ROBSON FERREIRA DE ALMEIDA

HOST TRANSCRIPTIONAL RESPONSES TO VASCULAR AND FOLIAR PHYTOPATHOGENIC FUNGI

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitotecnia, para obtenção do título de *Doctor Scientiae*.

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A Deus,

Aos meus pais, Ernandes Salgado (*in memoriam*) e Leonice Ferreira de Almeida,

À minha amada esposa pela força e incentivo

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RESUMO

ALMEIDA, Robson Ferreira de, D.Sc., Universidade Federal de Viçosa, Setembro de 2009. **Host transcriptional responses to vascular and foliar phytopathogenic fungi.** Orientador: Ney Sussumu Sakiyama. Co-orientadoras: Eunize Maciel Zambolim e Eveline Teixeira Caixeta.

O presente trabalho estudou mecanismos genéticos que controlam a resposta de plantas à uma espécie fitopatogênica do gênero *Verticillium*. *V. dahliae* (*Vd*) e *V. longisoporum* (*Vl*) são fungos de solo causadores de doenças vasculares de plantas herbáceas e plantas arbóreas, estas duas espécies são responsáveis por perdas anuais que somam bilhões de dólares. Este patógneo perde um alto grau de sua especificidade quanto ao hospedeiro e viabiliza a colonização e multiplicação em diferentes espécies de plantas. Até o presente momento, pouco se sabe a respeito da base genética e molecular da resistência/tolerância à *Verticillium spp.* Para preenchimento dessa lacuna, estudou-se a interação entre *Vl* e a planta modelo *Arabidopsis thaliana*. Experimentos de microarranjo e análises de metabólitos indicaram que isolados de *Vl* foram capazes de reprogramar a via metabólica do triptofano, resultando na redução do acúmulo do composto de defesa derivado do triptofano, glucosinolato indólico (IGS). Propõe-se com este estudo uma estratégia de patogenicidade utilizada por *Vl* que envolve o recrutamento do fator transcricional *WRKY* 70 como um regulador negativo da biosíntese de de IG. Esta parte do trabalho foi desenvolvida no laboratório da Dra. Paola Veronese da Universidade Estadual da Carolina do Norte (NCSU). Uma outra parte foi o estudo de genes envolvidos na resistência de café à ferrugem causada pelo fungo *Hemileia vastatrix* . Esta parte do trabalho foi desenvolvida no Biocafé/Bioagro/UFV (Universidade Federal de Viçosa). A metodologia utilizada para isolamento dos genes envolvidos na resistência à ferrugem foi a hibridização subtrativa supressiva (SSH), que se baseia na amplificação preferencial de seqüências diferencialmente,

representadas em duas populações de cDNA, possibilitada pela reação em cadeia da polimeras (PCR), e a prevenção da amplificação de sequências comuns pelo evento de supressão possibilitada. Foi utilizado neste trabalho o genótipo Híbrido de Timor CIFC 832/2 . Folhas deste genótipo foram inoculadas com uredóporos de *Hemileia vastatrix* (raça II). Após a inoculação, o RNA foi extraído das folhas 12 e 24 horas após a inoculação para proceder a hibridização subtrativa supressiva. Foi estudada a expressão de dois genes (cisteína protease e quitinase) através do RT-PCR quantitativo (real time-Polymerase chain reaction) em dois genótipos diferentes, CIFC 832/2 (resistente à *H. vastatrix*) e Catuaí (susceptível à *H. vastatrix*). O resultado obtido mostra dois padrões de expressão diferentes para os dois genes nos dois genótipos. A expressão dos dois genes no Híbrido de Timor CIFIC 832/2 foi mais elevada e antecipada quando comparada a expressão observada no Catuaí IAC 44.

ABSTRACT

ALMEIDA, Robson Ferreira de, D.Sc., Universidade Federal de Viçosa, September 2009. **Host transcriptional responses to vascular and foliar phytopathogenic fungi.** Adviser: Ney Sussumu Sakiyama. Co-advisers: Eunize Maciel Zambolim and Eveline Teixeira Caixeta.

We used a comprehensive approach for the dissection of genetic mechanims controlling plant responses to phytopathogenic species of the genus *Verticillium. V. dahliae (Vd)* and *V. longisporum (Vl)* are soil fungi causing vascular diseases in herbaceous plants and trees that result in billions of dollars in annual losses worldwide. The pathogens lack a high degree of host specificity and can colonize and multiply in several different plant species. At the present, there is little or no knowledge of the deterministic molecular and genetic basis of plant resistance/tolerance to *Verticillium spp*. To address this knowledge gap, we studied the interaction of *Vl* with the model plant *Arabidopsis thaliana.* Microarray experiments and metabolite analysis indicated that *Vl* but not *Vd* isolates were able of reprogramming the tryptophan (Trp) metabolic pathway resulting in lack of accumulation of the Trp-derived defense compounds indolic glucosinolates (IGs). We are proposing that the *Vl* pathogenicity strategy involves recruiting the transcription factor *WRKY 70* as negative regulator of the IG biosynthesis This part of the thesis was developed at Dr. Paola Veronese's lab at NCSU (North Carolina State University). We also studied the molecular mechanism involved in coffee's plant response against the phytopathogen *Hemileia vastatrix*.. We focused on the identification of genes involved or related with coffee resistance to orange rust caused by the fungi *Hemileia vastatrix* . This part of the thesis was developed at Biocafé/Bioagro/UFV (Federal University of Vicosa). The methodology used for the isolation of genes involved in resistance was the suppression subtractive hybridization (SSH), which is based on a preferential amplification performed by the polymerase chain

reaction (PCR) of differentially represented sequences in two cDNA populations, and prevention of the amplification of common sequences by suppression event. We used in the present work the Timor Hybrid CIFC 832/2 genotype. Leaves of this genotype were inoculated with uredospores of *Hemileia vastatrix* (race II). After inoculation, the RNA was extracted from leaves (at 12 and 24 hs) to proceed the suppression subtractive hybridization. We also studied the expression of two genes (cysteine protease and chitinase) by quantitative RT-PCR in two different genotypes, CIFC 832/2 (resistant to *H. vastatrix*) and Catuaí (susceptible to *H. vastatrix*). The result showed different patterns of expression of these two genes between these two genotypes. The expression of the two genes in the Timor Hybrid CIFC 832/2 was higher and earlier than was in the Catuaí IAC 44 genotype.

I. GENERAL INTRODUCTION

1.1. Vascular Pathogen

Similarly to the above-ground organs, roots can be attacked by a number of pathogenic and parasitic organisms. These include, in order of importance, fungi, nematodes, bacteria, virus, and parasitic higher plants. Compared to infection by foliar pathogens, there are many important differences in the ecology, epidemiology, life cycles, pathogenesis, and infection caused by root pathogens. Within the last few years, there have been major advances in the understanding of host-pathogen interaction, mostly involving foliar pathogens. Less well understood are the interaction and mechanisms of resistance to necrotrophic root pathogens; these do not have the high degree of host specificity that characterize most biotrophic foliar and root pathogens. *Arabidopsis thaliana* has become a model host plant, but few root pathogens have been used in this system (Paulitz, 2003).

The most significant pathogens of the roots of crop plants are either fungi or filamentous bacteria of the genus *Streptomyces* (Loria et al.; 2003). There are a few pathogenic soil-borne bacteria, such as *Ralstonia solanacerarum*, which causes a wilt, and the well-studied *Agrobacterium tumefaciens*, wich causes crow gall by genetic transformation of the plant (Gelvin. 2000; Schell, 2000).

Most root pathogens are necrotrophic, that is, they kill host tissue with toxins, peptide elicitors, or enzymes that trigger host cell lysis and death, thereby providing conditions favorable to pathogen growth. Classic examples are the Oomycete Phythium and the Basidiomycete *Rhizoctonia*. Although some species of these genera can infect above-ground parts under wet, humid conditions, they primarily attack roots and emerging shoots. Both genera show a preference for young, juvenile tissues with secondary wall thickenings, and both can attack germinating seeds in the soil, causing pre or post-emergence damping-off or seedling rot. They also can attack young root tips and feeder roots, since the newest tissue is formed at the root tip. These pathogens can directly penetrate the root epidermis, and thick-walled resistant survival structures have evolved that are capable of surviving environmental extremes in a dormant state in the absence of a susceptible host. Of the fungi that parasitize root systems, surprisingly few are biotrophic, that is, require a living plant to parasitize and obtain nutrients. Some, such as *Phytophthora sojae*, are hemibiotrophic and form haustoria or feeding structures in plant cells.

Another characteristic of most root necrotrophic pathogens is their wide host range. For example, *Pythium ultimum* has been recorded in over 100 genera of plants in the US (Farr et al., 1987).

In contrast to biotrophic pathogens, the majority of root necrotrophic pathognes do not appear to have closely co-evolved with a specific host, or to be distinguished by races that are virulent to specific genotypes, varieties, or

cultivars of domestic plants, and avirulent to closely-related genotypes (Paulitz, 2005).

The following is a generalized life cycle of necrotrophic root rotting soil-borne pathogenic fungi. Such fungi can survive in the soil in a dormant, quiescent state, when environmental conditions are not suitable for growth, or when the host is not present. They must also withstand microbial degradation and lysis, parasitism and predation, constituting an important trophic level in the soil ecosystem. Therefore, in many fungi, a thick-walled resistant spore or structure has evolved to serve this survival function. These survival structures are often dark-colored or melanized, making them more resistant to microbial degradation.

Once environmental conditions become favorable and a root emerges or grows in close proximity to the fungal propagule, the resistant structure will germinate to form hyphae that will grow toward the susceptible root or seed. Fungi have mechanisms of chemotaxis and chemotropism, and sense root exudates such as sugars, amino acids, organic acids and fatty acids (Deacon and Donaldson, 1993; Tyler, 2002).

Once the fungal hyphae or zoospore contacts the surface of the root, there probably is a recognition event on the part of both the fungus and plant. This also involves attachment of the fungus to the root. When a hypha contacts the root, it can form an appressorium, a swollen structure that attaches to the root and forms the infection hypha for penetration. In order to penetrate the host cell wall, fungal hyphae excrete cell-wall degrading

enzymes such as pectinases and other pectic enzymes; hemicellulases, cellulases, and proteinases (Campion *et al.*, 1997).

After gaining ingress, the pathogen grows intracellularly in the cortex of the root, killing the tissue ahead of the cortex of the advancing hyphae and colonizing the root. New infections can be initiated on adjacent roots by hyphae or zoospores produced on the killed tissue.

Diseases caused by soil-borne pathogens are considered to be monocyclic. New roots on the plant can become infected from initial primary infection, but there is no much plant-to-plant spread in a single season, because of the limited distance that the inoculums travels in the soil. This is unlike polycyclic foliar disease, which produces tremendous amounts of spore inoculums which spread from plant to plant by wind or rain in a exponential fashion in a single season (Paulitz, 2003).

One subset of necrotrophic pathogens, the wilt pathogens, has a more specialized life cycle with adaptation to growth in the vascular system. These include the forma specialis of *Fusarium oxysporum* and species of *Verticillium*. Wilt pathogens also colonize the cortex of the root, but gain access to the xylem in the zone of elongation before the vascular system is fully developed and differentiated, because the Casparian strip, suberized tissue in the endodermis surrounding the stele, presents a barrier to direct penetration of the vascular system in older parts of the roots. These fungal pathogens block the movement of water in the xylem by producing mycelia, spores, and high-molecular-weight polysaccharides in the xylem vessels, while degrading plant cell walls and releasing pectic substances and other

polymers that can clog the vascular system and reduce its water-transport efficiency to the leaves.

1.2.Leaf Pathogen

When a fungal pathogen lands on a plant leaf, the most obvious obstacle it faces is how to gain entry to the underlying tissue. Unlike bacteria, which have to circumvent the problem by locating stomata (pores in the plant epidermis), wounds, or other natural openings, many fungal species can rupture the cuticle (the tough outer layer of a plant) directly (Agrios, 1988). How they do so remains controversial (Schafer, 1993). In the case of some fungi, enzymatic action is clearly visible at the point of infection, suggesting that the plant cuticle is dissolved ahead of the infecting pathogen (Agrios, 1988). In other species, specialized infection structures called appressoria are formed that can generate high pressures, indicating a mechanical infection process (Schafer, 1993). Bechinger *et al.* 1999 reported that enormous invasive forces are applied by appressoria of a fungal pathogen, directly demonstrating for the first time that appressoria can exert sufficient pressure to enable mechanical infection of plants by fungi. By allowing appressoria to form on an optical waveguide, the forces exerted by the fungal penetration pegs could be visualized and quantified.

Many of the most severe and economically important plant diseases are caused by fungi, and the initial infection processes have been studied

extensively to develop effective disease control strategies. Fungi have evolved many methods for entering plants, including mechanisms for locating stomata and the ability to rapidly colonize wound sites (Schafer, 1993). But many fungal species simply penetrate plant cuticles directly, either as threadlike fungal cells called hyphae or, more frequently, by growing specialized appressoria (Schafer, 1993). Appressoria are swollen, dome-shaped, or cylindrical cells that differentiate from the end of fungal germ tubes and during maturation can further differentiate to produce a thick, rigid cell wall (Schafer, 1993). Appressoria allow tight adhesion to the plant surface, followed by rupture of the cuticle with a narrow hypha called a penetration peg. During this process, the fungus changes its axis of growth and reestablishes polarized growth as the penetration peg extends into the plant.

The infection process by appressoria can involve enzymatic action, and the external matrix around appressoria often contains cutinases, cellulases, and other nonspecific esterases to help soften the cuticle, thereby aiding adhesion and penetration (Agrios, 1988, Schafer, 1993). However, experimental proof of an absolute requirement for enzymatic activity has remained elusive. Fungi such as *Colletotrichum* and *Magnaporthe* species produce appressoria with tough melanin-pigmented cell walls (Howard *et al.*, 1996). Howard *et al.* previously showed that appressoria of *Magnaporthe grisea*, the causal agent of rice blast disease; generate very high internal pressure (turgor) (Howard, 1991). A cell collapse assay was used to predict the appressorial turgor of *M. grisea* by calculating the concentration of polyethylene glycol required to collapse an appressorium. Howard *et al.*

showed that *M. grisea* appressoria generate pressures of between 6 and 8 megapascals, the equivalent of 30 to 40 times the pressure of an average car tire, an astounding pressure for a cell to generate. Appressoria were also shown to be able to puncture artificial plastic membranes.

In some rust causer fungi, the appressorium develops only after the germ tube contacts the stomatal apparatus. The precise nature of the inductive signal provided by the stoma is still in question, but increasing evidence suggests that some physical component of the stomatal structure is responsible for triggering appressorium formation

Some pathogens depend on living host tissues for their growth and reproduction and form specialized structures called haustoria to allow intimate contact with living plant cells to obtain nutrients from host tissues. Rust fungi, for example, colonize tissues by spreading infection hyphae that form haustorial mother cells, which are involved in cell wall penetration and the production of the haustorium. Through the invaginated plant cell plasma membrane, the haustorium coordinates the uptake of host water and nutrients, and also the signaling between host and parasite to establish and maintain compatibility (Voegele, 2003; O’Connell, 2006).

Some studies of haustoria-forming plant pathogens suggest that the haustorium plays a cirtical role in delivering fungal effector proteins, including avirulence proteins, into the infected host cell O’Connell, 2006. Among these was the avirulent protein AvrL567, from the flax rust fungus (*Melampsora lini*),

which is recognized by the resistance proteins L5 and L6 of flax (*Linum usitatissimum*) triggering a necrotic resistance response (Dodds et *al*., 2004)

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II. CHAPTER 01

FUNCTIONAL GENOMIC ANALYSIS OF ARABIDOPSIS RESPONSES TO FUNGAL-INDUCED VASCULAR DISEASE

1. INTRODUCTION

Verticillium dahliae, *V. albo-atrum* and *V. longisporum* are soil-borne plant fungal pathogens causing *Verticillium* wilt diseases. The mycelium of these phytopathogenic species of *Verticillium* is hyaline, simple or branched, septet and multinucleate. They produce ovoid to elongate conidia on long philiades positioned in a whorl or spiral-like shape around the conidiophores. Branching of the conidiophores also occurs in whorls at several levels. The name of the fungal genus is indeed derived from this so-called ‘verticillate’ disposition of the conidiophores branches and philiades (Fradin, 2006).

Currently, there are no fungicides available to control *Verticillium* wilts once plants have been infected. Despite the economic importance of these pathogens and the considerable phytopathological attention they have obtained, little is known about the molecular genetic basis of host disease resistance and fungal pathogenicity (Fradin, 2006). The host range, epidemiology and infection process on various hosts of phytopathogenic *Verticillium spp.* have been intensely investigated (Schnathorst ,1981; Beckmann, 1987; Gold et al., 1996, Rowe and Powelson, 2002). After germination of the resting structures present in infested soils, which is induced by root exudates, fungal hyphae reach the root surface, penetrate the epidermal cells, transverse the root cortex and enter the immature xylem elements (Beckman, 1987; Mol, 1995). During the parasitic stage of their life cycles, the fungal pathogens are confined within the vascular system, which

is a nutrient-limited environment containing low amounts of sugars, inorganic salts, and amino acids, to which microorganisms are well adapted (Dixon and Pegg, 1972). In this liquid environment, *Verticillium spp.* exhibits both hyphal and a yeast-like growth, producing conidia budding directly from hyphae or formed on short phialides (Buckley et al., 1969). Transported by the transpiration stream, the released conidia are eventually trapped in bordered pits or at the vessel end walls. Colonization proceeds by formation of germ tubes, which penetrate into the upstream vessel elements, and subsequent production of new conidia (Beckman 1987; Gold et al. 1996). In the later stages of the disease cycle, as the foliage begins to senesce, the pathogen enters a limited saprophytic growth phase characterized by the differentiation of resting structures in the dying stem parenchyma. The resting structures are returned to the soil with the plant debris thus having a critical role as primary source of inoculum in the field (Schnathorst, 1981; Neumann et al., 2003).

So far, most monocotyledonous plants are considered to be non-host species. Many individual *Verticillium* spp. isolates are capable of causing a rather wide range of disease symptoms on several hosts, including chlorosis, anthocyanin accumulation, stunted growth, early flowering, defoliation, wilting and sudden death (Bhat and Subbarao, 1999; Subbarao *et al.*, 1995; Veronese et al., 2003). Despite the name *Verticillium* wilt, true wilt not always occurs as a consequence of the fungal colonization. Typically, wilting appears only on one half of an infected leaf, usually in the oldest shoots as invasion is acroptal (from base to apex) (Fradin, 2006). In tomato, the lower leaves turn yellow as tips and edges die, causing typical V-shaped lesion. Ultimately, the

whole leaf wilts and may abscise. Alternatively, leaves may develop yellow blotches that later turn necrotic and brown, and the veins may appear brown or purple (Fradin, 2006). In several plant species, wilting of young shoots can occur during warmer periods of the day with recovery at night. Drought or other conditions that reduce the plant's vitality can worsen disease development and symptom expression. Annuals often survive the season, but may be chlorotic, stunted, early senescent and have smaller yield. Often, a brown discoloration of the vascular tissues can be often found in stem sections. Overall, *Verticillium* diseases are difficult to diagnose on the basis of symptom expression particularly because several *Fusarium* species cause similar tissue damages (Fradin, 2006).

Infestation of plant roots by parasitic nematodes can enhance the occurrence and severity of diseases caused by other soil-borne microorganisms including *Verticillium* spp.. (Back *et al.*, 2002; Harrison, Johnson and Santo, 2001). This "disease-enhancing effect" has been attributed to several causes (*Back et.*, 2002). Firstly, the nematodes are inflicting wounding to the root system, which facilitates penetration by other pathogens. Secondly, wounding increases leakage of root exudates to the surrounding soil, which attracts other microorganisms (Grayston *et al.*, 1997) and, in particualr, stimulates the germination of *Verticillium* spores and resting structures. Finally, nematode infection leads to changes in xylem sap composition, which may promote pathogen proliferation (Katsantonis *et al.*, 2005).

Devastating outbreaks of *Verticillium* diseases characterized by almost complete yield losses have been reported, among the other crops, in cauliflower, cotton and sun flower (Church, 1995; Friebertshauser and De Vay, 1982; Koike et al., 1994). In Sweden, *Verticillium* wilt of *Brassicaceae* oil crops has been observed for the past 50 years .and is presently considered the major constrain for oilseed rape (*Brassica napus*) and turnip rape (*B. rapa*) production (Dixelius *et al.*, 2005). In Sweden and Germany, *Vertcillium* wilt disease is mainly caused by *V. longisporum* (Karapapa *et al.*, 1997), which is phylogenetically more closely related to *V. dahliae* (Fahleson *et al.*, 2004). Like *V. dahliae*, *V. longisporum* is cosmopolitan and causes wilt disease in many economically important crops, including cotton, curcubits, alfalfa, sunflower, eggplant, mint, strawberry, tomato and potato (Domsch *et al*. 1980; Subbarao *et al.*, 1995).

A differentiation of long-spored *Verticillium* isolates collected from horseradish, which were classified as *V. dahliae* var. *longisporum*, was first made in the early 1960s (Stark, 1961). Later, a detailed description of distinct morphological, physiological and molecular traits led to the proposition of treating *V. longisporum* as a distinct species (Karapapa et al. 1997b). To date, there is still controversy concerning the taxonomy of *V. longisporum* as a separate host-specific species of *Verticillium* due to the finding that *Brassica* crops can occasionally host short-spored *Verticillium* isolates and that *V. longisporum* is able to infect plant species outside the *Brassicaceae* family (Collins et al., 2003; Fahleson et al., 2003, Johannson et al. 2006).

1.1 MICROSCLEROTIUM FORMATION

Several fungal species, among which *Sclerotinia sclerotiorum* probably is the most well known (Bolton et al, 2006), produce sclerotia as durable vegetative resting structures. Microsclerotia consist of grape-like clusters of thick-walled and heavily melanized cells. These hyphal aggregates contain a dark (melanized) outer rind of several cell layers to shield the inner portion (medulla). They develop by swelling of the hyphae and septation of the cells that enlarge and produce lateral cells. Subsequently, melanin is deposited in the cell walls and intercellular spaces (Perry and Evert, 1982).

Melanins are dark pigments formed by the oxidative polymerization of phenolic or indolic compounds. Most fungal melanins are derived from the precursor 1,8-dihydroxynaphthalene (DHN) that is produced in the cytoplasm and subsequently deposited into the extracellular matrix where the enzymes laccases catalyze the final polymerization. In general, melanins have been thought to confer protection to environmental stress of various kinds including UV exposure (Butler, 1998). In *Verticillium* spp., melanins appears to contribute to long-term survival of resting structures as non-melanized microsclerotia quickly lose their availability (Hawke, 1995).

1.2 MECHANISMS OF PATHOGENICITY

It has been suggested that phytopathogenic *Verticillium* spp. can suppress host defense responses initiated near trapping sites for several hours, which provides the fungi with time for a lateral escape to adjacent

vessel (Gold and Robb, 1995; Lee *et al*., 1992). However, not much is known about the molecular control and crucial components in the pathogenicity of *Verticillium* spp.. Historically, the production of toxic or elicitor-like substances and the role of cell-wall-degrading enzymes have gained much attention. However, these are rarely fully characterized and often the unambiguous proof for a crucial role in pathogenicity is lacking.

1.3 CELL-WALL-DEGRADING ENZYMES.

Verticillium spp. produces several enzymes that may play a role in plant cell-wall degradation (Bidochka et al., 1999; Dobinson *et al*., 2004). Pectinolytic enzymes such as polygalacturonase, pectate lyase and pectinesterase have been among the most intensively studied mainly because the pathogen has to breach the pectin-containing pit membrane between vessel elements and at vessel ends in order to spread (Bishop and Cooper, 1983b). In addition, host depositions that play a role in the vascular occlusion are often rich in pectin and must be transversed by the pathogen for systemic colonization (Huang and Mahoney, 1999). Some of these enzymes have been shown to possess *in vitro* necrotizing activity on plant tissues and to induce wilting in shoot cuttings (Cooper and Wood, 1980). Furthermore, polygalacturonase, pectin lyase and cellulose production levels correlated with the degree of virulence in different *Verticillium* strains (Carder *et al*., 1987). *V. albo-atrum* pectinase deficientmutants were obtained through chemical mutagenesis and tested for virulence on tomato (Durrands and Cooper, 1988 a and b). The mutants were able to colonize the host at the

same extent as the wild type, but caused less production of pectinases (Durrands and Cooper, 1988b). Interestingly, one of the mutants was found to be also affected in the secretion of other cell-wall-degrading enzymes This mutant showed a drastic reduction in the systemic spread suggesting that *V. albo-atrum* utilizes a synergistically acting combination of cell-wall-degrading enzymes for the successful colonization of the host (Durrands and Cooper, 1988 a and b).

1.4 TOXINS AND ELICITORS.

In several studies, fungal crude culture filtrates have been used to study *Verticillium* spp. elicitation of plant defense responses and disease symptoms. However, the exact nature of the components of these extracts has been often unclear. Generally, they are found to contain high-molecular weight protein-lipopolysaccharide (PLP) complexes, glycoproteins and/or cell-wall-degrading enzymes. For instance, a high-molecular-weight PLP complex was isolated from a potato isolate of *V. dahliae* containing a glycopeptide toxin that was associated with the induction of disease symptoms in susceptible host genotypes. In particular, the PLP complex dissociated under non-reducing condition and the phytotoxic activity remained in the low-molecular-weight polypeptide fraction. This fraction displayed differential toxic activity correlated with the host-specificity of the pathogen isolate (Buchner et al., 1982). Immuno-colocalization experiments demonstrated accumulation of this toxin on the cell wall of the xylem elements in stems and tubers of

susceptible plants (Nachmias *et al.*, 1985). Furthermore, the toxin was not produced by a non-pathogenic mutant of the same isolate (Buchner *et al.*, 1982). These findings appear to support the hypothesis that this toxin complex is required for *V. dahliae* pathogenicity (Buchner *et al.*, 1982). Another 65-kDA glycoprotein complex was isolated from *V. dahliae,* which was also able to induce defense responses in in cotton and soybean cells. Subsequently, the protein component (53-KDA deglycosylated protein) of the complex was found to be responsible for the eliciting activity (Davis *et al.*, 1998). Other partially purified *V. dahliae* secreted compounds were isolated by other research groups and shown to induce chlorosis and necrosis on leaves of susceptible tomato and potato cultivars (Mansoori *et al.*, 1995).

These results suggest that phytopathogenic *Verticillium* spp. may produce a broad spectrum of phytotoxins and/or elicitors of host cell death leading to compatible interactions with the hosts.

Interestingly, susceptible cotton cultivars were found more sensitive to crude fungal extracts which were able to cause ion leakage (Meyer *et al.*, 1994). This observation supported the hypothesis that the wilting symptom is the result of the activity of fungal toxin rather than to vessel occlusion.

However, stunting can also be explained by the reduction of photosynthesis that occurs upon stomatal closure caused by water stress (Flexas and Medrano, 2002).

Finally, the observation that resistant or tolerant plant species are often insensitive to *Verticillium* spp. toxic activity (Buchner *et al.*, 1989) has led to

the suggestion that toxin insensitivity forms the basis of resistance or tolerance.

Despite all these suggestions and claims, definitive evidences for a major role of a “toxin” in causing *Verticillium* wilt diseases is still missing (Cooper, 2000; Pegg and Brady, 2002). First of all, questions can be asked regarding the purity of the extracts and the exact components causing the phenomena that have been observed. Furthermore, data from other studies implicated vessel occlusion as primary cause of water stress. This occlusion can be due to physical blockage of the plant’s xylem by the pathogen itself, by embolism caused by pathogen-derived macromolecules or due to host defense responses that are aimed at vessel plugging (Van Alfen, 1989). Although this has not been demonstrated for *Verticillium* spp., large PLP complexes and/or polysaccharides could be in fact physically involved in vessel blockage (Cooper, 2000).

In several different fungi, oomycetes and bacteria, proteins have been identified that appear to belong to a newly recognized family of elicitor molecules called NLPs (for Nep1-like proteins), named after the first member Nep1 (for necrosis and ethylene inducing peptide) which was isolated from *Fusarium oxysporum* (Bailey, 1995). An NLP member named VdNep has been recently isolated from *V. dahliae* able to induce a hypersensitive response-like cell death in various plant species and wilting in cotton leaves (Wang *et al*. 2004a). However, little is known about the expression of NLP members during in plant interaction and their role in virulence has not been established yet (Wang *et al*. 2004a).

1.5 PHYSIOLOGY OF PLANT DEFENSE AGAINST VERTICILLIUM SPP. INFECTION

Typically, host tolerance rather than resistance has often been associated with *Verticillium* wilt diseases. Whereas susceptible plants are characterized by extensive fungal colonization and intense symptoms, resistant plants are characterized by little colonization and symptom expression. Tolerant plants, however, show few symptoms despite extensive fungal colonization. There is no clear consensus on the definition of tolerance, and literature describing tolerance and resistance against plant pathogens is a semantic mine-field (Clarke, 1986). Tolerance can be defined as enduring the effect of a pathogen infection while still producing a good crop (Agrios, 1997).

The emerging consensus is a conceptual distinction between the activity on the pathogen itself and the activity on the detrimental effects of the pathogen population. In other words, resistance reduces pathogen populations while tolerance plants reduce fitness loss of the host without altering pathogen development (Restif and koekka, 2004). In several studies it has been suggested that tolerance is caused by host plant insensitivity to *Verticillium* toxin complexes (Buchner *et al.*, 1982, Nachmias *et al.*, 1985).

Several different processes operating either at the pre-vascular phase of *Verticillium* infection or at the vascular phase have been found to contribute to wilt resistance. It has been determined that many infections stay in the pre-vascular phase, with the fungus growing inter- and intra-cellularly only in the cortex and the plants affected only to a limited extent (Huisman, 1988).

The endoderm, which is suberized during normal root development, acts as a natural barrier against *Verticillium* spp. infection (Talboys, 1958). In addition, lignin is quickly deposited in the walls of epidermal and cortical cells as well as around the penetrating hyphae to form so-called lignin tubers (also called apposition or papillae) and trap the fungus (Griffiths, 1971). The production of phytoalexins and related compounds with potential antimicrobial activity represent another of the earliest host defensive mechanisms. Most of *Verticillium* infection attempts are stopped in a pre-vascular colonization phase. However, when the pathogen overcomes the pre-vascular defense responses and reaches the xylem vessels, the vascular stage of the wilt disease starts. *Verticillium* spp. colonization occurs in a cyclical manner, alternating between phases of fungal growth and fungal elimination by the host defense machinery (Heinz *et al*., 1998). While in a resistant host genotype, the pathogen is contained in the root and/or at the crown level as a result of rapid mounting of defense responses, in a susceptible plant, the fungus escapes or delays the onset of the antimicrobial repertoire (Pegg and Brady, 2002). Rapid deposition of suberin and other coating material on vascular cell walls, including the trapping sites, forms a barrier against fungal penetration and thus may prevent horizontal spread (Robb *et al.* 1989). At this stage, the infection commonly results in vessel occlusion by gums, gel and other deposits secreted by the neighboring parenchyma cell and tyloses (Benhamou, 1995). All these obstructions block transport through the vessel element and movement of the fungus. The effect of this occlusion on the pathogen depends on the rate and level of its formation. If it occurs in

advance of the pathogen and before conidia are released into the sap stream, the pathogen may be contained and the occlusion contributes to resistance. However, if many vessels are affected at the same time and cannot be compensated for by the production of new vessel elements, plants may collapse (Talboys, 1972).

1.6 THE VE RESISTANCE GENES

Polygenic resistance to *Verticillium* spp. has been identified in several plant species including alfalfa, cotton, potato and strawberry (Bolek *et al.*, 2005; Simko *et al.*, 2004b). Single dominant resistance genes have been identified in cotton, sunflower, potato and tomato species (Jansky et al., 2004). In tomato (*Solanum lycopersicon*), the *Ve* locus that provides resistance against *Verticillium wilt* has been used by plant breeders for 60 years and is introduced in most cultivated tomatoes. Isolates of *V. dahliae* and *V. albo-atrum* that are contained by the *Ve* locus are designated as race 1 while all others are assigned to race 2 (Pegg, 1974). Although some studies do not record any fungal growth in *Ve*-carrying tomato plant (Williams *et al.*, 2002), others note that initial colonization of resistant and susceptible tomato cultivars with race 1 *V. dahliae* is similar in extent (Chen *et al.*, 2004). According to recent studies, the fungus enters the xylem and attempts to spread. At this stage, in resistant tomato, a rapid coating response prevents fungal spread (Chen *et al.*, 2004). However, whereas in susceptible plants the fungus recovers and starts spreading again, resulting in a cyclical

colonization (Heinz *et al*., 1998), in resistant plants the fungus does not substantially overcome the elimination (Chen *et al*2004) The tomato *Ve* locus has been fully characterized (Kawchuk *et al*., 2001). Positional cloning revealed the presence of two closely linked genes, *Ve1* and *Ve2*, encoding leucine-rich repeat (LRR) proteins that belong to the class of so-called receptor-like proteins (RLP) (Kruijt *et al*, 2005). When separately expressed in a susceptible potato cultivar, both *Ve* genes were found to confer resistance against a race 1 isolate of *V. albo-atrum* (Kawchuk *et al*., 2001). Whether the two genes are involved in perception of the presence or activity of the same pathogen factor is presently not known, nor where *Ve*-mediated resistance is established in the plant. Because the fungus is present in the xylem even in resistant plants, it is tempting to speculate that the Ve resistance proteins exert their activity in the parenchyma cells surrounding the xylem vessels. For the tomato 1-2 gene that provides resistance against the vascular pathogen *Fusarium oxysporum*, expression was demonstrated in these parenchyma cells, suggesting a correlation with the resistance response (Mes *et al*., 200).

Ve gene homologues have been identified in the wild tomato species *Solanum licopersicoides* (Chai *et al.,* 2003) and the wild potato species *Solanum torvum* (Fei *et al*., 2004). In tetraploid potato (*Solanum tuberosum*), a *Verticillium* resistance quantitative trait locus (QTL) was identified using the tomato *Ve1* gene as a probe. This locus was found to contain at least 11 genes, all putatively encoding LRR receptor-like proteins (Simko, *et al*., 2004a). The tomato and potato genomes are highly collinear and the QTL locus was mapped to a chromosome 9 region that is syntenic to the short arm

of the tomato chromosome 9 that carries *Ve1* and *Ve2* (Simko *et al.*, 2004a). This suggests that the *S. tuberosum* QTL indeed harbors a resistance determinant. However, so far it has not been demonstrated that any of the *Ve* gene homologues in any species are active resistance genes (Chai *et al.*, 2003; Fei *et al.*, 2004; Simko *et al.*, 2004a).

Apart from the various *Ve* genes, the RLP class of resistance genes also harbors the tomato *Cf* resistance genes that provide resistance against the fungal pathogen *Cladosporium fulvum* and the tomato *LeEix* genes that play a role in perception of the ethylene-inducing xylanase elicitor (EIX) from the biocontrol fungus *Trichoderma viride* (Kruijt *et al.*, 2005, Thomma *et al.*, 2005). They all encode extracellular LRRs that are putatively involved in elicitor recognition, a membrane-spanning domain and a short cytoplasmic tail that generally lacks obvious signaling domains. Nevertheless, many RLP genes are predicted to contain conserved mammalian endocytosis signatures within their short cytoplasmic domains (Kruijt *et al.*, 2005; Ron and Avni, 2004). Remarkably, the EIX elicitor is internalized by the host upon direct interaction with the receptor-like protein LeEix (Ron and Avni, 2004).

Not much is known about downstream signaling components for the tomato *Ve* genes. Only recently, a gene was identified through reverse genetics that is required for *Ve*-mediated resistance (Hu et al., 2005). This gene was found to act upstream of SA accumulation and PR-gene induction upon pathogen challenge, and is required for basal defense against virulent pathogens as well as resistance mediated by several resistance (*R*)-genes of the TIR-class (displaying homology to Drosophila Toll and mammalian

Interleukin-1 Receptor domain). Based on structural and functional similarities this gene was identified as the tomato homologue of the *Arabidopsis EDS1* (enhanced disease susceptibility 1) gene, which is also primarily involved in resistance mediated by the TIR-class of *R*-genes (Aarts *et al*., 1998; Hu *et al*., 2005; Parket *et al*., 1996).

In *Arabidopsis*, which is a susceptible host for *V. longisporum*, more susceptible disease phenotypes include induction of early flowering, senescence and dying. In 2003, Veronese and collaborators identified a genetic source of disease tolerance in the accession C-24 characterized by pathogen-induced delay of transition to flowering and mild chlorosis symptoms. Genetic analysis indicated that a single dominant locus, which is referred to as *VERTICILLIUM TOLERANCE 1* (*VET1*), controlled the more tolerant disease phenotype likely functioning also as a negative regulator of the pathogen-induced transition to flowering. *VET1* was mapped on chromosome IV. Interestingly, the differential symptom responses observed between the tested *Arabidopsis* accessions were not correlated with different rates of fungal tissue colonization or with differential transcript accumulation of *PR-1* and *PDF1.2* defense genes, molecular markers of the induction of salycilic acid (SA)- and ethylene (ET)/jasmonic acid (JA)-dependent defense pathways, respectively, whose activation was no detected during the *Arabidopsis-V. longisporum* interaction (Veronese et al., 2003).

2. RESEARCH OBJECTIVES

To gain further insights on the molecular genetics of basal defense responses to *Verticillium* spp., we have studied the compatible interaction between the model plant *Arabidopsis thaliana* and a cauliflower isolate of *V. longisporum*, which is pathogenic on cruciferous and non-cruciferous crop plants. In particular, we have first aimed to characterize global genome transcriptional changes occurring in the root system during early stages of the fungal infection using microarray experiments. Subsequently, our goal has been the functional analysis of genes with putative regulatory functions on the most distinctive host defense responses identified *via* transcription profiling.

3. MATERIALS AND METHODS

3.1 PLANT GROWTH AND INOCULATION.

Arabidopsis thaliana ecotype Columbia, which is the reference ecotype for the Arabidopsis scientific community, was used in the microarray experiments. For the inoculation experiments, it was used an *in vitro* inoculation method that allows the analysis of hundreds of seedlings in relatively small and fully controlled environment (Veronese *et al*, 2003). Briefly, Columbia seeds were rinsed with 70% ethanol and surface-sterilized with 2% sodium hypochlorite. Before sowing, the seeds were stratified at $4^{o}C$ 2 days to ensure a more uniform germination. Sowing was done in square Petri dishes on the surface of a cellophane membrane (cat.1650963 Bio-Rad, Richmond, CA, USA) laid over Murashige and Skoog medium (Sigma chemical Co., St, Louis, MO, USA) solidified with 0,8% agar and supplemented with 2% sucrose. Sixteen seeds were placed in each plate. Seedlings were grown in a growth chamber at $22 \pm 1^{o}C$ under a 12h light/12h dark photoperiodic cycle. When 2-weeks–old, the root system of each individual seedling was inoculated with a 2µl drop of conidial suspension (10^{4} conidia/ml) containing 0.1% agar to avoid scattering of the suspension. Control seedlings were mock-inoculated with 01% agar solution in sterile distilled water. The cauliflower isolate of *V. longisporum* VlBob.70 was used throughout this study. Cultures of the isolate were grown on potato dextrose agar (PDA, Difco, Detroi, MI, USA). Conidia were harvested from 4 to 5 day-

old plates by flooding the surface of the plates with sterile distilled water and filtering through two layers of sterile cheesecloth. Conidia were counted using a calibrated hemocytometer .

3.2 RNA ISOLATION, MICROARRAY HYBRIDIZATION AND STATISTICAL ANALYSIS.

Plant material for the transcription profiling analysis was harvested at 24, 48 and 72 hours post-infection (hpi). The root system of mock- and fungal-inoculated plants was collected in 2.0 ml centrifuge tubes and immediately frozen in liquid nitrogen. Total RNA was isolated using RNeasy Total RNA isolation KIT (Qiagen Inc., Valencia, CA, USA). RNA concentration was calculated using a NanoDrop spectrophotometer (Thermo Scientific). The cDNA synthesis, labeling, hybridization to GeneChip Arabidopsis ATH1 genome arrays, data acquisition and preliminary data quality control were performed by the company Expression Analysis (www.expressionanalysis.com; Durham, NC, USA). The facility provided Veronese's laboratory with row microarray data in excel spread sheets. Data were statistically analyzed in collaboration with Dr. Steffen Heber, Assistant Professor at NCSU Computer Science Department and member of the Bioinformatic Research Center. Briefly, all data analysis was performed in R (R Development Core Team, 2006). After a successful quality assessment (Heber and Sick, 2006), normalization and background correction was performed using the Robust Multichip Average (Irizarry *et al.*, 2003). Subsequently, a linear model was fitted to the data using the limma package (Smyth, 2004). This method is thought to be especially suitable for

experiments with small numbers of arrays since it uses an empirical Bayes method to mitigate standard errors of the estimated log-fold changes. The contrasts of interest were extracted and P values for differentially expressed genes were computed. To control the proportion of false positives in our results, a Benjamini and Hochberg multiple testing correction (Benjamini,Y. and Hochberg,Y., 1995) with false-discovery rate of 0.05 (unless otherwise noted) was applied. R code is available upon request.

3.3 VALIDATION OF MICROARRAY DATA BY QRT-PCR.

The expression of selected genes differentially regulated upon fungal infection and identified *via* microarray analysis was validated by quantitative real-time polymerase chain reaction (qRT-PCR). New inoculation experiments were performed in triplicate according to the same methods used for generating material for transcriptome analysis. Total RNA was isolated using RNeasy Total RNA isolation KIT (Qiagen Inc., Valencia, CA, USA). The cDNA samples were prepared using the Taqman ® Reverse Transcription Kit protocol (ABI, Foster City, CA), 1µl of total RNA at the concentration of 200ng/µl and random hexamer primers. Gene specific PCR primers were designed using Intergrated DNA Technologies (IDT) Oligo Design and Analysis Tools. To ensure consistent tissue collection, RNA extraction, and RT, we used the housekeeping gene control Actin1 primer. PCR was performed using an ABI7900HT sequence detection system set to the following conditions: Taq 95°C for 10 min, denaturing 40 cycles at 95°C for 15s, and annealing and extension at 60°C for 1 min. The following reagents were combined with each sample and control: 12.5µl of SYBR Green master

mix (ABI), 3.75 µl of primers (0.2 µM), and 5.0 µl of cDNA (0.001 µM). Data were analyzed using ABI SDS software (ABI), and gene expression data were calculated using the ΔΔCT method (Livak and Shmittgen, 2001).

Table 1- Primers sequence used in validation by qRT-PCR

Gene	AGI	Primer sequence
CYP71A13	AT2G30770	F 5'-TAA AGA GGT GCT TCG GTT GCA TCC-3' R 5'-CCC ATA TCG CAG TGT CTC GTT GGA -3'
PR1	AT2G14610	F 5'-ACA CGT GCA ATG GAG TTT GTG GTC-3' R 5'-TAC ACC TCA CTT TGG CAC ATC CGA-3'
WRKY33	AT2G38470	F 5'-ACC ATC GGT TCT CCA GTG AGG AAA-3' R 5'-AAG ACG AAT CCT GTG GTG CTC TGT-3'
PAD3	AT3G26830	F 5'-TCC GGT GAA TCT TGA GAG AGC CAT-3' R 5 -ACA GTT TCT CTT ATG ACC GCT-3'
ACT2	AT3G18780	F 5'-ATG GAA GCT GCT GGA ATC CAC-3' R 5'-TTT GCT CAT ACG GTC AGC GAT-3'
MYB51	AT1G18570	F 5'-TCG TTG ATG TGA CGC CGG TCT AT-3' R 5'-TTG ACA TGG TCA CGT GGG ATT CAT CG-3'
MYB122	AT1G74080	F 5'-GAT GTC CGT TGA GTC TTG TTT GGA G-3' R 5'-ACT TCA TTG ATC GGC GTC ACG TAG-3'
ASA1	AT5G05730	F 5'-GAT GCA AGC ACT GAC TTT CTC TCG-3 R 5'-CGG AGA AAC AGA GAC GCA TTT AAT C-3'
CYP83B1	AT4G31500	F 5'-GGC AAC AAA CCA TGT CGT ATC AAG-3' R 5'-CGT TGA CAC TCT TCT TCT CTA ACC G-3'
CYP79B2	AT4G39950	F 5'-AAC AAA AAG AAA CCG TAT CTG CCA C-3' R 5'-TCC TAA CTT CAC GCA TGC TAT CTC-3'
MYB34	AT5G60890	F 5'-CAC GAC TGT CGA TAA TTT TGG GTT T3' R 5'-CAT ATT GTC ATC TTC GTT CCA GGA A-3'
TSB1	AT5G54810	F 5'-AGC CTC AGG CAC CTC TGC TAC TT-3' R 5'-GGA GAC GGA GAA GGA TGA TGA CTT-

		3’
UGT74	AT1G24100	F 5’-TCC TAA TCG AGA AAT TCA AAT CCA C-3’ R 5’-CGC AAC ACA GAA CAA ACA GTG AGA T-3’
WRKY70	AT3G56400	F 5’- TGGTTCGTCCACGGAGAATGCCTT-3’ R5’- CGCCGCTAAAGCTCAAATCGCTTT-3’
ITS		F5’- CAGCGAAACGCATATGTAG-3’ R5’- AGATTTGGGGGATGTTCGG-3’

AGI- Arabidopsis Genome Initiative

3.4 METABOLITE ANALYSIS.

For quantification of indolic glucosinolates, 100 mg mock- and fungal-inoculated root tissues were extracted for 10 minutes in 1.5 ml boiling water containing 10nM of the internal standard sinigrin hydrate (Sigma-Aldrich) and 30 mM lead acetate. Samples were then grinded using 0.5mm Zr/Si glass beads and a BeadBeater. After microcentrifugation, 1ml of the supernatant was collected and loaded onto a DEAE25 Sephadex column (packed in a Pasteur pipette) of bed depth of 3 cm. The sample was allowed to fully enter the gel bed and washed with 2 ml of distilled water. A mix of 300µl aryl sulfatase (5000 units per 10 ml) and 200 µl of 100mM sodium sulfate (pH5.5) was added to the Sephadex column, the top of the column was sealed with parafilm and allowed to lay overnight at room temperature. The desulfated glucosinolates were then eluted from the column in 3ml of 60% methanol and the eluant evaporated in a CentriVap and re-constituted in 200 µl of 10% acetonitrile for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) performed in collaboration with Dr.

Neigel Deighton at the NCSU Genomic Sciences Laboratory (http://gsl.cals.ncsu.edu/).

For camalexin analysis, 500µl of 60% methanol:water solution containing 2µM reserpine (SIGMA) were added to 50mg of root tissues. The tissues were disrupted using a minibeadbeater (0.5mm and 1.0mm Zr/Si). After 2 cycles of 2 minute shaking, samples were centrifuged (5 minutes at 12000 rcf). The supernatant was then transferred to an auto sampler vial ready for analysis. The camalexin analysis was performed at the NCSU Genomic Sciences Laboratory.using a LC-UV-ESI-MS/MS on a Thermo LTQ linear ion trap mass spectrometer with electrospray interface. Statistical analysis was performed using SAS software version 8.2 (SAS Institute Inc, Cary, NC).

3.5 HISTOLOGICAL ANALYSIS OF THE ROOT FUNGAL COLONIZATION PROCESS

For visualization of the fungal colonization process, the root system of inoculated seedlings was collected at different times after the artificial inoculation and boiled for 1 min in a lactophenol solution (1 part lactic acid: 1 part glycerol: 1 part liquid phenol: 1 part distilled water) containing 10 µ/ml trypan blue. The samples were cleared in lactophenol solution without trypan blue, rinsed with 50% ethanol solution in water and then transferred onto a slide to be analyzed in a Olympus BX60 transmitted and reflected microscope.

3.6 FUNGAL BIOMASS QUANTIFICATION.

The root system of inoculated seedlings was harvested at 12, 24, 36, 48, 60 and 72 hpi. Genomic DNA extraction was performed using DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Fungal biomass was quantified by determining the concentration of fungal DNA in infected plant extract. First, total fungal genomic DNA was extracted from axenic cultures, quantified using a NanoDrop spectrophotometer and serial dilutions were used to build a calibration curve using Verticillium ITS-specific primers and qRT-PCR. The amount of *V. longisporum* DNA in the infected tissues was estimated comparing qRT-PCR results obtained using ITS primers with the calibration curve data.

4.0 RESULTS AND DISCUSSION

Hystological analysis of the root system stained using trypan blue allowed the detection of fungal hyphae inside of xylem after 48 hpi. After *V. longisporum* entered the root, hyphae of *V. longisporum* grew following the grooves of the junction of the epidermal cells, as observed at 72 hpi. At 24 hpi, it was not possible to visualize the hyphae inside the xylem, however, hyphae were observed attached on the root surface (Figure-1).

Studying differential interaction of *Verticillium longisporum* and *V. dahliae* with *Brassica napus*, Eynck and collaborators observed first penetration of *V. longisporum* at 60 hpi (Eynck, 2007). The fungus entered the root tissue by direct penetration of the epidermal cells without forming any conspicuous infection structures such as appressoria. Only slight hyphae are formed before entering epidermal cells, which is probably due to the accumulation of cytoplasm in the hyphal tip as a response to the mechanical resistance of the plant tissue (Eynck, 2007).

During *V. longisporum* penetration, plant cell walls are perforated by a thin penetration peg. In the lumen of the epidermal cells, the hyphae regain their regular diameter. Subsequently, hyphae grow intracellularly and intercellularly in the root cortex, more or less directed towards the center cylinder (Eynck, 2007).

4.1 MICROARRAY ANALYSES

In this study, 3272 genes were up-regulated *Arabidopsis thaliana* cv. Columbia in response to *V. longisporum* when considering fold change 1.5, as shown in the Venn diagram.

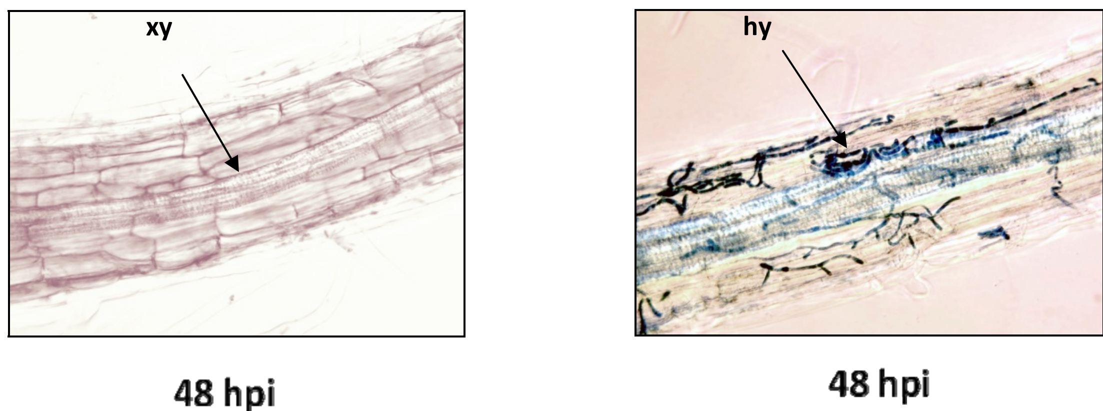

Figure 1- Trypan blue staining image of *V. longisporum* infection in *Arabidopsis thaliana* cv. Columbia, 48 hpi during root colonization, xy = xylem, hy= hyphae.

The number of up-regulated genes increase during the time course experiment, reaching the strongest overall at 72 hpi. Using cutoff value of 1.5 the number of up-regulated genes are three times higher, as shown in the Venn diagram; 50 genes were up-regulated throughout the experiment. At 24 hpi, 65 genes were already up-regulated in response to Verticillium, although, as previously shown, *Verticillium* was not visualized inside of the root system.

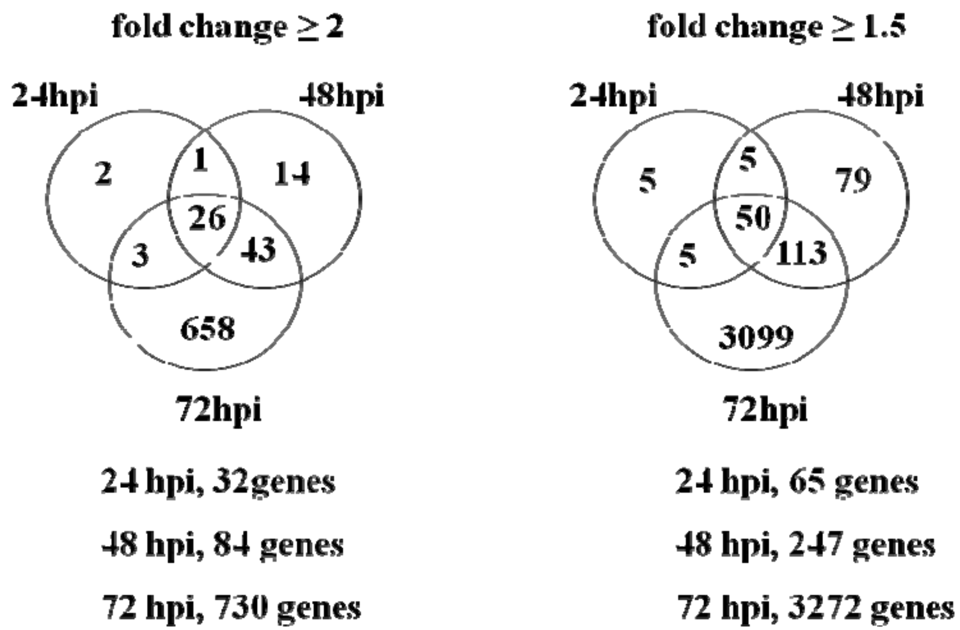

Figure 2- Venn diagram of the numbers of overlapping and non-overlapping induced genes on the array with fold change higher than 1.5 and 2.0 after 24, 48 and 72 hour post-inoculation with *Verticillium longisporum* in *Arabidopsis thaliana* cv. Columbia.

The number of genes up-regulated at 48 hpi was 247, but the highest number of genes up-regulated was observed at 72 hpi. The number of genes up-regulated at that time was 3272.

As shown in figure 3, *V. longisporum* was able to grow in Columbia, corroborating the microscopy results that show the increase of hyphae content in plants throughout the experiment. The amount of *V. longisporum* continued to increase during the experiment, showing that Columbia was able to reduce the growth of *V. longisporum* until 72 hpi.

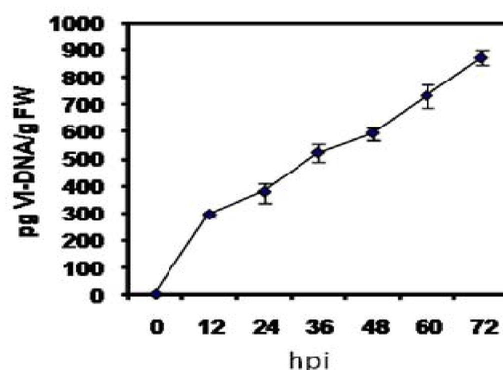

Figure 3- Estimation of *Vl* growth by real-time PCR using root tissue of seedlings with *Vl*. For this analysis, samples were collected at 0, 12, 24, 36, 48, 60 and 72 hpi.

Figure 4 shows a summary of the functional categorization of the up-regulated genes from samples collected at 24, 48 and 72 hpi. Most cellular processes are spatially restricted to defined regions of the cell. The results of this study shows a high number of genes codified for protein located in the extracellular space at 24 and 48 hpi. However, at 72 hpi, a larger number of genes codifying proteins with nuclear location was identified.

This higher number of genes codifying proteins with nuclear locations may be related with the higher level of transcription factor active at 72 hpi. According to data shown in Figure 4, an increase on transcription factor activity was visualized at 72 hpi. This transcription factor regulating the expression of genes, and transcription factor and DNA associated and DNA-binding proteins are nuclear, for example the MYB and WRKY families.

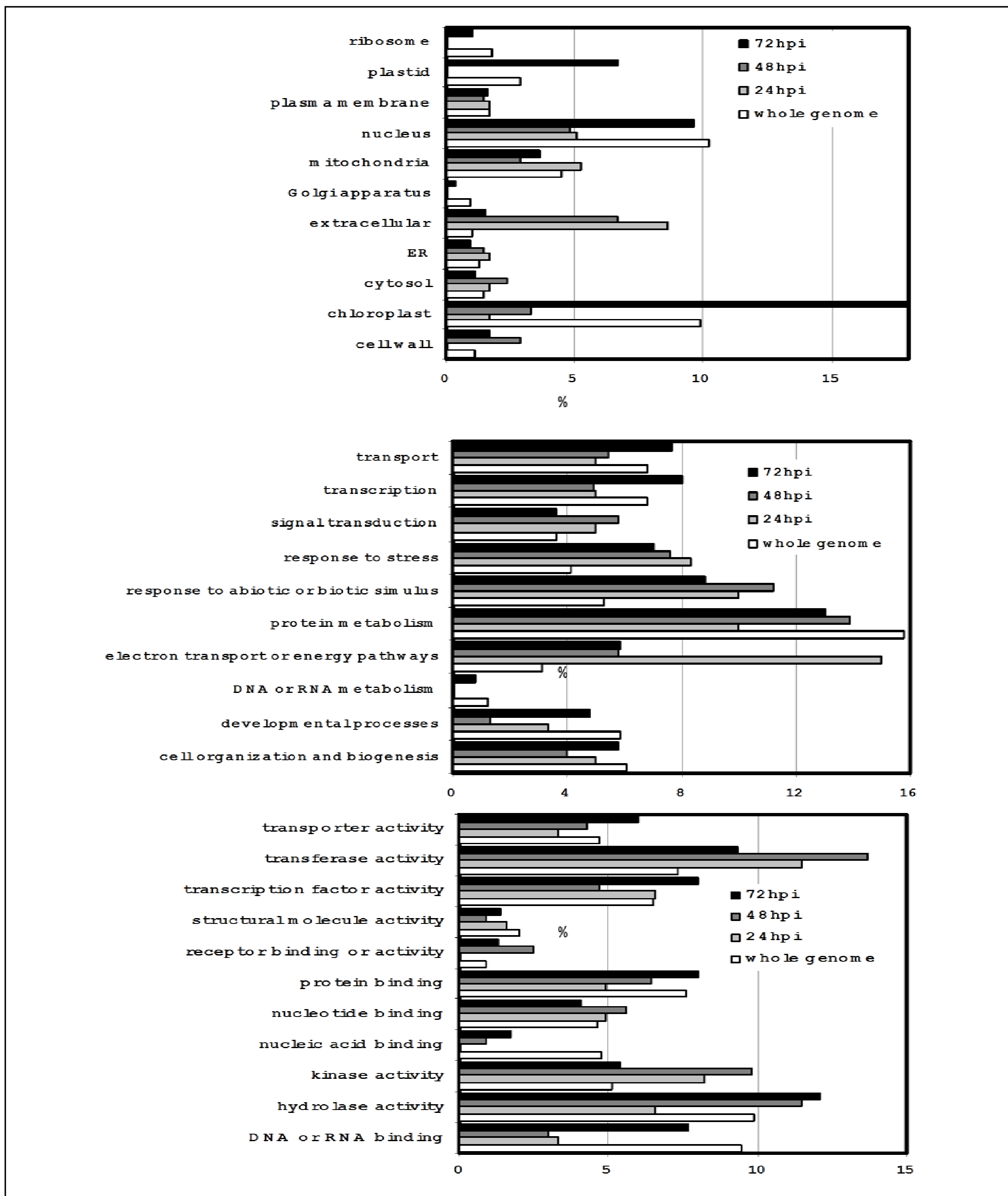

Figure 4- Functional distribution of responsive genes in *Arabidopsis thaliana* cv. Columbia. The x and y axes indicate the percentage of genes and functional categories, respectively. The genes were categorized for the three time-points , 24, 48, 72 hpi and whole genome

Genes with predicted function of response to abiotic and biotic stimulus were higher at 24 hpi and, subsequently, there was a reduction of percentage of gene predicted to be involved in response to abiotic and biotic stimulus, culminating with the lowest level at 72 hpi. A shift in plant defense strategy reflecting from *V. longisporum* suppressing defense in *Arabidopsis* was previously reported, where PR-1, PR-4 and PDF1.2 were induced early in this pathogen interaction and suppressed at later stages of infection (Johansson, 2006).

Among the up-regulated genes indentified in microarray data, eight genes were involved in tryptophane metabolic pathway, ASA1, TSB2, CYP79B2, CYP71A13, CYP71B15, besides the transcription factors MYB34, MYB51 and MYB122. This trypthophe metabolic pathway leads to indolic glucosinolate and camalexin as well.

Synthesis of indolic glucosinolates in *A. thaliana* starts with the conversion of precursor amino acids to their corresponding aldoximes by substrate-specific cytochromes P450 belonging to the CYP79 family. The post-aldoxime enzymes, although specific for the (Glucosinolates) GS pathway, have low substrate specificity for the amino acid side chain and efficiently convert even exogenous aldoximes to the corresponding GSs (Mikkelse *et al.*, 2002).

Several studies have suggested that GSs or rather their degradation products are involved in plant defense against insects and pathogens. Upon tissue disruption, as a consequence of wounding or pathogen attack, the GSs, which are stored in the vacuole, are hydrolyzed by endogenous β-

thioglucosidases (myrosinases) (Rask *et al*., 2000) to primarily nitrilas and isothiocyanates (ITCs).

Less documentation exists on the role of GSs in plant-plathogen interaction. *In vitro* studies have demonstrated that ITCs can inhibit growth of fungal and bacterial pathogens (Brader et al., 2001). It has been demonstrated that volatile compound released from glucosinolate affect the microsclerotium germination of *in vitro Verticillium longisporum* using macerate from broccoli, cauliflower and Indian mustard (Debode, 2005).

The incorporation of extract of *Brassica napus* with high content of glucosinolate into the soil showed to be effective in reducing the incidence (81%), wilt severity (80%), and vascular discoloration (70%) in tomato plants inoculated with *Verticillium dahliae.*

Specific breakdown glucosinolate products can be important to inhibit *Verticillium* infection. Glucosinolates can be converted to volatile compounds, differential responses of volatiles from the true leaves of broccoli, cauliflower and Indian mustard (Brassica juncea) on mycelium growth and germination of microsclerotia of *V. longisporum* have been reported (Debode *et al*., 2005). In this *in vitro* assay, Indian mustard showed the most antifungal effects, especially on microsclerotium germination, compared with the two *B. oleracea* genotypes.

Indolyl glucosinolate, a compound generated during hydrolysis of glucosinolate, at more acid pH can form indolyl-3-acetonitrile and elemental sulphur (S) (Mithen, 2001). This elemental sulphur is well documented in certain specialized prokaryotes, but has rarely been detected in eukaryotes.

Elemental S was first identified in laboratory as a novel phytoalexin in the xylem of resistant genotypes of *Theobroma cacao*, after infection by the vascular, fungal pathogen *Verticillium dahliae* (Resende, 1996).

4.2 VALIDATION BY qRT-PCR

Microarray data was validated by qRT-PCR and several genes of the tryptophan pathway were analyzed at three time points, 24, 48 and 72 hpi. For qRT-PCR, samples of root tissue inoculated and non-inoculated with *Verticillium longisporum* were harvested to proceed RNA extraction.

In response to *Vl* inoculation genes of the tryptophane pathway were up- regulated. This pathway leads to expression of two secondary metabolites, camalexin and glucosinolates. In Figure 5, it is possible to monitor the expression of genes that leads to camalexin accumulation. Genes leading to camalexin were up-regulated during the time course experiment,. All these genes, ASA1, TSB1, CYP79B1, CYP71A13, CYP71B15, were up-regulated, reaching at least 4.9 fold changes, as illustrated in Figure 5.

Interestingly, the same magnitude observed in genes leading to camalexin accumulation was not the same as observed in the branch that leads to glucosinolate accumulation, CYP83B1 is an enzyme that is responsible for the synthesis of indolic glucosinolate from indol-3-acetaldoxime (IAOX). A co-regulation of indolic glucosinolate pathway enzymes and tryptophan biosynthetic enzymes has been observed by analyzing microarray data derived from different stress experiments (Gachon *et al.*, 2005). Hence, the activation of the tryptophan biosynthetic pathway seems to be required for providing sufficient levels of the precursor

tryptophan for the increased formation of indolic glucosinolates in response to stress (Gachon *et al.*, 2005).

Some transcription factors have been identified as regulators of tryptophane pathway and indolic glucosinolate as well. Overexpression of MYB122 transcription factor in the wild-type background led to the enhanced transcription of several tryptophan pathway genes, including ASA1, TSB1, CYP79B2, CYP79B3 and CYP83B1, along with elevated auxin levels (Gigolashvili, 2007).

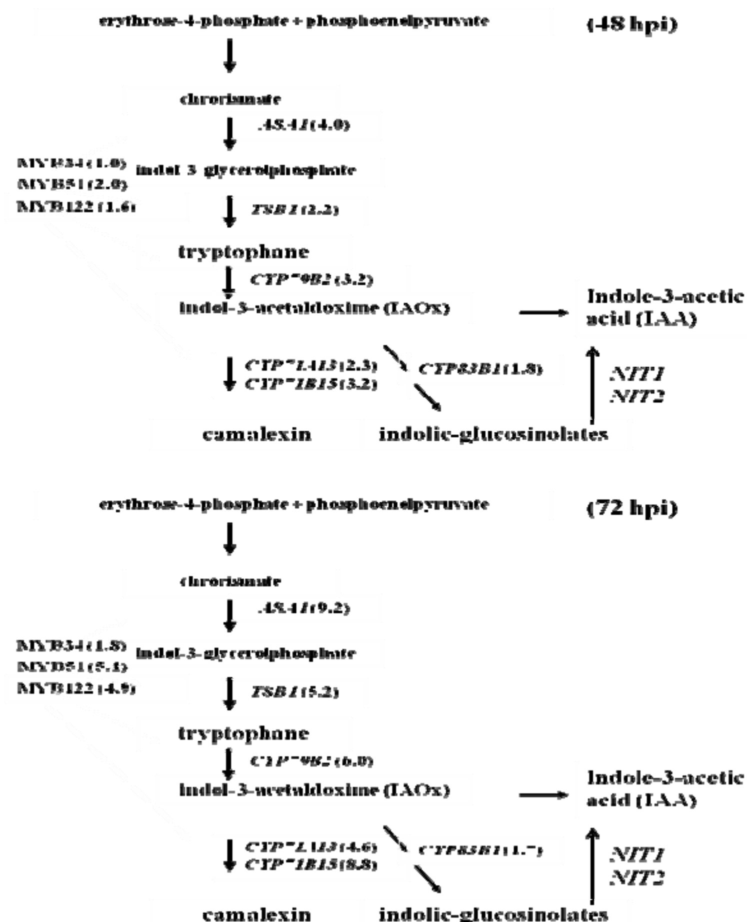

Figure 5- Effects of *Verticillim longisporum* on tryptophane metabolic pathway genes. Gene expression fold changes upon *Vl* infection as detected by qRT-PCR in parenthesis. Analysis of gene expression in root tissue collected at 24, 48, 72 hpi. Adapted from Tamara Gigolashvili 2007.

All genes mentioned before, except *ASA1*, were activated by MYB51, another transcription factor that positively regulates indolic glucosinolate biosynthetic pathway genes. MYB51 can activate genes both upstream and downstream of IAOx, leading to increased indolic glucosinolate levels without a significant effect on IAA (indolic-3-acetic acid) contents. Besides these two transcription factors, MYB34 is also involved in tryptophan pathway induction and high levels of IAA (Gigolashvili, 2007).

Recently, a mutant *pad3* (CYP71B15), in which camalexin production is completely abolished, did not show any changed response, which shows that camalexin is not a key component in this defense system (Johansson, 2006).

Tjamos and associates reported the isolation of a plant-growth-promoting rhizobacterium (PGPR) strain, which was identified as *Paenibacillus alvei* and designated K165, with biocontrol activity against *V. dahliae* in glasshouse and field experiment. In this experiment, phytoalexin deficient *pad3* (camalexin deficient) was inoculated with K165 and the percentage of diseased leaves as compared with the controls was reduced significantly. Thus, camalexin was not involved in protection against *V. dahliae* mediated by strain K165 (Tjamos, 2005).

4.3 METABOLITE ANALYSES

To confirm data validated by qRT-PCR, analysis of secondary metabolites was conducted by collecting samples of root tissue from Col-0 inoculated with *Verticillium longisporum* at 24, 48 and 72 hpi. The samples were analyzed by LC-MS/MS for indolic glucosinolate and camalexin.

According to qRT-PCR results, seedlings inoculated with *Vl* showed induction of genes leading to camalexin and repression of CYP83B1 that results in indolic glucosinolate accumulation (Figure 6).

Due to the lower level of induction in the genes of camalexin biosynthesis at 24hpi, it was not possible to quantify any camalexin at this time point. However, at 48 hpi some camalexin was detected, although at 48 hpi the fold change of genes of camalexin biosynthesis showed a fold higher than 2.0, which may be due sensitivity of the method.

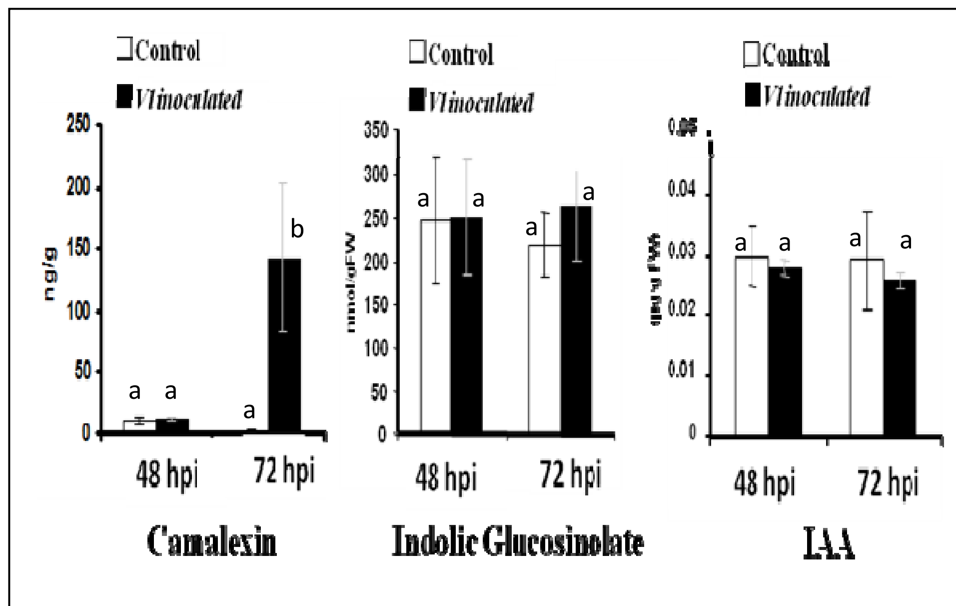

Figure 6- Tryptophan metabolites content of *A. thaliana* Col-o in response to *Vl* inoculation after 48 and 72 hpi. White bars correspond to samples collected from non-inoculated seedlings and black bars correspond to samples collected from inoculated seedlings. Values correspond to the mean of three replicates $\pm$ standard error. IAA (Indole acetic acid). Means followed by the same letter are not statistically different (p> 0.05)

On the other hand, at 72 hpi a strong induction of camalexin was detected in response to *Vl*, this result is in agreement with qRT-PCR data,

that showed induction of genes leading to camalexin accumulation. The level of camalexin in inoculated seedlings was statistically different from non-inoculated seedlings, showing an alteration of camalexin content exerted by the pathogen. As mentioned before, camalexin does not confer any resistance to *Verticillium*. Differently, camalexin inhibits *in vitro Botrytis cinerea* growth in a dose-dependent manner, and completely blocked it at a concentration of 50µg $ml^{-1}$of purified form (Ferrari, 2003)

In contrast, no statistical difference was found between inoculated and control seedlings for indolic glucosinolate accumulation at both time points. However, transcription factors, such as MYB51, MYB122 and MYB34, were induced in response to *Vl* in comparison with non-inoculated seedlings. Assume that there is another control involved in response to *Vl* preventing indolic glucosinale accumulation.

We also show in this study the quantification of typical indole glucosinolates, indole-3-yl-methil (I3M), 1-methoxy-indole-3-yl-methyl (1MO-I3M) and 4-methoxy-indole-3yl-methyl (4MO-I3M) (Figure 7). I3M has been described as the most abundant indole glucosinolate in *Arabidopsis* (Pfalz et al., 2009)

Although it has not been measured in this study, 4-hydroxy-indole-3-yl-methyl (40H-I3M), an indolic glucosinolate, has been demonstrated to be generated from the conversion of I3M by the CYP81F2 gene-encoded enzyme. CYP81F2 was also identified in microarray results, this gene was induced by *V. longisporum*. The CYP81F2-encoded enzyme is responsible for a previous step of myrosinase action (PEN2).

The myrosinase-mediated degration of glucosinolates gives rise to an unstable thiohydroximat-*O*-sulphonate which, on release of sulphate, can result in the production of isothiocyanates, thiocyanates, nitriles and elemental sulphur, depending on the concentration of H^+ and other factors (Bones, 1996)

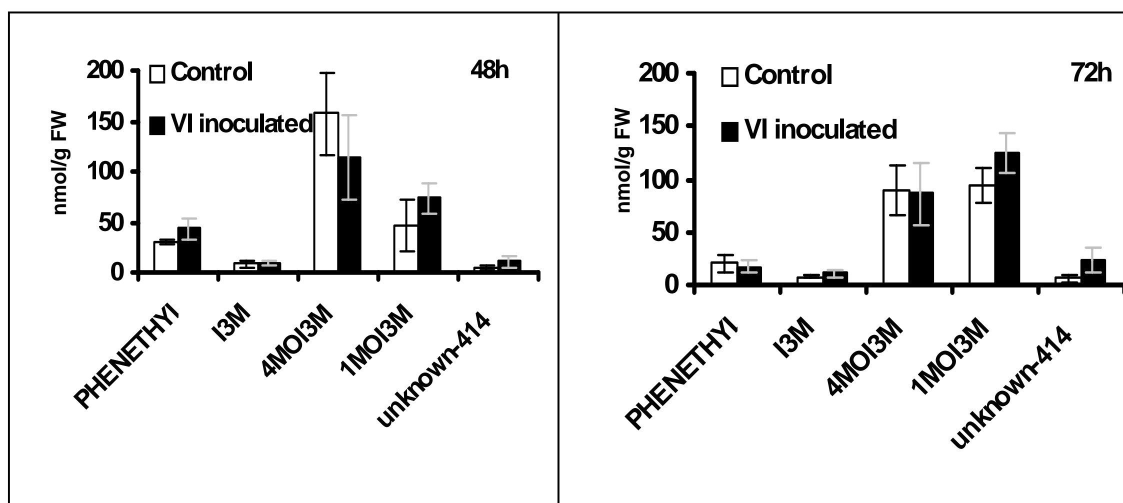

Figure 7- Indolic glucosinolates contents in *Arabidopsis thaliana* (Col-0) inoculated and non-inoculated with *V. longisporum*. I3M (indol-3-yl-methyl-GS, 4MOI3M (4-methoxyindol-3-ylmethyl-GS, 1MOI3M (1-methoxyindol-3-ylmethyl-GS).

Low level of indolic glucosinolate can be the cause of *Vl* infection in Col-o seedlings. Recently, sulphur elements (S) have been involved in defense against *Verticillium*. Glucosinolates are a class of sulphur-containing secondary metabolites present in all cruciferous.

An investigation of chemical defenses in resistant lines of *Theobroma cacao* against the xylem-invading fungal pathogen *Verticillium dahliae* revealed a typical multiple phytoalexin response comprising four antifungal compounds (Cooper *et al.*, 1996 ; Resende *et al.*, 1996). Extracted xylem

contained a terpenoid and two phenolics. The most hydrophobic and fungitoxic compound unexpectedly proved to be elemental sulphur.

S^0 appeared in *T. cacao* and tomato in a relevant tissue and cellular location, at the right time and in sufficient amounts to be implicated in a defense role, mediated by inhibition of the pathogen *V. dahliae* (Cooper *et al*., 1996).

Subsequently, S^0 production has been reported to be linked with active defense in another three important plant families, and potentially with preformed defense in another family: Leguminosae, Malvaceae, Solanaceae, and Cruciferae (Williams *et al*., 2002; Williams and Cooper, 2003).

S^0 was detected from xylem excised from plants inoculated with fungal vascular pathogens: cotton and tomato to *V. dahliae*, French bean and tobacco to *Fusarium oxysporum* forma specialis, and tomato to the bacterial vascular pathogen *Ralstonia solanacearum* (William and Cooper, 2003). The pattern and kinectics of S^0 formation mirror that of many organic phytoalexins, with a faster and greater production in disease-resistant genotypes than in susceptible lines, and with no or negligible amounts in control tissue (Mansfield, 2000; Kuhn and Hargreaves, 1987).

With the exception of members of the Cruciferae, the response may be specific to xylem. S^0 was absent from leaves of six species showing hypersensitivity to incompatible isolates of *Pseudomonas syringae* pathovars. Other than lettuce, barley, and cabbage, these included species which had produced S^0 in xylem challenged by vascular pathogens, i.e. tomato, tobacco, and French bean.

Genes expressed specifically in xylem are well documented and those of possible relevance include group 1 and group 2 sulphate transporters, the former in response to *Verticillium* (Howarth *et al*., 2003, Smith et al., 1995; Takahashi *et al*., 2000) and the I-2 gene in tomato for resistance to *F. oxysporum* (Mes *et al*., 2000).

Similarly to glucosinolate, IAA was not induced in response to *V. longisporum*, as shown in Figure 6, no statistical difference for IAA accumulation was observed between 48 and 72 hpi. This may be due to part of IAA being generated from indolic glucosinolate once it was induced, then the expectancy that IAA would not accumulate at a high level as well.

To answer how *V. longisporum* was altering the tryptophan pathway, two transcription factors were selected to be tested. The two transcription factors supposed to be involved in tryptophan pathway regulation are WRKY33 and WRKY70.

The *wrky33* mutants exhibit enhanced susceptibility to two necrotrophic pathogens, *Botrytis cinerea* and *Alternaria brassicicola*, whereas overexpression of WRKY33 increases resistance, suggesting a specific role of WRKY33 in plant defense against necrotrophic pathogens (Petersen, 2008)

A screening for putative target genes of WRKY33 was done, for this, four transcripts that accumulated in response to BRH (SA analogue) treatment in wild type, but not in *wrky33*. Two of these, PAD3 and CYP71A13, are strongly co-regulated and are required for the synthesis of antimicrobial

compound camalexin, which is important for resistance against necrotrophic pathogens (Petersen, 2008).

Selected to be involved in tryptophan regulation, WRKY70 was identified as negative regulator of indolic glucosinolate (IGS) accumulation. Earlier reports demonstrated a JA-dependent induction of biosynthesis of indolic glucosinolate in *Arabidopsis* (Brader *et al.*, 2001).

To examine the effect of WRKY70 on the regulation of, Li and collaborators assayed IGS contents in the WRKY70-over-expressing line S55 and control plants following MEJA treatment. A substantial reduction in MEJA-induced accumulation of IGS was detected in the WRKY70-over-expressing line, indicating that WRKY70 negatively regulates JA-induced production of IGS (Li, 2006).

To investigate the role of WRKY33 and WRKY70 in the modulation of tryptophan genes, the expression of CYP71B15 (PAD3) and CYP83B1 was analyzed in *wrky*33 and *wrky*70 mutants and Col-0 (Wild type).

As shown in Figure 8, the expression of PAD3 was statically the same in Col-0 and the *wrky*70 mutant. Loss of function in *wrky* 70 was not responsible for any alteration in PAD3 expression, as expected. However, loss of function in *wrky* 33 transcription factor showed a strong reduction of PAD3 expression.

This result confirms WRKY33 as a positive regulator of PAD3, a key enzyme that affects camalexin synthesis. In some way, not discovered yet, *Vertlicillium longisporum* recruits WRKY33 to induce camalexin accumulation whose function is not related with tolerance to *Vl*.

In contrast, to elucidate the control of the branch leading to indolic glucosinolate expression, the expression of CYP83B1 was analyzed in Col-0, WRKY33 and WRKY70. In this case, WRKY70 was shown to act not only regulating the expression of CYP83B1 but also the expression of PAD3.

The analysis of the *wrky*70 mutant allowed the visualization of an increase in CYP83B1 expression; the data demonstrate that WRKY70 is a negative regulator of CYP83B1, and consequently a negative regulator of indolic glucosinolate accumulation. The analysis of CYP83B1 shows some positive regulation by WRKY33, since the loss of function in WRKY33 reduced the expression of CYP83B1, at a level even lower than in Col-0.

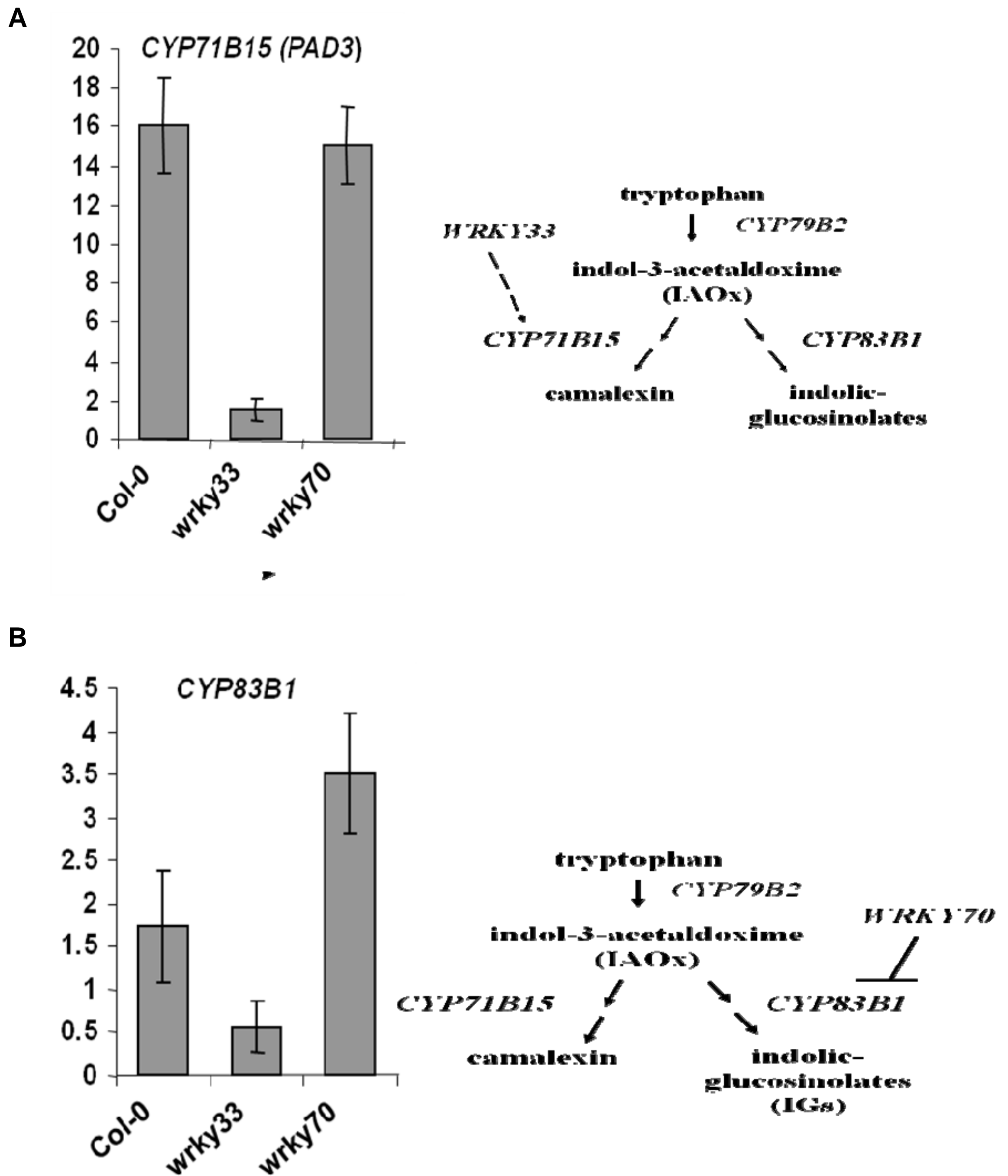

Figure 8- Absolute quantification done at real time. A - absolute expression of CYP71B15 (PAD3) . B - absolute expression of CYP83B1. Col-0 (wild type), *wrky33* (knockout in WRK33 genes) and *wrky70* (knockout in WRK70 gene).

5.0 CONCLUSIONS

Global transcription profiling and metabolite analysis of *Arabidopsis* root system upon colonization by the soil-borne vascular fungus *Verticillium longisporum* has lead to the identification of pathogen-induced effects on the host Trp metabolic pathway. In particular, in response to fungal colonization we observed strong activation of the transcription of Trp and camalexin biosynthetic enzyme genes and significant accumulation of camalexin. Remarkably, we did not detect induction of genes involved in the biosynthesis of the Trp-derived secondary metabolites IGs and IAA. Furthermore, metabolite profiling confirmed lack of pathogen-induced accumulation of both IGs and IAA.

Transcription profiling indicated that during pathogen challenge the transcription factor genes *WRKY33* and *WRKY70* were up-regulated as early as 24 hpi. The analysis of the expression of Trp, camalexin and IG biosynthetic enzyme genes in Col-0 wild type and *wrky33* and *wrky70* mutant knock-outs, confirmed *WRKY33* as a positive regulator of camalexin accumulation and *WRKY70* as a negative regulator of IG biosynthesis. We propose the capability of modulating the accumulation of the Trp-derived defense related secondary metabolites as a *V. longisporum* pathogenicity mechanism. As camalexin accumulation was shown not to play a major role in basal resistance against phytopathogenic *Verticillium* spp., the pathogen appears to be able to recruit *WRKY70* to selectively impair the induction of the accumulation of IGs.

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III CHAPTER 02

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED DURING INCOMPATIBLE INTERACTION

1. INTRODUCTION

Coffee leaf rust, caused by the fungus *Hemileia vastatrix* (Berlely & Broome) is a major disease of *Coffea arabica* L. Naturally rust resistant coffee varieties display a specific plant resistance response called hypersensitive reaction (HR), a form of a programmed cell death analogous to animal apoptosis (Lam, 2001; Martins, 1996). Resistance mechanisms associated with HR appear to be efficient particularly against biotrophic pathogens, such as rust fungi, which depend on the living host cells for their reproduction (Heath, 1997; Johal, 1995)

In coffee, the HR is manifested by death of subsidiary and guard cells of stomata where the fungus penetrates (Martins, 1996). In plant-pathogen interactions, activation of HR and plant defense response is mainly triggered through the enhanced transcription of numerous genes coding for proteins displaying a wide array of biological activities (Shenk, 2000). These transcriptional changes can be usually monitored within the first hours following pathogen inoculation (Sheideler, 2002).

During the HR, several genes have been shown to be activated leading to the establishment of a highly antimicrobial environment in and around the infected cell (Grant, 1999). Accordingly, plant defense genes against different pathogens have been associated with HR, including those involved in phytoalexin biosynthesis, production of pathogenesis-related (PR) proteins and lignin deposition (Tiburzy, 1990; Kemp, 1999; Snyder, 1990).

Cytological and biochemical studies have shown that coffee cultivars display an HR to *H. vastatrix* that is associated with callose haustoria

encasement, deposition of phenolic-like compounds and host cell wall lignification (Silva, 1999). The involvement of oxidative enzymes such as lipoxygenase and peroxidase and the stimulation of the phenylpropanoid pathway enzyme PAL and PR proteins, such as β-1,3-glucanases and chitinases have also been associated with the expression of resistance in some coffee cultivars (Guerra-Guimarães, 1993; Guzzo, 1996).

Enhancing *C. arabica* resistance to the rust fungus has become a goal for economic and sustainable coffee production. Híbrido de Timor (HT) was reported to be a natural hybrid of *C. arabica* and *C. canephora* that was spotted in an arabica coffee field in Timor Island. Some plants of the interespecific hybrid HT belong to the physiological group A, this group correspond to plants resistant to all the known races of *H. vastatrix*. This hybrid is a source of resistance to coffee leaf rust and other coffee diseases and has been extensively used in breeding programs, including the two genotypes, CIFC-832/1 and CIFC-832/2.

The aim of the present study was to identify genes from HT involved in the resistance to the fungus using the suppression subtractive hybrization (Diatchenko *et al.*, 1996).

2.0 MATERIALS AND METHODS

Four plants of HÍbrido de Timor CIFC 832/2 were grown in greenhouse conditions. This genotype belongs to the physiological group A and is resistant to all the known races of *H. vastatrix*. Two young coffee leaves fully developed were inoculated by spreading urediospores of *H. vastatrix* race II with a camel hairbrush on the abaxial surface. Distilled water was then sprayed on the inoculated surface and the plants were kept in a dark moist chamber at 22^0C to allow spore germination (D'Oliveira, 1957). Two leaves from another plant were mock-inoculated by spraying distilled water on the abaxial surface and used as control.

Leaves were removed from the moist chamber at 12 and 24 hours post-inoculation (hpi). Leaves were frozen by immersion in liquid nitrogen and stored at -80^0 until RNA extraction.

2.1 RNA EXTRACTION AND CDNA SYNTHESIS

Following tissue grinding, total RNA was extracted using Plant RNA Purification Reagent (Invitrogen), according to the manufacturer´s protocols. Quality and concentration of RNA were checked on agarose gel and by absorbance measurements at 230, 260 and 280 nm. The mRNA was obtained using the Dynabeads® mRNA Purification Kit (Dynal Biotech-Invitrogen) following the manufacturer's instructions.

The cDNA was synthesized from mRNA using the SMART-PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions.

2.2 SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION (SSH)

SSH libraries (Diatchenko et al., 1996) were carried out using the Select cDNA Subtraction Kit (Clontech) according to manual instructions. Libraries were constructed using cDNA from RNA samples collected at 12 and 24 hpi. Two libraries, forward and reverse, were constructed for each time point. Forward libraries were constructed by collecting RNA from inoculated HT (Tester) and subtracting from non-inoculated HT (Driver). We also performed a reverse subtraction, using non-inoculated plants as the Tester and inoculated plants as the Driver, in order to confirm differentially expressed genes.

We named these four libraries as follows: HT 12 F, corresponding to the library obtained from mRNA extracted from HT inoculated plants collected at 12 hpi (HTi12) and subtracted from mRNA collected from HT non-inoculated 12 hpi (HTni12) ; HT 12 R, corresponding to the library obtained from mRNA extracted from HTni12 plants and subtracted from mRNA extracted from HTi12 plants. For 24-hpi libraries we used the same kind of description, where: HT 24 F corresponds to the library obtained from HTi24 RNA samples subtracted from HTni24 RNA samples; and HT 24 R corresponds to the library obtained from HTni24 RNA samples subtracted from HTi24 RNA samples.

All PCR amplifications were carried out using the Advantage cDNA PCR Kit (Clontech). After digestion with *RsaI*, the tester cDNA preparation was divided in two subpopulations, which were ligated to different adaptors. The two subpopulations were hybridized with an excess amount of driver cDNA, after which they were combined and hybridized again in the presence of driver cDNA, without denaturing the DNA before the second hybridization. Following the second hybridization, two rounds of PCR were performed to enrich and amplify the differentially expressed sequences.

PCR products from subtracted cDNA were cloned into pGEM-T easy vector (Promega) and used for the transformation of *Escherichia coli* DH5α by heat shock transformation. Transformed colonies were selected in LB (Luriaa-Bertani) medium containing ampicilin (200mg/ml), X-GAL (20mg/ml) and IPTG (Isopropyl-β-D-thiogalactoside). Subsequently, the white colonies were transferred to a liquid medium using a toothpick and agitated overnight at 37°C. The stock colony was prepared by transferring 300ml of culture to a 1.5ml Eppendorf tube together with 300ml of glycerol 50%. The mixture was quickly frozen in liquid nitrogen and stored in a freezer at -80 ºC.

2.3 PURIFICATION OF PLASMID DNA

To extract plasmid DNA, a small piece of stock colony was transferred, using a toothpick, to 5 ml of LB medium containing ampicilin (200mg/ml) and agitated for 24 hs. In order to obtain plasmid DNA, the Wizard DNA Extraction Kit (Promega) was used according to the manufacturer's recommendation. We performed PCR in candidate clones to detect the presence of DNA inserts

in the plasmid. Presence and size of cloned fragments were examined by PCR using the primers SP6 and T7 from the pGEM- Teasy vector.

2.4 SEQUENCING AND CLONE ANALYSIS

The inserts of differentially expressed clones were amplified by PCR using the M13 universal primers and sequenced with MegaBace 1000. To target pGEM-T fragments easily, DNA sequences were subjected to UniVec database (NCBI VecScrem). BLASTX (Basic Local Alignment Search Tool) searchs were performed at the Genomics and Expression Laboratory (LGE) – the Brazilian Coffee Genome Project (Vieira *et al*., 2006).

To identify redundant genes in the forward and reverse libraries, a local BLAST was performed. All genes from forward were BLASTed against genes from the reverse library, then only the non–redundant genes were classified as differentially expressed. Sequences were also classified into functional categories using Blast2GO and MIPS (Munich Information Center for Protein Sequences)

2.5 VALIDATION OF DIFFERENTIAL GENE EXPRESSION BY QUANTITATIVE RT-PCR

We performed quantitative real-time PCR (qRT-PCR) on selected genes to validate differential expression data. In this experiment we collected samples from HT plants and the rust susceptible *C. arabica* ‘Catuaí IAC 44’ plants. In both cases, samples from inoculated and non-inoculated plants were collected. The samples were collected at 12, 24, 48 and 72 hpi by immerging the leaves in liquid nitrogen.

For these experiments, we chose candidate genes involved in response to pathogen by consulting the recent publications. Specific gene primers were designed using DNA sequences from the Brazilian Coffee Genome Project - LGE .

RNA was isolated using Plant RNA Purification Reagent (Invitrogen). Template cDNA samples were prepared according to the Taqman ® Reverse Transcription Kit protocol (ABI, Foster City, CA) using 1µl of total RNA 200ng/ µl and random hexamer as primer.

Quantitative PCR was run according to the manufacturer's protocol (SYBR Gren, Invitrogen). The ubiquitin gene was used as reference, as it showed no variation in expression level in both treated and untreated leaves. The cDNAs from first-strand reaction were used as a template for amplification in qRT-PCR with specific primers that were designed based on the coffee sequences obtained by SSH. The specific coffee primers (forward-F and reverse-R) were synthetisized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The cycling conditions comprised: 1 cycle at 94^0 for 2 min, followed by 45 cycles at 94^0C for 10s, annealing at 60^0C for 30 s and extension at 72^0C for 20 s. Reaction specificity was confirmed by obtaining dissociation curves for each reaction. Two biological replicates were used, and all samples were amplified in triplicate from the same RNA preparation.

The threshold cycle (Ct) values of the triplicate PCRs were averaged and relative quantification of the transcript levels calculated using the ΔΔCt method (Livak and Schmittgen, 2001), where ΔΔCT= (ΔCT sample-ΔCT

calibrator) = [(CT sample – CT housekeeping gene) – (CT calibrator – CT housekeeping gene)]. Sample corresponds to samples collected from inoculated plants and calibrator to samples collected from non-inoculated plants. The housekeeping gene corresponds to a gene constitutively expressed, in this case we used ubiquitin, since during the time course of the experiment no expression alteration was observed for this gene. The relative quantification data obtained for each target gene was used to calculate the standard error.

3.0 RESULTS

Four SSH libraries were constructed with RNA, the libraries were named,HT 12 F, HT 12R, HT 24 F and HT 24 R. To identify differentially expressed genes, leaf tissue was collected at 12, 24 hpi and, simultaneously, leaf tissue was harvested from control (mock-inoculated) plants. For each library a different number of candidate clones to represent a differential expressed gene was obtained (Table 1). The size of the fragments identified ranged from 175 bp to 1340 bp. The reverse libraries were constructed to confirm differentially expressed genes.

Table 1. Number of clones obtained from each SSH library

Library (F/R[a])	Description[b]	Number of candidate clones
HT 12 F	HT 12hs inoculated subtracted from HT 12hs non-inoculated	104
HT 12 R	HT 12hs non-inoculated subtracted from HT 12hs inoculated	142
HT 24 F	HT 24hs inoculated subtracted from HT 24hs non-inoculated	151
HT 24 R	HT 24hs non-inoculated subtracted from HT 24hs inoculated	131

[a]F corresponds to Forward and R Reverse

[a] HT 12 F (corresponds to SSH forward library obtained at 12 hs post-inoculation with *H. vastatrix*)

[a] HT 12 R (corresponds to SSH reverse library obtained at 12 hs post-inoculation with *H. vastatrix*)

[a] HT 24 F (corresponds to SSH forward library obtained at 24 hs post-inoculation with *H. vastatrix*)

[a] HT 24 R (corresponds to SSH reverse library obtained at 24 hs post-inoculation with *H. vastatrix*)

[b] HT 12 hs (RNA sample collected from Híbrido de Timor leaves 12hs post-inoculation or mock-inoculation). [b] HT 24 hs (RNA sample collected from Híbrido de

Timor leaves 24hs post-inoculation or mock-inoculation) with *H. vastatrix*, and colleted 12hs and 24 hs after inoculation; HTNI 12hs and HTNI 24hs- cDNA obtained from Híbrido de Timor leaves mock inoculated.

Of the 104 candidate clones from the HT 12 F library, 96 contained inserts confirmed by PCR and 90 of them were successfully sequenced. Sequence analyses of those clones with insert revealed that 58 (64.4%) showed similarity with genes found in the database, whereas 32 (35.6%) showed no significant gene homology. Sixty-eight (68) clones from the HT 12 R library were analyzed by PCR and 57 showed harbored inserts. Based on sequence results and BLAST analysis, 29 (50.8%) clones showed significant homology with genes deposited in the database and 28 (49.2%) showed no significant homology (Table 2).

Table 2. Summary of number of clones analyzed, sequenced and identified in the database

Library	Number of clones analyzed	Clones with insert	Sequenced	Identified on BLAST X
HT 12 F	96	90	90	58
HT 12 R	68	57	57	29
HT 24 F	78	76	76	46
HT 24 R	54	54	54	43

Among the HT 24 F library, 76 clones of 78 had inserts: all of them were sequenced and 46 (60.5%) showed similarity with genes deposited in the

database, whereas 30 (39.5%) sequences showed no similarity with genes deposited in the database. The number of clones analyzed from the HT 24 R library was 54, all of them had inserts: 43 (79.6%) had similarity with genes deposited in database, whereas 14 (20.4%) clones had no similarity with genes deposited in the database analyzed in our study (Table 2).

A total of 28 non-redundant and differentially expressed genes were identified in the HT 12 F library (Table 3). Many of the differentially expressed genes were redundant and in these cases only one representative was selected. To identify these redundant genes, we used the Local Blast tools in each library. We also used the Local Blast to identify genes expressed in both libraries, forward and reverse. Genes present in both forward and reverse libraries were considered non-differentially expressed.

Among the 28 differentially expressed genes identified in the HT 12 F library, ESTs matched with genes involved in plant cell defense reaction, such as chitinase, cystein protease, metallothionein, ADP-Rybosilation factor and others. All the sequence is described in Table 3. Since we used different data base, different gene annotation was observed for a same gene. There is, in the tables, a correspondence between annotation and e-value to identify which annotation was found in a specific data base. Hypothetical protein comprised 46.4% of the genes identified in the HT 12 F library.

Table 3. Genes differentially expressed identified in the HT 12 F library and classified by MIPS

Number of ESTs	Blast X identification	Identities (%)
Oxidative burst		

01	Catalase	94.0
02	Cysteine protease 1 [Plantago major]	48.0
Stress response		
03	Fiber protein Fb25 [Gossypium barbadense]	92.0
04	Metallothionein I homolog - spotted monkey flower	85.0
05	metallothionein-like protein [Citrus unshiu]	96.0
Antimicrobial protein		
06	acidic class III chitinase SE2 [Beta vulgaris]]	91.0
Cell maintenance and development		
07	23kDa polypeptide of the oxygen-evolving complex of photosystem II [Cucumis sativus]	96.0
08	ADP-ribosylation factor [Arabidopsis thaliana]/	99.0
09	sedoheptulose-1,7-bisphosphatase	86.0
Protein Synthesis		
10	60S ribosomal protein L24 [Prunus avium]	94.0
11	60S RIBOSOMAL PROTEIN L34	98.0
Metabolism of lipids		
12	Chloroplast fatty acid desaturase 6 [Olea europaea) Glycine max	98.0
13	RNA polymerase II subunit (hsRPB10)-related [Arabidopsis thaliana]	73.0
Cell Biogenesis		
14	Endoxyloglucan transferase [Daucus carota]	95.0
Unclassified		
15	Hypothetical protein	96.0
16	Hypothetical protein (Arabidopsis thaliana)	75.0
17	Hypothetical protein	69.0
18	hypothetical protein F7H19.70	96.0
19	Hypothetical protein	100.0
20	Hypothetical protein	85.0
21	Hypothetical protein	96.0
22	Hypothetical protein	100.0
23	Hypothetical protein	61.0
24	Hypotetical protein	99.0
25	CCR4-associated factor 1-related protein	98.0
26	Hypothetical protein	93.0
27	Hypothetical protein	100.0

28	Hypothetical protein	93.0

In the HT 24 F library, 18 differentially expressed genes were identified. Genes related with plant response to pathogen were identified *in silico* (Table 4). For instance, chitinase, acid endochitinase precursor, poliubiquitina, serine carboxipeptidase and endoxyloglucan transferase were identified in this library. Chitinase was also identified in response to *H. vastatrix*, besides being identified at the HT 12 F library (Table 4).

Table 4. Differentially expressed genes identified in the HT 24 F library and classified by MIPS

ESTs	Blast X identification	Identities (%)
Antimicrobial protein		
01	Acidic Chitinase Classe III	99.0
02	Acidic chitinase protein precursor	100.0
03	Acidic endochitinase precursor remover	100.0
Stress response		
04	Aquaporina	98.0
Regulated protein degradation		
05	Polyubiquitin	96.0
Detoxification		
06	Glyoxalase I	100.0
07	Thioredoxin	98.0
Transporter		
08	purine permease, putative	100.0
Cell wall maintenance		
09	GBR5/glycine-rich protein	100.0
Metabolism		
10	acyl CoA synthetase	96.0
11	Aldo/keto reductase/	84.0
12	Hypothetical protein	79.0
Light response		
13	Photosystem II 5 kDa protein, Ankyn,	82.0
Unclassified		
14	Hypothetical protein	100.0
15	Hypothetical protein	97.0
16	Hypothetical protein	100.0
17	Hypothetical protein	98.0
18	Serina carboxipeptidase	100.0

Cysteine protease was not isolated from 24 hpi libraries, however, a putative cysteine proteinase inhibitor was identified in HT 24 F, which may

explain the reason why cysteine proteinase was not identified in 24 hs libraries.

All the non-redundant genes identified in these four libraries were classified into functional categories using Blast2Go. We were able to assign putative function to 63 genes. The other 42 ESTs encode proteins with insufficient sequence similarity to those with known function, therefore they were regarded as unclassified genes

The genes of known function were classified into 18 categories whose distribution is represented as a pie chart in Figure 1. The largest group obtained with known function in the HT 12 F library was allocated to stress response (11%) and cell maintenance (11%) followed by antimicrobial protein oxidative burst/apoptosis (7%) and protein synthesis (7%). In contrast, genes involved in transcription (4%), cell biogenesis (4%) and metabolism of lipids (3%) were the smallest groups (Figure 1).

In the HT 24 F library the three largest groups were antimicrobial protein (17%), metabolism (17%) and regulated protein degradation (17%). Moreover, detoxification, transporter and stress response comprised 7% each group. The other two groups, cell wall maintenance (6%) and light response (6%) were the smallest group (Figure 2).

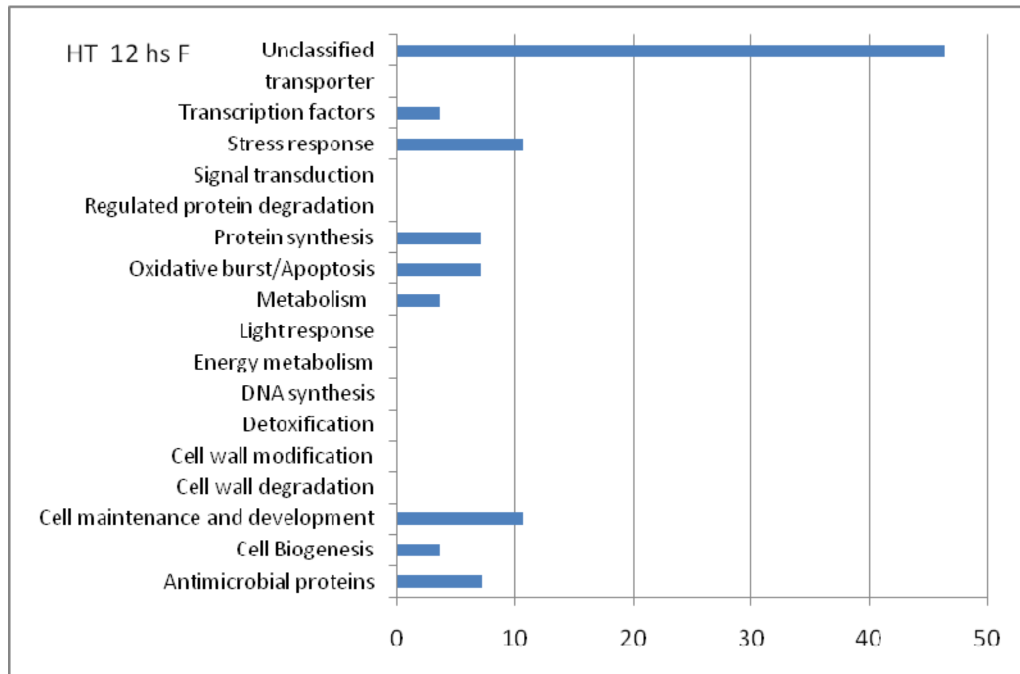

Figure 1. Classification of ESTs isolated from the subtractive cDNA library. ESTs from the HT 12 F library.

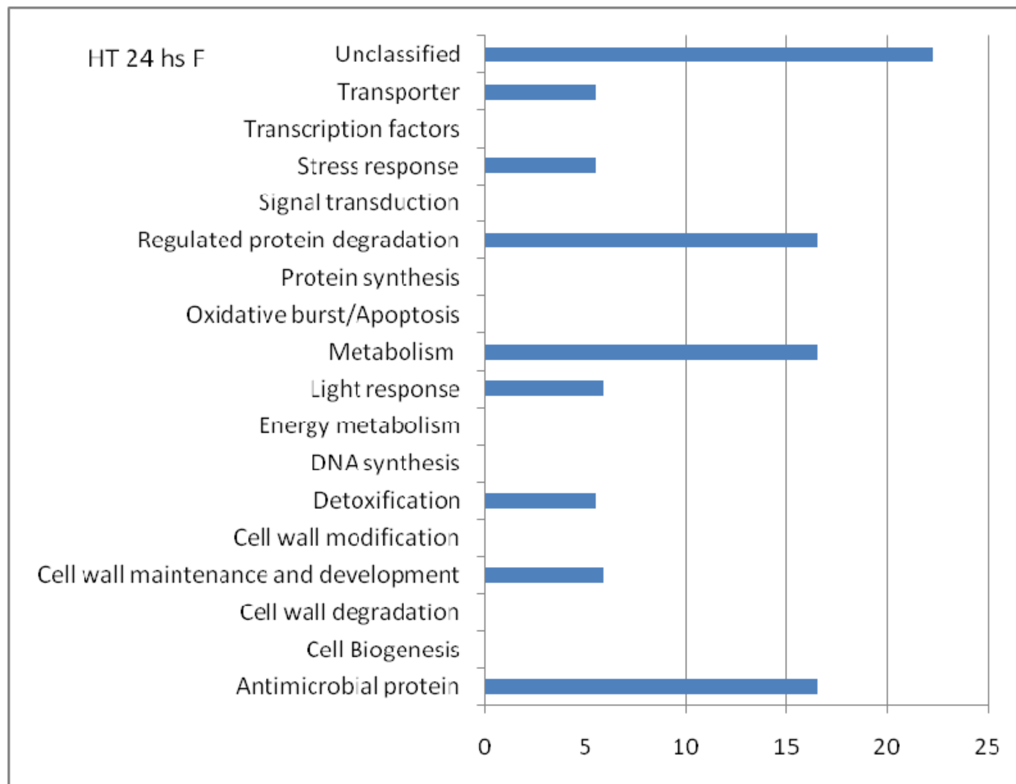

Figure 2. Classification of ESTs isolated from the subtractive cDNA library. : ESTs from the HT 24 F library.

3.1 VALIDATION BY QUANTITATIVE RT-PCR

Quantitative expression analysis by quantitative RT-PCR was performed for two selected genes, cysteine proteinase and chitinase. Cysteine proteinase was identified in the HT 12 F library and chitinase was identified in both, HT 12 F and HT 24 F libraries. These two genes were chosen because of their function in response to pathogen and for their function has already been confirmed in other pathosystem.

The temporal expression of the chitinase gene was quantitatively analyzed in four different time points, 12, 24, 48 and 72 hpi. The incompatible interaction between HT 832/2 and *H. vastatrix* showed the highest level at 24

hpi, the fold change at this time was 544.5. At 48 hpi, a strong reduction was observed, the relative expression at this time was around 6. Finally, the lowest relative expression level for chitinase was displayed at 72 hpi (Figure 3A).

However, for the compatible interaction between Catuai and *H. vastatrix,* the expression profile differed from the incompatible interaction. In this case the induction of chitinase gene was observed only at 48 hpi, although the induction occurred, the magnitude was not the same. At 48 hpi the fold change was 4.3, reducing to 3.5 at 72 hpi.

The other gene analyzed by quantitative RT-PCR was cysteine protease, with the maximum expression level in incompatible interaction being detected at 24 hpi (30 fold). The induction of cysteine protease occurred in both, compatible and incompatible interaction, however, a weak induction, in comparison with incompatible interaction, was observed at 48 hpi for compatible interaction and kept weak at the same level at 72 hpi (Figura 3 B).

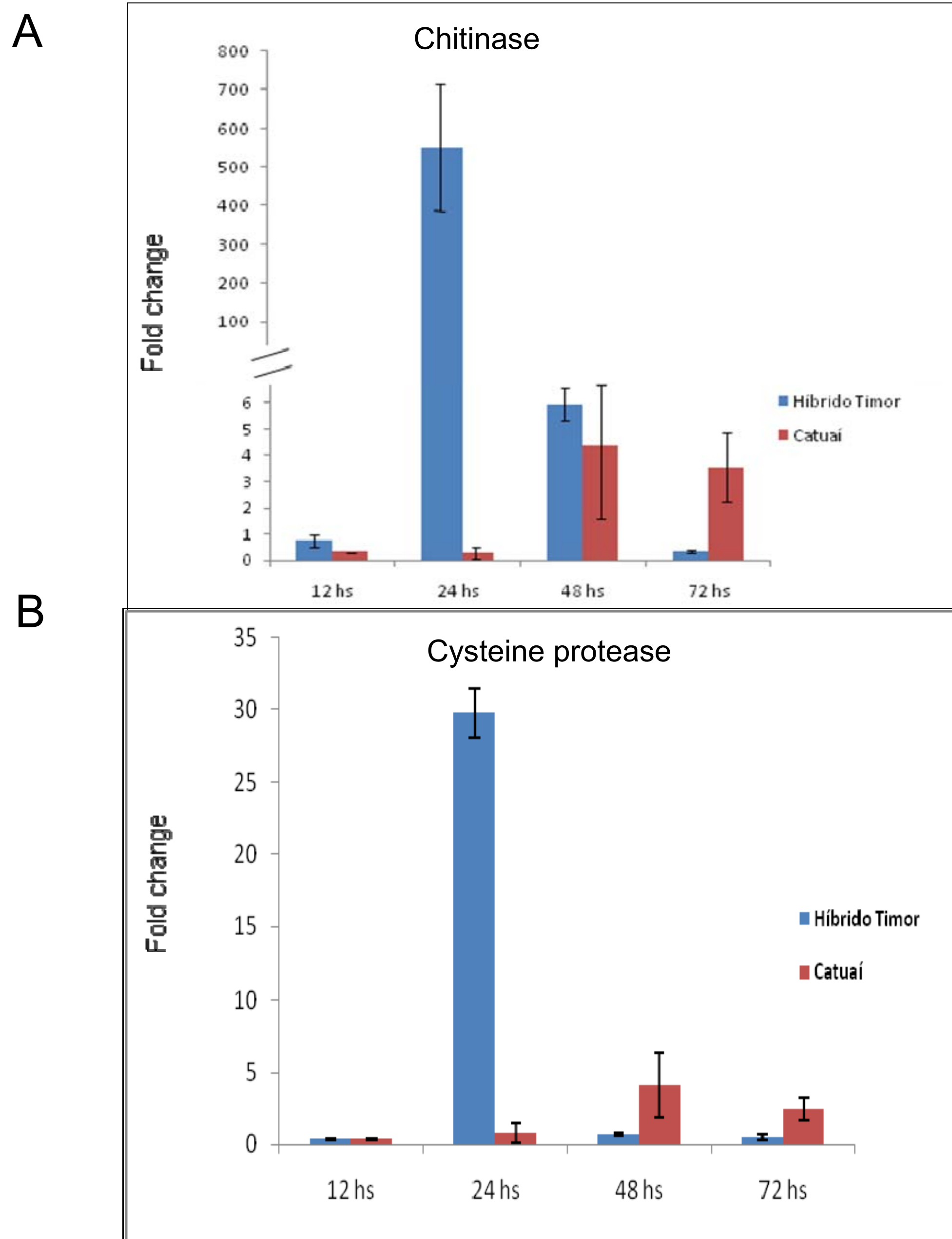

Figure 3. Quantification of relative expression level of coffee genes by real-time PCR evaluated in Hibrido de Timor (resistant) and Catuai (susceptible) at 12, 24, 48 and 72 hpi. Expression values are relative to meam control values (water-treat leaves ($2^{\Delta\Delta CT}$). A Expression analysis of chitinase gene. B Expression analysis of cysteine gene.

4.0 DISCUSSION

In this work, we attempted to carry out a global analysis of gene expression during an incompatible interaction between the rust fungus *H. vastatrix* and Híbrido de Timor, an interspecific hybrid of *C. arabica* x *C. canephora*, since differences in gene expression are responsible for both morphological and phenotypic diversities as well as indicative of cellular responses to environmental stimuli and perturbations (Eisen et al., 1998). The temporal gene expression profiles of coffee genes during the infection can provide evidence of genes responsible for the resistance and a basis for further looking into the plant-pathogen interaction and gene function.

Among the non-redundant clones obtained in the HT 12 F library at least 7% may be involved in oxidative burst/apoptosis and 7% antimicrobial protein. However, at 24 hpi, 17% of the analyzed genes encoded antimicrobial protein, higher percentage than observed at 12 hpi.

Some genes are involved in oxidative burst/apoptosis, such as catalase. The inhibition of catalase would lead to an increase in the concentration of hydrogen peroxide (H_2O_2) or active oxygen species. These compounds arise during respiration, photosynthesis, photorespiration or during the hypersensitive response against pathogen.

H_2O_2 may have a direct antibiotic activity against invading pathogen. H_2O_2 and its derivatives may also act as intermediates in the signaling cascade for the expression of genes related to defense (Chen et al., 1993; Durner et al., 1997).

Among the genes involved in cell maintenance and development ADP ribosylation factor 1 has been involved in resistance to bacterium. Recently, It

has been shown that Legionella, in a dot/Icm-dependent manner, secretes a guanine nucleotide exchange factor (RaIF) into the host cell. Dot/Icm proteins comprise a specialized secretion system that serves to transfer bacterial proteins into the cytosol of the target host cell (Vogel et al., 1998).

RaIF apparently alters the activity of host ADP ribosylation factor 1 (ARF1), thus allowing the bacterium to recruit ER-derived membranes as part of its strategy to remodel its vacuole (Nagal et al., 2002).

Cell biogenesis genes, for instance, endoxyloglucan transferase, was identified in our libraries. Xyloglucans are interlaced with cellulose microfibrills and act as a network providing tensile strength for the cell wall. In the context of the plant-pathogen interaction, the brackdown of the xyloglucan by fungal cellulases and xylosidases, spanning the space between cellulose microfibrills, could weaken the wall and provide increased access for fungal cellulases to degrade the cellulose microfibrills (Juge, 2006; Miedes et al., 2004).

Apart from the xyloglucan degrading enzymes, endoxyloglucan transferase has been proposed to have a dual role integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan, or restructuring existing cell wall material by catalyzing transglucosylation between previously wall bound xyloglucan molecules. An increase in endoxyglucan transferase accumulation during the defense reaction associated with the incompatible tomato- *Cuscuta reflexa* interaction has been reported (Alber et al., 2004)

A cell wall maintenance glycine rich protein gene was also identified in our library. The expression of glycine rich protein genes (AtGRP5 and

AtGRP23) were enhanced by SA (Salicylic Acid) and ABA (Abscisic acid) (Fauth et al., 1998). The enhanced expressions of AtGRP5 and AtGRP23 by HPA (15-hydroxypalmitic acid), one of the major components of cutin in plant tissues, has been demonstrated. It has been revealed that several cutin monomers, including HPA and their derivatives, effectively induced the accumulation of H_2O_2 in cucumber leaves (Fauth et al., 1998). These two GRPs are suggested to be involved in defense mechanism of *Arabidopsis* thaliana against pathogens mediated by cutin monomers.

The manner by which GRPs contribute to the mechanism of defense against pathogenic attack remains in question. AtGRP3 has been shown to bind to the extracellular domain of Wak1, a cell wall associated receptor kinase, and it has been suggested that the interaction of AtGRP3 with Wak1 occurs through a pathogenesis-related process (Park et al., 2001). In tobacco, a cadmium-induced GRP protein exerted its inhibitory effects against *Tobamovirus* by enhancing callose deposits in the vasculature, thus blocking the systemic movement of the virus (Ueki et al., 2002). Similar callose deposition was observed with an increase of LsGRP1 gene expression against a fungal pathogen, *Botrytis elliptica*, in lily plants (Lu et al., 2007). The most abundant antimicrobial protein found at 12 and 24 hpi was chitinase, of the class III acid chitinases, which are characterized by lysozyme activity. These chitinases can be considered as molecular markers of SAR response as they are known to be upregulated in response to bacterial or fungal infection (Busam et al. 1997; Lawton et al. 1994).

Indeed, class III acidic chitinases were differentially expressed in *C. arabica* during infection by *H. vastatrix* (Fernandez et al., 2004). Chitinase was previously associated with protection of plants against coffee leaf rust in susceptible coffee plants (cv. Mundo Novo) when treated with ASM (acibenzolar-*S*-methyl). ASM is an inducer of local and systemic resistance (Guzzo et al., 2004). Controversially, classe III chitinase was not identified in plants of Híbrido de Timor CIF 832-1 inoculated with *H vastatrix* race II (Guzzo et al., 2009).

An early increase in chitinase and glucanase activity in coffee leaf rust incompatible interactions, but not in the compatible ones, was observed by Maxemiuc-Naccache et al. (1992). Similar results were obtained when studying chitinase activity in intercellular fluids of incompatible coffee-rust interactions (Guerra-Guimarães et al., 2003).

Nevertheless, basic chitinase isoforms, from intercellular fluids of coffee leaves, were present in both compatible and incompatible ones. Immunodetection analyses performed with antibodies specific to class I chitinase revealed the importance of these isoforms in the incompatible interactions (Guerra-Guimarães et al., 2003).

Chitinases catalyze the hydrolysis of chitin oligomers and are generally induced in response to fungal pathogens or abiotic stress, or during plant development (Collinge et al., 1993). Chitinase enzymes are grouped into five different classes (I to V) based on their amino acid sequences and protein primary structures (Beitema, 1994; Collinge et al., 1993). It is shown that overexpression of a combination of chitinase and β1-4 glucanases results in

fungal resistance in tobacco, potato, and wheat (Bieri et al., 2003; Jach et al., 1995; Lorito et al., 1998). In grapevine (*Vitis vinifera*), the two investigated chitinase genes (*PR-3*, *PR-4*) were demonstrated to be important to confer resistance to downy mildew caused by *Plasmopara viticola* (Kortekamp, 2006).

Cysteine protease is another gene identified in our suppression subtractive hybridization library. Its function in plant is involved with programmed cell death (PCD) or apoptosis that is controlled by a multistep signaling pathway (McConkey and Orrenius, 1994, Stewart, 1994). Recently, it has been demonstrated that cysteine proteases also are induced in plant systems undergoing PCD, such as tracheary element differentiation in *Zinnia elegans* (Minami and Fukuda, 1995; Ye and Varner, 1996). Importantly, in the Zinnia system, the terminal differentiation that ends in cell death could be blocked by the addition of a specific cysteine protease inhibitor.

Results suggest that plants have the ability to control PCD by inhibition of cysteine proteases that regulate the expression of specific protease inhibitor genes. In plants, the proteases and the inhibitor proteins are regulated by different stimuli (Johnson et al., 1989; Doares et al., 1995; koiwa et al. 1997).

In tomato, resistance to *Cladosporium fulvum* was demonstrated to be dependent on a cystein protease. *Cf-2* confers *Avr2*-dependent resistance to *C. fulvum*. Mutation in a cysteine protease (*Rcr3*) suppress *Cf-2* function (Kruger, 2002).

In our work, the maximum level of expression of chitinase and cysteine protease genes was observed at 24hpi, using quantitative RT-PCR. This results are in agreement with pattern of expression observed for lipoxygenase, β-1,3-glucanase and ABC transporter, whose maximum level of expression was observed at 24hpi (Guzzo et al., 2009).

5.0 CONCLUSIONS

This investigation has provided insights into the nature of pathogen-responsive genes, specially in rust resistant HT-CIFC 832/2 plants. Several different events and distinct defense pathways appear to be involved in the expression of resistance in the resistant genotype HT-CIFC 832/2. The isolated cDNAs presented here were involved in oxidative burst, HR response, transduction, synthesis of antimicrobial proteins and several genes with unknown functions.

The study of the expression of two genes (cysteine protease and chitinase) revealed different patterns between HT-CIFC 832/2 and Catuaí. It was observed in HT-CIFC 832/2 a stronger and earlier induction of chitinase in comparison with the expression observed in Catuaí IAC 44. Although in a different magnitude, the pattern of expression of cysteine protease was the same as observed for chitinase.

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