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*, *Physiol. Mol. Plant Pathol.*, 58:119-131.

Mengiste T., Chen, X., Salmeron, J. and Dietrich, R. (2003). The *Botrytis susceptible 1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*, *Plant Cell*, 15:2551-2565.

Meuwly, P., Molders, W., Buchala, A. and Métraux, J.P. (1995). Local and systemic biosynthesis of salicylic acid in infected cucumber plants, *Plant Physiol*., 109:1107-1114.

Mohr, P.G. and Cahill, D. (2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to P*seudomonas syringae* pv. *tomato* and *Peronospora parasitica*, *Funct*. *Plant Biol*., 30:461-469.

Mol, L. and Vanriessen, H.W. (1995). Effect of plant-roots in the germination of microsclerotia of *Verticillium dahliae*, *Eur*. *J*. *Plant Pathol*., 101:673-678.

Montesano, M., Brader, G. and Tapio Palva, E. (2003). Pathogen derived elicitors: searching for receptors in plants, *Mol*. *Plant Pathol*., 4:73-79.

Morel, J.B. and Dangl, J.L. (1997). The hypersensitive response and the induction of cell death in plants, *Cell Death Differ*., 4:671-683.

Morel, J.B. and Dangl, J.L. (1999). Supressors of the *Arabidopsis lsd5* cell death mutation identify genes involved in regulation disease resistance responses, *Genetics*, 151:305-319.

Mou, Z., Fan, W. and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes, *Cell*, 113:935-944.

Nachmias, A., Buchner, V. and Krikun, J. (1982). Comparison of protein-lipopolylsaccharide complexes produced by pathogenic and non-pathogenic strains of *Verticillium dahliae* Kleb. from potato, *Physiol*. *Plant Pathol*., 20:213-221.

Nandi, A., Kachroo, P., Fukushige, H., Hildebrand, D.F., Klessig, D.F. and Shah, J. (2003). Ethylene and jasmonic acid signaling affect the NPR1-independent expression of defense genes without impacting resistance to *Pseudomonas syringae* and *Peronospora parasitica* in the *Arabidopsis ssi1* mutant, *Mol*. *Plant-Microbe Interact*., 16:588-599.

National Sunflower Association. (2008). Health and Nutrition. http://www.sunflowernsa.com/health/default.asp?contentID=45

Nelson, P.E. and Wilhelm, S. (1958). Thermal death range of *Verticillium albo-atrum*, *J*. *Phytopathol*., 48:613-616.

Nicholson, R.L. and Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance, *Annu*. *Rev*. *Phytopathol*., 30:369-389.

Orozco-Cardenas, M., Narvaez-Vasquez, J. and Ryan, C. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin and methyl jasmonate, *Plant Cell*, 13:179-191.

Paniego, N., Heinz, R., Fernandez, P., Talia, P., Nishinakamasu, V. and Hopp, H. (2007). Sunflower, Genome Mapping and Molecular Breeding in Plants (Kole, C. eds.), Springer Berlin Heidelberg, pp. 153-177.

Park, J., Gu, Y., Lee, Y. and Yang, Z. (2004). Phosphatidic acid induces leaf cell death in *Arabidopsis* by activating the Rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation, *Plant Physiol*., 134:129-136.

Pegg, G.F. (1974). *Verticillium* diseases, *Rev*. *Plant Pathol*., 53:157-182.

Pegg, G.F. and Brady, B.L. (2002). *Verticillium* wilts. 1st ed. CABI Publishing, Wallingford, Oxon OX10 8DE, UK.

Pegg, G.F. and Cronshaw, D.K. (1976). Relationship of in vitro to in vivo ethylene production in *Pseudomonas solanacearum* infection of tomato, *Physiol*. *Plant Pathol*., 9:145-154.

Pemberton, C.L. and Salmond, G.P.C. (2004). The Nep1-like proteins: a growing family of microbial elicitors of plant necrosis, *Mol*. *Plant Pathol*., 5:353-359.

Peña-Cortés, H., Willmitzer, L. and Sanchez-Serrano, J. (1991). Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family, *Plant Cell*, 3:963-972.

Pieterse, C.M.J., Van Wees, S.C.M., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and Van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*, *Plant Cell*, 10:1571-1580.

Pirrung, M.C. (1999). Histidine kinases and two-component signal transduction systems, *Chem*. *Biol*., 6:R167-R175.

Preston, C.A., Lewandowski, C., Enyedi, A.J. and Baldwin, I.T. (1999). Tobacco mosaic virus inoculation inhibits wound-induced jasmonic acid-mediated responses within but not between plants, *Planta*, 209:87-95.

Putt, E. (1978). History and Present World Status. Sunflower Science and Technology (Carter, J.F. eds.), Amer. Soc. Agro., Crop Sci. Sco. Amer., Soil Sci. Soc. Amer. Madison, WI. pp. 1-25.

Qutob, D., Kamoun, S. and Gijzen, M. (2002). Expression of P*hytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy, *Plant J*., 32:361-373.

Radman, R., Saez, T., Bucke, C. and Keshavarz, T. (2003). Elicitation of plants and microbial cell systems, *Biotechnol*. *App*. *Biochem*., 37:91-102.

Rashid, K.Y., Desjardins, M.L. and Kaminski, D.A. (2006). Disease of sunflower survey in Manitoba in 2006. In: Sunflower. Agriculture and Agri-Food Canada, research station.

Raskin, I., Skubatz, H., Tang, W. and Meeuse, B.J.D. (1990). Salicylic acid levels in thermogenic and nonthermogenic plants, *Ann*. *Bot*., 66:369-373.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*, *Plant Physiol*., 97:1342-1347.

Reinbothe, S., Mollenhauer, B. and Reinbothe, C. (1994). JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens, *Plant Cell*, 9:1197-1209.

Robinson, M.M., Shah, S., Tamot, B., Pauls, K.P., Moffatt, B.A. and Glick, B.R. (2001). Reduced symptoms of *Verticillium* wilt in transgenic tomato expressing a bacterial ACC deaminase, *Mol*. *Plant Pathol*., 2:135-145.

Rojo, E., Titarenko, E., León, J., Berger, S., Vancanneyt, G. and Sánchez-Serrano, J.J. (1998). Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in *Arabidopsis thaliana*, *Plant J*., 13:153-65.

Ryals, J., Neuenschwander, U., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996). Systemic acquired resistance, *Plant Cell*, 8:1809-1819.

Ryerson, D.E. and Heath, M.C. (1996). Cleavage of nuclear DNA into oligosomal fragments during cell death induced by fungal infection or by abiotic treatments, *Plant Cell*, 8:393-402.

Sackston, W.E. (1957). Leaf mottle or *Verticillium* wilt of sunflower, *Plant Dis*. *Rep*., 41: 337-343.

Sanmartin, M., Jaroszewski, L., Raikhel, N.V. and Rojo, E. (2005). Regulation cell death since the origin of life, *Plant Physiol*., 137:841-847.

Sasabe, M., Takeuchi, K., Kamoun S., Ichinose, Y., Govers, F., Toyoda, K., Shiraishi, T. and Yamada, T. (2000). Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture, *Eur*. *J*. *Biochem*., 267:5005-5013.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H. and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in *Arabidopsis* by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways, *DNA Res*., 8:153-161.

Schenk, P.M., Kazan, K., Wilson, I. anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. (2000). Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 97:11655-11660.

Schnathorst, W.C. (1981). Life cycle and epidemiology in *Verticillium*. Fungal wilt diseases in plants (Mace, M.C., Bell, A.A and Beckman, C.H. eds.), Academic Press, New York, pp. 81-112.

Shah, J., Kachroo, P. and Klessig, D. (1999). The *Arabidopsis ssi1* mutation restores PR gene expression in SA-Insenstive (*npr1*) plants and renders *PDF1.2* (Defensin) gene expression SA dependent, *Plant Cell*, 11:191-206.

Shah, J., Tsui, F. and Klessig, D.F. (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsisthaliana* identified in a selective screen utilizing the salicylic acid-inducible expression of the *tms2* gene, *Mol*. *Plant-Microbe Interact*., 10:69-78.

Shettty, K.G., Subbarao, K.V., Huisman, O.C. and Hubbard, J.C. (2000). Mechanism of broccoli-mediated *Verticillium* wilt reduction in cauliflower. *J. Phytopathol*., 90:305-310.

Showalter, A.W. (1993). Structure and function of plant cell wall proteins, *Plant Cell*, 5:9-23.

Smit, F. and Dubety, I.A. (1997). Cell wall reinforcement in cotton hypocotyls in response to a *Verticillium dahliae* elicitor, *Phytochemistry*, 44: 811-815.

Solano, R. and Ecker, J.R. (1998). Ethylene gas: Perception, signaling and response, *Curr*. *Opin*. *Plant Biol*., 1:393-398.

Soylu, E.M., Soylu, S. and Mansfield, J.W. (2004). Ultrastructural characterization of pathogen development and host responses during compatible and incompatible interactions between *Arabidopsis thaliana* and *Peronospora parasitica*, *Physiol*. *Mol*. *Plant Pathol*., 65:67-78.

Stevens, S., Stevens, A.B., Gast, K.L.B., O'Mara, J.A., Tisserat, N. and Bauerfeind, R. (1993). Commercial Specialty Cut Flower Production. Sunflower. Cooperative Extension Service. Kansas St Univ. Manhattan, KS. MF-1084. pp. 1-7.

Stuiver, M.H., Custers, J.H.H.V., Sela-Buurlage, M.B., Melchers, L.S., Van Deventer-Troost, J.P., Lageweg, W. and Ponstein, A.S. inventors. (2000). Antifungal proteins, DNA coding therefore, and hosts incorporating same. Australian Patent No. AU718274.

Summermatter, K., Sticher, L. and Métraux, J.P. (1995). Systemic responses in *Arabidopsis thaliana* infected and challenged with *Pseudomonas syringae* pv *syringae*, *Plant Physiology*, 108:1379-1385.

Takahashi, H., Chen, Z., Du, H., Liu, Y. and Klessig, D.F. (1997). Development of necrosis and activation of disease resistance in transgenic tobacco plants with severely reduced catalase levels, *Plant J.*, 11:993-1005.

Takahashi, H., Kanayama, Y., Zheng, M.S., Kusano, T., Hase, S., Ikegami, M. and Shah, J. (2004). Antagonistic Interactions between the SA and JA Signaling Pathways in *Arabidopsis* Modulate Expression of Defense Genes and Gene-for-Gene Resistance to *Cucumber Mosaic Virus*, *Plant Cell Physiol*., 45:803-809.

Talboys, P.W. (1957). The possible significance of toxic metabolites of *Verticillium alboatrum* in the development of hop wilt symptoms, *Trans*. *Br*. *Mycol*. *Soc*., 40:415-427.

Talboys, P.W. and Busch, L.V. (1970). Pectic enzymes produced by *Verticillium* wilt species, *Trans*. *Br*. *Mycol*. *Soc*., 55:367-381.

Ten, L.N., Mukhimedshanov, S.Z. and Otroshchenko, O.S. (1981). Structure of the peptide moiety of verticillin, *Khimiya Prirodnykh Soedinenii Tashkent*, 4:476-479.

Ten, L.N., Tyshchenko, A.A., Stepanichenko, N.N., Gusakova, S.D., Mukhamedzhanov, S.Z., Otroshchenko, O.S. and Kas'yanenko, A.G. (1977). Metabolites of the

pathogenic fungus *Verticillium dahliae* VI. Pentaketide metabolites and neutral lipids of virulent and avirulent strains, *Khimiya Prirodnykh Soedinenii Tashkent*, 5:632-635.

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. (1995). Function of the oxidative burst in hypersensitive disease resistance, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 92:4158-4163.

Thaler, J.S., Owen, B. and Higgins, V.J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles, *Plant Physiol*., 135:530-538.

Thomma, B.P., Penninckx, I.A., Broekaert, W.F. and Cammue, B.P. (2001). The complexity of disease signaling in *Arabidopsis*, *Curr*. *Opin*. *Immunol*., 13:63-68.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B.,Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens, *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*., 95:15107-15111.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.J. and Broekaert, W.F. (1999). Requirement of functional Ethylene-Insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*, *Plant Physiol*.,121:1093-1101.

Thordal-Christensen, H. (2003). Fresh insights into process of nonhost resistance, *Curr*. *Opin*. *Plant Biol*., 6:351-357.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J. and Katinakis, P. (2005). Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent

K-165 and pathogenesis-related proteins gene expression, *Mol*. *Plant-Microbe Interact*., 18:555-561.

Tolmsoff, W.J. (1973). Life cycles of *Verticillium species*. *Verticillium* Wilt of Cotton. Proceedings of a Work Conference of the National Cotton Pathology Research Laboratory, College Station, pp. 20-38.

Ton, J., Pieterse, C.M.J. and van Loon, L.C. (1999). Identification of a locus in *Arabidopsis* controlling both the expression of rhizobacteria-mediated induced systemic resistnace (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*, *Mol*. *Plant-Microb Interact*., 12:911-918.

Triant, D.A. and Whitehead, A. (2009). Extraction of High-Quality RNA and DNA from Small Tissue Samples, *J*. *heredity*, 100:246-250.

Turner, J.G., Ellis, C. and Devoto, A. (2002). The jasmonate signal pathway, *Plant Cell*, 14:S153-S164.

Uppal, A.K., El Hadrami, A., Adam, L.R. and Daayf, F. (2007). Pathogenic variability of *Verticillium dahliae* isolates from potato fields in Manitoba and screening of bacteria for biocontrol, *Can*. *J*. *Plant Pathol*., 29:141-152.

Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J. (1998). Systemic resistance induced by rhizosphere bacteria, *Annu*. *Rev*. *Phytopathol*., 36:453-483.

Van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994). Recommendations for naming pathogenesis-related proteins, *Plant Mol*. *Biol*. *Rep*., 12:245-264.

Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C. and Pieterse, C.M.J. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on known defense-genes but stimulates the

expression of the jasmonate inducible gene *Atvsp* upon challenge, *Plant Mol*. *Biol*., 41:537-549.

Veit, S., Wörle, J.M., Nürnberger, T., Koch, W. and Seitz, H.U. (2001). A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, *Arabidopsis*, and tobacco, *Plant Physiol*., 127:832-841.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction, *Plant Cell*, 6:959-965.

Veronese, P., Narasimhan, M.L., Stevenson, R.A., Zhu, J.K., Weller, S.C., Subbarao, K.V. and Bressan, R.A. (2003). Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*, *Plant J*., 35:574-587.

Vidhyasekaran, P. (2004). Concise Encyclopedia of Plant Pathology, Haworth Press, Binghamton, NY, p. 619.

Voth, V., Munnecke, D.E., Paulus, A.O., Kolbergen, M.J. and Bringhurst, R.S. (1973). Effect of tarp thickness and dosage on response of California strawberries to fumigation, *Calif*. *Agric*., 27:14.

Wang, H., Li, J., Bostock, R.M. and Gilchrist, D.G. (1996). Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development, *Plant Cell*, 8:375-391.

Wang, J.Y., Cai, Y., Gou, J.Y., Mao, Y.B., Xu, Y.H., Jiang, W.H. and Chen, X.Y. (2004). VdNEP, an elicitor from *Verticillium dahliae*, induces cotton plant wilting, *Appl*. *Environ*. *Microbiol*., 70:4989-4995.

Wang, K.L.C., Li, H. and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks, *Plant Cell*, 14:S131-S151.

Wang, L., Pei, Z., Tian, Y. and He, C. (2005). OsLSD1, a rice zinc finger protein, regulates programmed cell death and callus differentiation, *Mol*. *Plant-Microbe Interact*., 18:375-384.

Ward, E.W.B., Cahill, D.M. and Bhattacharyya, M.K. (1989). Abscisic acid suppression of phenylalanine ammonia-lyase activity and mRNA, and resistance of soybeans to *Phytophthora megasperma* f.sp. *glycinea*, *Plant Physiol*., 91:23-27.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A. and Beer, S.V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*, *Science*, 257:85-88.

Wendehenne, D., Lamotte, O., Frachisse, J.M., Barbier-Brygoo, H. and Pugin, A. (2002). Nitrate efflux is an essential component of the cryptogein signalling pathway leading to defense responses and hypersensitive cell death in tobacco, *Plant Cell*, 14:1937-1951.

White, R.F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco, *Virology*, 99:410-412.

Wikipedia. (2009). http://en.wikipedia.org/wiki/Sunflower

Wilhelm, S. (1955). Longevity of the *Verticillium* wilt fungus in the laboratory and in the field, *J. Phytopathol*., 45:180-181.

Xiao, S., Brown, S., Patrick, E., Brearley, C. and Turner, J. (2003). Enhanced transcription of the *Arabidopsis* disease resistance gene *RPW8.1* and *RPW8.2* via

salicylic acid-dependent amplification circuit is required for hypersensitive cell death, *Plant Cell*, 15:33-45.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998). *COl1*: An *Arabidopsis* gene required for jasmonateregulated defense and fertility, *Science*, 280:1091-1094.

Xu, Y., Chang, P.F.L., Liu, D., Narasimhan, M.L., Raghothanma, K.G., Gasegawa, P.M. and Bressan, R.A. (1994). Plant defense genes are synergistically induced by Ethylene and Methyl Jasmonate, *Plant Cell*, 6:1077-1085.

Xu, Y.C., Cao, Y.L., Guo, P., Tao, Y. and Zhao, B.L. (2004). Detection of nitric oxide in plants by electron spin resonance, *J. Phytopathol.,* 94:402-407.

Yang, Z., Rogers, L.M., Song, Y., Guo, W. and Kolattukudy, P.E. (2005). Homoserine and asparagine are host signals that trigger in planta expression of a pathogenesis gene in *Nectria haematococca*, *Proc. Natl. Acad. Sci. U.S.A.*, 102:4197-4202.

Zeise, K. and von Tiedemann, A. (2002). Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*, *J. Phytopathol.*, 150:112-119.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X.A. (2003). Gain-of-Function Mutation in a Plant Disease Resistance Gene Leads to Constitutive Activation of Downstream Signal Transduction Pathways in *suppressor of npr1-1*, constitutive 1, *Plant Cell*, 15:2636-2646.

Zheng, H.Z., Cui, C.I., Zhang, Y.T., Wang, D., Jing, Y. and Kim, K.Y. (2005). Active changes of lignification-related enzymes in pepper response to Glomus intraradices and/or Phytophthora capsici, *J. Zhejiang Univ. Sci.*, 68:778-786.

Zuppini, A., Navazio, L., Sella, L., Castiglioni, C., Favaron, F. and Mariani, P. (2005). An endo-polygalacturonase from *Sclerotinia sclerotiorum* induces calcium-mediated signaling and programmed cell death in soybean cells, *Mol*. *Plant-Microbe Interact*., 18:849-855.