VELVETLEAF - *COLLETOTRICHUM COCCODES* PATHOSYSTEM: MOLECULAR MONITORING OF THE PATHOGEN AND GENE EXPRESSION ANALYSIS DURING PLANT PATHOGEN INTERACTION

by

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ABSTRACT

Colletotrichum coccodes strain DAOM 183088 is considered a potential bioherbicide for velvetleaf (*Abutilon theophrasti*), a devastating weed in North American corn and soybeans. Risk assessment studies have created a demand for an accurate and robust method to monitor this strain, and to distinguish it from indigenous background population of microorganisms present in the field. Safe biological control management of velvetleaf also requires comprehensive understanding of the pathogenicity determinants employed by this host-specific fungus to establish infection on velvetleaf, an aspect central to a safe biocontrol strategy task. In this study, molecular markers were designed that allow strain specific identification of the bioherbicide strain of *C. coccodes* and its identification within complex plant and soil matrices. An assay was developed to quantify *C. coccodes* from deliberate release field soil samples, in which biases caused by soil-originating PCR inhibitors were monitored on a sample per sample basis. The developed external control assay allowed for the estimation of target *C. coccodes* DNA quantities with normalization for the presence of PCR inhibitory compounds. Kinetic growth curves of disease development were performed for *C. coccodes* wild-type and T20-a (genetically engineered for hypervirulence with the *NEP1* (necrosis and ethylene inducing peptide) gene) strains on velvetleaf leaves over a period of 14 days after *C. coccodes* infection. The wild-type strain was more efficient at infecting velvetleaf than the transgenic T-20a strain, while expression of *NEP1* could not be detected suggesting that the introduced gene

may not be transcriptionally active in the transformed strain, a result in conflict with previous observations. Velvetleaf and *C. coccodes* genes specifically up-regulated at 12 and 24 h after fungal infection were cloned and differentially screened by microarrays. The resulting EST collection was sequenced and assigned to putative functions. Early gene up-regulation was confirmed by QRT-PCR analysis for type 3 metallothionein, EREB, WRKY, and bZIP transcription factors, reticuline oxidase, ascorbate peroxidase, and ACC oxidase gene candidates. In addition, type 2, type 3 metallothionein, and bZIP gene expression profiles were investigated over a period of 14 days after *C. coccodes* infection, and the results indicated that *C. coccodes* altered the expression of all three gene analyzed.

RÉSUMÉ

Le *Colletotrichum coccodes* (DAOM 183088) est un agent de lutte biologique (ALB) contre l'Abutilon (*Abutilon theophrasti*), une mauvaise herbe problématique pour les cultures de soja et de maïs en Amérique du Nord. L'évaluation des risques liés à la libération du *C. coccodes* en champs requiert un outil performant permettant le suivi environnemental de l'ALB. La compréhension des mécanismes de pathogénicité et de résistance est également un aspect crucial de la lutte biologique contre l'Abutilon. Des amorces de PCR spécifiques au seul isolat pathogène de l'Abutilon ont été développées et ont permis la détection du *C. coccodes* dans différents sols et plantes d'Abutilon. Un contrôle externe a également été élaboré de façon à pouvoir quantifier l'ALB dans des sols provenant de champs traités avec l'ALB tout en normalisant, pour chacun des échantillons analysés, les quantités d'ALB aux contenus en inhibiteurs de PCR. La croissance de 2 isolats de *C. coccodes*, sauvage et T-20a (transformé génétiquement avec le gène *NEP1* (necrosis and ethylene inducing peptide) à des fins de virulence accrue), a été suivie sur les feuilles d'Abutilon pendant 14 jours suivant l'infection des champignons. Le *C. coccodes* T-20a était moins virulent que le *C. coccodes* sauvage, et l'expression du gène *NEP1* n'a pu être détectée chez le T-20a, ce qui suggère que le transgène ne soit plus transcriptionnellement actif chez cet isolat. Les gènes du *C. coccodes* et de l'Abutilon sur-exprimés 12 et 24 h après l'infection du *C. coccodes* ont été clonés par SSH, analysés grâce à un criblage différentiel par biopuces, puis séquencés et leurs fonctions identifiées. La sur-expression des gènes de métallothioneine de type 3, facteurs de transcription

EREB, WRKY, et bZIP, réticuline oxidase, ascorbate peroxidase, et ACC oxidase, 12 et 24 h après l'infection du *C. coccodes*, a été confirmée par PCR en temps réel. L'étude de l'expression des gènes de métallothioneine de types 2 et 3, et du facteur de transcription bZIP sur une période de 14 jours suivant l'infection du *C. coccodes* a également permis de démontrer que le champignon affectait l'expression de ces 3 gènes chez l'*Abutilon.*

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LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CFU	Colony forming unit
DAI	Day after infection
EC	External control
ER	Enrichment ratio
ITS	Internal transcribed spacer
PCR	Polymerase chain reaction
QRT-PCR	Quantitative reverse-transcription-polymerase chain reaction
RAPD	Random amplified polymorphic DNA
SCAR	Sequence Characterized Amplified Region
SSH	Suppression subtractive hybridization

CONTRIBUTIONS OF AUTHORS

This thesis is written in the form of manuscripts according to the "Guidelines Concerning Thesis Preparation". The thesis contains five chapters (III to VII) representing five different manuscripts, all of which have been published (chapters III and IV), accepted for publication (chapter V), or under review (chapters VI and VII). Each co-author is mentioned, along with his/her corresponding address, at the beginning of each chapter. Below is a general description of the contribution of every co-author, and detailed description of what each co-author contributed can be found in the connecting statements preceding each chapter.

My role in all of the chapters was to design and conduct all of the work involved in all of the experiments including data analysis, data mining and preparation of the first draft of each manuscript. My supervisor, Dr. S. Jabaji-Hare provided supervision and funds throughout this research, and made available technical assistance over the duration of this study. She also provided valuable suggestions in the design of most experiments and corrected several versions of the manuscripts. Dr. A. Watson provided operating funds for chapters III and IV, useful background information on the bioherbicide *C. coccodes* and reviewed the final versions of the manuscripts in chapters III, IV and V. Dr. P. Seguin provided invaluable advise and guidance in the choice of the most suitable statistical method for analyzing the data and corrected the final version of the manuscripts found in Chapters IV and V. Dr. B. Ahn assisted in fungal plant

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CHAPTER I

Introduction

Colletotrichum coccodes (Wallr.) Hughes is considered a potential bioherbicide for velvetleaf (*Abutilon theophrasti* Medic.) (Wymore *et al.*, 1988), a devastating weed in corn and soybean in North America. Risk assessment studies involving the release of *C. coccodes* (183088) into the environment has created a demand for an accurate and robust method not only to monitor this strain, but to distinguish it from indigenous background population of microorganisms present in the field, including other pathogenic strains of *C. coccodes*. In order to study the effect of targeted strains of *C. coccodes* on the disease development in velvetleaf, and to evaluate persistence and survival of the released target strain in the environment, *C. coccodes* (183088) must be re-isolated and accurately identified. This procedure traditionally requires collection of a large number of *C. coccodes* cultures, isolation on selective media (Farley, 1976) and microscopic examination of morphological traits (Sutton, 1980) is the only method of identification and detection, despite the fact that it is not able to distinguish *C. coccodes* (183088) from other *C. coccodes* strains nor it is able to accurately quantify the target organism.

Safe biological control management of velvetleaf requires comprehensive understanding of the mechanisms involved in pathogenicity. Because of its restricted host range, *C. coccodes* (183088) becomes an appealing candidate for controlling velvetleaf infestations. In the last decade, research on *C. coccodes* biocontrol properties had focused on studies targeted to optimize inoculum

production (Yu *et al.*, 1997), application (Hodgson *et al.*, 1988; Wymore and Watson, 1989), and efficiency (Ahn *et al.*, 2005a, 2005b; Amsellem *et al.*, 2002). However, little is known on the pathogenicity determinants employed by this host-specific fungus to establish infection on velvetleaf, an aspect that is central to the task of a safe biocontrol strategy. Several molecular methods are available to isolate sets of genes expressed in particular conditions such as plant infection related genes, or interaction specific genes, among which suppression subtractive hybridization (SSH) has the advantage of being non-targeted and restrictive. The isolated genes can then be screened by microarrays, their up-regulation verified by real-time reverse transcription (RT)-PCR, and their functions inferred by comparison to sequence data banks.

1.1. Hypotheses

In light of the above observations, this thesis is based on several hypotheses:

(i) Strain-specific PCR primers can be developed and are able to selectively identify *Colletotrichum coccodes* (183088) among heterogeneous organisms including other strains from the same species;

(ii) Development of a method in which accurate quantification of the target strain without the bias estimation of PCR inhibitory compounds co-extracted with the target organism's DNA is achievable in deliberate-release field trials;

(iii) The effect of *C. coccodes* strains on the disease development in velvetleaf is estimable by molecular growth kinetics;

(iv) The pathogenic relationship between *C. coccodes* and velvetleaf is conditioned by differential expression of plant and fungal genes at the early stages of infection;

(v) The expression ratios of selected plant genes, namely type 3 metallothionein, type 2 metallothionein, and bZIP transcription factor are temporally altered in response to *C. coccodes* infection.

1.2. Objectives

Based on the limited knowledge available on the mechanisms of survival and pathogenicity of *Colletotrichum coccodes*, the general objectives of this work were to design and implement molecular diagnostic tools to monitor *C. coccodes* after its release as a bioherbicide in the environment, and to improve our understanding of the molecular determinants involved in the pathogenic relationship between velvetleaf and *C. coccodes*. To achieve these global objectives, specific targeted objectives were formulated:

(i) To develop strain-specific molecular markers and test them for their specificity, accuracy and sensitivity in detecting *C. coccodes* from plant and soil samples;

(ii) To monitor and trace the amount of *C. coccodes* in deliberate field-release soils using a robust assay that accurately quantifies target DNA and takes into account the effects of PCR inhibitory compounds present in soil samples;

(iii) To establish molecular growth kinetics curves for the wild-type and transgenic strains of *C. coccodes* during infection of velvetleaf leaves;

(iv) To isolate and characterize hundreds of cDNAs that are differentially expressed during velvetleaf-*C. coccodes* interaction using suppression subtractive hybridization and microarray differential screening;

(v) To monitor the temporal gene expression of type 2, type 3 metallothionein, and bZIP transcription factor during infection of velvetleaf by *C. coccodes*.

CHAPTER II

LITERATURE REVIEW

2.1. *Colletotrichum coccodes*, a mycoherbicide for velvetleaf

2.1.1. The weed: velvetleaf, *Abutilon theophrasti* Medic.

Abutilon theophrasti Medic., known as velvetleaf, is a troublesome weed in soybean (*Glycine max* (L.) Merr.) and corn (*Zea mays* L.) cropping systems in several parts of the USA, Canada, Europe and the Mediterranean region (Andersen *et al.*, 1985; Sattin *et al.*, 1992; Spencer, 1984). In Canada, the weed has settled from Southern Ontario throughout Quebec and Nova Scotia (Andersen *et al.*, 1985; Doyon *et al.*, 1986; Warwick and Black, 1988). Its rapid growth and prolific seed production (Warwick and Black, 1988), the long viability and ease of dispersion of the seeds (Akey *et al.*, 1991; Dekker and Meggitt, 1983), all confer to velvetleaf characteristic features of a group of weeds that are tenacious and therefore difficult to control (Grime, 1979). As much as $ 343 million are spent annually to chemically control velvetleaf. As a result of weed competition, soybean and corn cultures suffer from dramatic yield losses (40 and 51% decrease in corn and soybean yields, respectively, at weed densities of 25 weedy plants/m^2 (OMAFRA, 2002a, 2002b).

Diverse management practices have been commonly attempted to sustain effective control of *A. theophrasti* infestations. These include multiple herbicide applications combined with or without crop rotations, row spacing or tillage practices, as part of integrated management programs. Unfortunately, none of these alone or together are efficient to reduce velvetleaf presence or its

propagation to reasonable levels (Sattin *et al.*, 1992; Warwick and Black, 1988). In the last two decades, the potential control of velvetleaf with inoffensive biological methods has been examined. *Colletotrichum coccodes* (Watson *et al.*, 2000) and *Fusarium lateritium* (Boyette and Walker, 1986) have both been proposed as potential biological control agents for velvetleaf. Although *F. lateritium* was abandoned because of lack of host-specificity, prospects of integrating *C. coccodes* with other weed management practices have been envisaged.

2.1.2. The mycoherbicide: *Colletotrichum coccodes* (DAOM 183088)

Several weeds such as *Capsella bursa-pastoria*, *Convolvulus arvensis*, *Oxalis stricta*, *Solanum nigrum*, and *Abutilon theophrasti* are hosts of *C. coccodes* strains (Raid and Pennypacker, 1987), on which the species causes either foliage anthracnose or necrotic lesions on the roots. Other *C. coccodes* strains are well-known pathogens of economically important crops including tomato (Byrne *et al.*, 1997) and potato (Lees and Hilton, 2003) species, on which they cause anthracnose and black dot diseases, respectively.

Gotlieb and co-workers (Gotlieb *et al.*, 1987) were the first to isolate *C. coccodes* (Wallr.) Hughes strain (DAOM 183088) from velvetleaf leaves in Vermont, USA. On this weed, the strain causes leaf blight, characterized by chlorotic spots that become water-soaked and further develop into grey-brown lesions. Leaf desiccation and premature drop are the end symptoms for velvetleaf (Gotlieb *et al.*, 1987). Studies on the host-range of *C. coccodes* (DAOM 183088) revealed that velvetleaf was identified as the only susceptible plant among the 42

different plant species rated for susceptibility to *C. coccodes* highly suggesting that the strain was host-specific (L.A. Wymore, personal communication). Although the same strain was isolated from lesions developed on leaves of some tomato cultivars, it did not cause disease or symptoms typical of what strain 183088 causes on velvetleaf. The host-range of *C. coccodes* was considered narrow enough to envisage safe biological control of velvetleaf.

Biological control has been applied for many years to protect against weed infestations (Babu *et al.*, 2003; Li *et al.*, 2003). Among the different biocontrol strategies developed is the use of mycoherbicides, also referred to as an inundative strategy. This strategy involves the deliberate use of natural enemies such as *C. coccodes* to suppress the growth or reduce the population of a weed species (Watson, 1993). Inoculum of weed pathogens is applied in a manner analogous to chemical herbicide application (TeBeest and Templeton, 1985; Templeton, 1982). Unlike chemical herbicides, biological control agents (BCA) can only be used against a specific target plant since their host-specificity is a safety requirement.

In the last decade, continued research on the mycoherbicide *C. coccodes* had focused on optimizing the inoculum production (Yu *et al.*, 1997), application (Hodgson *et al.*, 1988; Wymore and Watson, 1989), and on improving efficiency (Ahn *et al.*, 2005a, 2005b; Amsellem *et al.*, 2002). *C. coccodes* can severely damage or kill velvetleaf when applied to young seedlings bearing only cotyledons or at most one true leaf (Wymore *et al.*, 1988), however at later growth stages, velvetleaf plants continue to grow after shedding their infected leaves. Recent

attempts have focused on increasing the virulence of *C. coccodes* through genetic transformation (Amsellem *et al.*, 2002).

2.1.3. The genetically transformed T-20a *Colletotrichum coccodes*

Molecular biology of weed control is perceived as a promising approach to efficiently control weed infestations thanks to aggressive host-specific pathogens (Duke, 2003; Gressel, 2000, 2001). With this end in view, the *NEP1* gene (necrosis and ethylene inducing peptide) encoding a *Fusarium oxysporum* phytotoxic protein was used for genetic transformation of the wild-type strain of *C. coccodes* (DAOM 183088) (Bailey *et al.*, 2000; Jennings *et al.*, 2000). The resulting transgenic strain of *C. coccodes,* T-20a, was found to be 9 times more virulent than the wild-type against velvetleaf at the three true-leaf stage when applied as chopped mycelium (Amsellem *et al.*, 2002). Despite this success, T-20a host range was not limited to velvetleaf, as is the case of the wild-type strain, but was extended to include tobacco and tomato plants causing 67 to 100% seedling mortality, respectively (Amsellem *et al.*, 2002).

2.2. Importance of *Colletotrichum* species

The genus *Colletotrichum* is one of the most important worldwide fungal pathogen and is commonly known as the anthracnose pathogen (Latunde-Dada, 2001). Because of its importance, several studies dealing with plant-pathogen interactions, fungal differentiation and plant defence responses have been documented (Bailey *et al.*, 1980; Mahe *et al.*, 1993; O'Connell and Bailey, 1988).

For example, the first study that established race-cultivar specificity between pathogens and their hosts were conducted with *Colletotrichum* (Barrus, 1911).

Many species of this genus have been studied for use as mycoherbicides (Table 2.1). Among them is the strain DAOM 183088 of *C. coccodes*. Some *Colletotrichum* mycoherbicide strains have been successfully used for years against several weeds, while others are still hindered with formulation or commercialization difficulties (Hallett, 2005) while others are now considered as model mycoherbicide systems.

Table 2.1. *Colletotrichum* strains as mycoherbicides.

Commercial names	Latin names	Target plants	Life cycle of target plant
BioMal®	*C. orbiculare*	Round-leaved mallow (*Malva pusilla*) and safflower (*Carthamus tinctoris*)[a]	annual to a short-lived perennial / annual
Burr anthracnose®	*C. orbiculare*	Spiny cockleburr (*Xanthium spinosum*)[b]	annual
Collego®	*C. glooeosporioides* f.sp. *aeschynomene*	Northern joinvetch (*Aeschynomene virginica*)[b]	annual
Hakea	*C. gloeosporioides*	Silky needlebush (*Hakea sericea*)[c]	perennial
Lubao II	*C. glooeosporioides* f.sp. *cuscutae*	Dodder spp. (*Cuscuta chinensis*, *C. australis*)[d]	annual
Velgo®	*C. coccodes*	Velvetleaf (*Abutilon theophrasti*)[a]	annual
NA	*C. dematium* f.sp. *epilobii*	Fireweed (*Epilobium angustifolium*)[e]	perennial
NA	*C. graminicola*	Barnyard grass (*Echinochloa crus-galli*)[f]	annual
NA	*C. truncatum*	Scentless chamomile (*Matricaria perforate*)[g]	annual, biennial, sometimes perennial
NA	*C. truncatum*	Hemp sesbania (*Sesbania exaltata*)[h]	annual

[a] Watson et al., 2000; [b] Templeton, 1992; [c] Morris, 1989; [d] Hallet, 2005; [e] Leger *et al*, 2001; [f] Yang *et al.*, 2000; [g] Peng *et al.*, 2005; [h] Boyette, 1991; NA, not applicable.

2.3. Molecular Diagnostics

Our ability to precisely identify and monitor mycoherbicide strains after their release in the environment is an asset for any sustainable weed biocontrol strategy. A wealth of methodologies exist for the detection of microorganisms, including traditional quantification of fruiting structures (Miller, 1996), scoring of disease symptoms (Pei *et al.*, 2003), genetic transformation with marker proteins such as the green fluorescent protein (GFP) and the ß-glucoronidase (GUS) genes (Aly *et al.*, 2001; Chen *et al.*, 2003; Green and Jensen, 1995), biochemical (Bochner, 2003; Gessner and Schmitt, 1996; Schmitz *et al.*, 1991), and microbiological methods (Carnegie *et al.*, 2003). Recently, polymerase chain reaction (PCR)-based methods have gained an astonishing popularity in the field of diagnostics (Bej, 2003; McCartney *et al.*, 2003), because of their sensitivity, specificity and ease of implementation.

Since the initial report of specific DNA amplification using PCR by Kary Mullis and co-workers in 1985, the number of different applications of the technique has grown exponentially. One of the first applications of PCR in mycology was described in 1990 by White and co-workers and dealt with the amplification and direct sequencing of ribosomal DNA (rDNA) to establish the taxonomic and phylogenetic relationships among fungi (White *et al.*, 1990). The advent of PCR has enabled the development of powerful molecular markers for the detection or discrimination of fungi, either at the species or at the strain level, and extensive applications have largely been found in mycology, including taxonomy, phylogeny, and diagnostics. PCR-based detection of pathogenic fungi has been reported for several important systems such as *Phytophthora ramorum*,

the sudden oak death pathogen (Kong *et al.*, 2004), ochratoxin A-producing *Aspergillus* spp. (Patino *et al.*, 2005), mycotoxin-producing *Fusarium* spp. (Li *et al.*, 2005; Mule *et al.*, 2004; Nicholson *et al.*, 2004), black-dot disease causing *Colletotrichum coccodes* strains (Cullen *et al.*, 2002), as well as anastomosis groups of *Rhizoctonia solani* causing stem canker and black scurf on potato (Lees *et al.*, 2002).

2.3.1. Molecular markers

There are two main approaches for the development of molecular markers: (i) the use of conserved genes for which sequence data are available and (ii) the screening of random DNA sequences that are specific for the target organism. The choice of the most favorable approach is often dictated by the availability of large sequence data sets for a given gene in the target organism and related organisms, and by the level of specificity one wants to achieve.

One of the most documented DNA regions for the design of specific primers is the ribosomal DNA (Chen *et al.*, 2000). Such regions have been used to detect important species of *Colletotrichum* based on the variability of the internal transcribed spacer (ITS) regions that separate ribosomal genes. For instance, Cano and co-workers (Cano *et al.*, 2004) developed an ITS-based detection tool to differentiate five *Colletotrichum* species that are of clinical interest (*C. coccodes, C. crassipes, C. dematium, C. gloeosporioides,* and *C. graminicola*). Another diagnostic test was also developed using sequences flanking the ITS region that was targeted to detect a single *C. coccodes* species (Cullen *et al.*, 2002). Other fungal genes successfully used in detection assays include actin (Mahe *et al.*,

1992), β-tubulin (Fraaije *et al.*, 2001) or ascomycete mating type genes (Foster *et al.*, 2002).

Molecular markers (PCR amplified products) can also be designed from random regions of DNA in the target genome by screening the latter with degenerated PCR primers. The most commonly used screening tool was, for a long time, restriction fragment length polymorphism (RFLP). RFLP markers have been used in several biological control systems for the differentiation of *Pochonia chlamydosporia* var. *catenulata* strains, the biocontrol agents of root-knot nematodes (Atkins *et al.*, 2003b); the estimation of genetic variability among the mycoherbicide isolates of *Cercospora caricis* against the purple nutsedge (*Cyperus rotundus*) (Inglis *et al.*, 2001), and among *Colletotrichum gloeosporioides* isolates causing anthracnose on Brazilian lucerne (*Stylosanthes guianensis*) (Kelemu *et al.*, 1999). Although RFLP markers were successfully used in all of the above studies, they require large amounts of purified DNA and their implementation is relatively labour- and time-consuming.

Other techniques available for the screening of fungal genomes include universally primed PCR (UP-PCR; (Bulat and Mironenko, 1996) that make use of semi-random 15 to 20 bp long primers to amplify mostly intergenic DNA regions, the inter simple sequence repeat (ISSR)-PCR (Zietkiewicz *et al.*, 1994) which targets microsatellite DNA regions thanks to repeated primer sequences, and the random amplified polymorphic DNA (RAPD)-PCR (Williams *et al.*, 1990), that randomly amplify anonymous DNA regions thanks to 10 mer randomly designed primers.

2.3.2. RAPD / SCAR markers

When sequence information for primer design is not available, RAPD markers provide an ideal solution for detection thanks to single short PCR primers of arbitrary nucleotide sequences. Initially developed to detect polymorphism between organisms of unknown DNA sequences (Williams *et al.*, 1990), RAPD is also known as arbitrary primed PCR (AP-PCR; (Welsh and McClelland, 1990)) and DNA amplification fingerprinting (DAF; (Caetano-Anollès *et al.*, 1991)).

The markers generated by RAPD are generally used for genetic mapping, as well as for diagnostic purposes. Different races of *Fusarium* f.sp. *vasinfectum* (Assigbetse *et al.*, 1994; Manulis *et al.*, 1994), or isolates of *Fusarium* spp. (Wilson *et al.*, 2004), *Cercospora caricis* (Inglis *et al.*, 2001), *Beauveria bassiana* (Castrillo *et al.*, 2003), *Rhizoctonia solani* AG-2 (Toda *et al.*, 2004), AG-3 (Bounou *et al.*, 1999), binucleate *Rhizoctonia* isolates AG-G (Leclerc-Potvin *et al.*, 1999), *Stachybotrys elegans* (Taylor *et al.*, 2003) and *Trichoderma* (Abbasi *et al.*, 1999; Zimand *et al.*, 1994) have all been successfully identified by this method.

The products of RAPD or the amplicons generated by the PCR reaction depend on the sequence and the length of the oligonucleotide used for priming, as well as the reaction conditions. The PCR products obtained are easily affected by the quality of the template mixture, the DNA polymerase and the thermocycler used. Because of non-specific annealing temperature and the tendency of short primers to generate complex DNA electrophoretic patterns, RAPD profiles tend to be difficult to reproduce.

To overcome those constraints, a DNA fragment generated by RAPD can be cloned and further sequenced to provide a sequence-characterized amplified region (SCAR) for the target isolate (Paran and Michelmore, 1993). Traditionally, from this sequence, new primers are designed and referred to as SCAR primers and have been successfully used to detect several fungal biological control agents including *Chondrostereum purpureum* on *Sitka alder* and trembling aspen (Becker *et al.*, 1999), *Beauveria bassiana*, used against insects (Castrillo *et al.*, 2003), a strain of *Trichoderma atroviride*, against soilborne fungal plant pathogens (Hermosa *et al.*, 2001), *Stachybotrys elegans*, against *Rhizoctonia solani* (Taylor *et al.*, 2003), and *Aureobasidium pullulans* to control postharvest diseases (Schena *et al.*, 2002). In addition, SCAR markers were used to detect several fungal pathogens including *Rhizoctonia solani* AG-3 isolates pathogenic on potato (Bounou *et al.*, 1999), non-pathogenic binucleate *Rhizoctonia* isolates (AG-G) (Leclerc-Potvin *et al.*, 1999), and the head-blight cereal pathogen *Fusarium* spp. (Nicholson *et al.*, 1998).

Among the advantages of SCAR primers over RAPD primers are their reliability, their specificity for a given locus, and the fact that their detection is quantifiable. In our study, RAPD screening combined with SCAR primer design was chosen as a strategy to specifically detect DNA sequence unique to *C. coccodes* DAOM 183088 among a large collection of other heterogeneous organisms (Chapter 3).

2.3.3. Real-time PCR DNA quantification

Monitoring the establishment and spread of introduced mycoherbicides is essential to evaluate the success of biological control programs. Among the quantitative tools available, quantitative PCR, which consists in measuring the amount of amplicon by reference to the dilution series of an external standard (Cross, 1995), rarely gives accurate DNA quantifications since small variations in sample preparation or processing are substantially magnified during the amplification (Coutlee *et al.*, 1995; Luque *et al.*, 1994). Competitive PCR (Clementi *et al.*, 1995; Zimmermann and Mannhalter, 1996) which involves the co-amplification of a specific target sequence with known concentrations of an internal standard in one reaction tube allows for more accurate results since both the target sequence and the standard share primer recognition sites and have the same amplification efficiency. The most recent and revolutionizing DNA quantification method is quantitative real-time PCR (QPCR) (Bustin, 2002; Wilhelm and Pingoud, 2003). Quantitative detection of amplified DNA is performed at a few cycles of the amplification in which the amount of DNA increases exponentially from the background to the plateau (exponential phase). Therefore, quantification is not affected by any reaction components that may cause bias against the more abundant template and make any quantification based on measurements of overall product yield intrinsically unreliable.

Different kinetic PCR methodologies can be used in real-time PCR, all of which involve fluorescence detection of the amplified products and allow an accuracy reaching the DNA copy number determination. Several chemistries are available to detect the amplified product. The simplest chemistry makes use of

SYBR Green, a fluorescent dye that binds specifically to double-stranded DNA, while others (*e.g.* molecular beacons, hybridization and hydrolysis probes) rely on hybridization of fluorescent probes to the correct amplicon. Pioneer uses of real-time PCR for quantification of cellular DNA or RNA include viral (Jalava *et al.*, 1993), bacterial (Kolk *et al.*, 1994) and fungal (Loeffler *et al.*, 2000) nucleic acids. This method is becoming the method of choice for direct fungal DNA quantification from complex environments such as plants and soil.

2.3.3.1. Quantification of fungal DNA *in planta*

Real-time quantitative PCR (QPCR) has become the method of choice in plant pathology to monitor pathogen colonization through DNA quantification in plant tissue (Alkan *et al.*, 2004; Boyle *et al.*, 2005; Gao *et al.*, 2004; Hietala *et al.*, 2003; Luchi *et al.*, 2005; Reischer *et al.*, 2004; Vandemark and Barker, 2003; Waalwijk *et al.*, 2004; Winton *et al.*, 2002). For instance, Boyle and colleagues monitored *Melampsora-larici-populina* and *Melampsora medusae*, causing poplar leaf rust, in compatible, incompatible, and non-host interactions at various times following infection (Boyle *et al.*, 2005). A TaqMan-based quantification assay successfully detected *Biscogniauxia mediterranea*, the causal agent of charcoal disease in symptomless oak tissues (Luchi *et al.*, 2005). Overall, real-time QPCR offers many advantages and its applications for the quantification of pathogens *in planta* include, but are not limited to, pathogen quantification as part of quarantine programs, ecological and epidemiological studies as well as research dealing with the understanding of fungal pathogenicity and plant disease management.

2.3.3.2. Quantification of fungal DNA in soil

Soils are one of the most challenging matrixes from which to extract good quantity and quality DNA. This is because they vary greatly in chemical and organic composition, and contain abundant humic and fulvic acids, lignin-associated and phenolic compounds, polysaccharides, tannins, and heavy metals that are potentially inhibitory to the PCR amplification (Wilson, 1997). Successful detection, characterization and quantification of fungal DNA in soil require adequate DNA purification from the co-extracted contaminants that inhibit PCR. With this end in view, numerous methods have been developed over the years to circumvent the problem of co-extracted PCR inhibitors (Braid *et al.*, 2003; Jiang *et al.*, 2005; Moreira, 1998; Tebbe and Vahjen, 1993; Torsvik, 1980; Tsai and Olson, 1991; Young *et al.*, 1993; Zolan and Pukkila, 1986). Since all these methods can also affect the yield of DNA extraction, and because the success of a given method is often dependent on the type of soil from which DNA is to be isolated (Frostegard *et al.*, 1999; Martin-Laurent *et al.*, 2001; Steffan *et al.*, 1988), it becomes a challenge to find a standard DNA purification method that is efficient and reliable. As a consequence, several specific soil DNA extraction methods have been reported based on the type of soil under investigation (Anderson and Cairney, 2004; Atkins *et al.*, 2003a; Cullen *et al.*, 2005; Schena and Ippolito, 2003; van de Graaf *et al.*, 2003; Zhang *et al.*, 2005).

Current methods used for soil DNA quantification do not take into account the biases caused by PCR inhibitors, and because different soil samples contain different amounts of PCR inhibitors, the resulting data are affected by PCR efficiencies varying from one sample to another. Although several internal control

tools have been developed to detect PCR inhibitors (Burggraf and Olgemoller, 2004; Najioullah *et al.*, 2001; Poon *et al.*, 2004; Rajeev *et al.*, 2005; Stranska *et al.*, 2004; Ursi *et al.*, 2003), these are only qualitative and their use to quantify biases in PCR efficiency from soil DNA extract samples remains limited.

To circumvent these limitations, quantitative internal controls have also been developed for QPCR quantification but unfortunately, their use is limited to the medical field. These include internal controls that are endogenous to the samples analyzed (Meijerink *et al.*, 2001; Rosenstraus *et al.*, 1998) or heterogeneous internal controls (Broccolo *et al.*, 2002; Stocher and Berg, 2002; Stocher *et al.*, 2002) that are spiked into the samples analyzed. Current methods used to quantify DNA from environmental samples, such as soil, do not take into account the biases caused by PCR inhibitors.

2.4. *Colletotrichum* infection process

A potential pathogen not only possesses the necessary «factors» for disease development but requires an ability to recognize features on a plant that signal its suitability for parasitism (Parker, 2003). During plant infection, the pathogen undergoes different physiological and morphological changes that would not be induced without the appropriate host. In *Colletotrichum*, those developmental stages are brought about by the activation of specific genes (Idnurm and Howlet, 2001; Latunde-Dada, 2001) conferring the degree of virulence, and the ability or inability of the fungus to infect the given host or non-host plant, respectively.

2.4.1. Adhesion

Attachment of *Colletotrichum* conidia to the host-cuticle involves hydrophobic interactions as it is the case for many other plant pathogenic fungi (Mercure *et al.*, 1994; Nicholson and Epstein, 1991). In some *Colletotrichum* species, a second phase of adhesion may occur during which active metabolic processes can occur. The secretion of a protein exudate that spreads outward from the spore on the leaf surface (Sela-Buurlage *et al.*, 1991; Young and Kauss, 1984) has been correlated with this phase, however timing and location of protein(s) release varies among *Colletotrichum* species, which makes unclear whether or not the secretion of these materials contributes to spore adhesion.

2.4.2. Germination and Penetration

Colletotrichum species penetrate their hosts through natural openings, *e.g.* stomata, wounds and by direct penetration, which seems to be the most common way (Bailey *et al.*, 1992). The mechanism that *Colletotrichum* applies to directly penetrate plants can be through (*i*) mechanical force alone, (*ii*) secretion of cutin degrading enzymes, or (*iii*) a combination of both processes (Bailey *et al.*, 1992). In most *Colletotrichum* species, differentiation of an appressorium is a prerequisite for host-penetration and the appressorium develops from the tip of the swelling germ-tube formed by the septated conidia (O'Connell *et al.*, 2000). Maturation of the appressorium involves formation of a penetration pore at the base of the cell, deposition of melanized wall layers (Perfect *et al.*, 1999) and secretion of extra-cellular matrix materials (Bailey *et al.*, 1992).

2.4.3. Infection and colonisation: necrotrophs and hemibiotrophs

Two main infection strategies are exhibited by *Colletotrichum* species: hemibiotrophy and necrotrophy. Physiological and morphological development of both groups of *Colletotrichum* is similar until penetration of the plant cuticle. The infection typically starts with conidial adhesion and germination on plant surfaces, the production of germ tubes and their differentiation into appressoria, which penetrate the cuticle (Perfect *et al.*, 1999).

Necrotrophic pathogens kill in advance of mycelial spread, by producing cell-wall degrading enzymes and/or phytotoxins (Agrios, 1997). They hydrolyse plant cuticles and grow intra- and intercellularly (refer to Fig. 2.1A thereafter), obtaining nutrients from dead host-cells (Thrower, 1966), probably thanks to the secretion of toxins and cell-wall degrading enzymes (Perfect *et al.*, 1999). Typical necrotrophic pathogens include *C. capsici* on cowpea (*Vigna unguiculata*) (Bailey *et al.*, 1992; Pring *et al.*, 1995), and cotton (*Gossypium hirsutum* L.) (Roberts and Snow, 1984), *C. circinans* on onion (*Allium cepa* (Walker, 1921), and *C. musae* on *Musa* sp. (Swinburne and Brown, 1983).

The majority of *Colletotrichum* species including strains of *C. coccodes* that are pathogenic on tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*) (Latunde-Dada, 2001), are intracellular colonizers, hemibiotrophic pathogens that generally feed on living host cells before switching to necrotrophy, though the biotrophic stage may be relatively short, and may vary from 24 h up to 3 days depending on the species (O'Connell *et al.*, 2000).

Hemibiotrophic species of *Colletotrichum* (refer to Fig. 2.1B thereafter) enter host epidermal cells after hydrolyzing the plant cuticle (Bailey *et al.*, 1992).

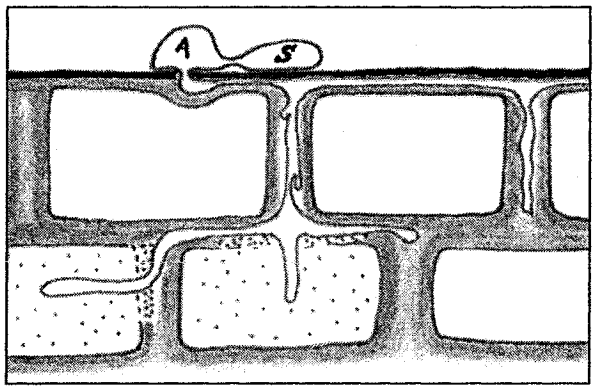
Thin penetration hyphae swell, form infection vesicles and broad primary hyphae develop in the cell that remains alive during this biotrophic phase (Elliston *et al.*, 1976). The primary hyphae grow within the cell lumen by surrounding the plasma membrane that invaginates, but do not penetrate the host protoplasm (O'Connell *et al.*, 1985). The presence of an interfacial extracellular matrix separating the fungal cell wall from the invaginated plant plasma membrane has been reported in several interactions that display hemibiotrophy (Bailey *et al.*, 1992; O'Connell, 1987) and seems absent at later stages of infection development (O'Connell, 1987) and in *Colletotrichum* species that do not have a biotrophic stage (Bailey *et al.*, 1992). Further evidence of the role of this matrix in pathogenicity comes from studies establishing the presence of a similar matrix in the hemibiotrophic *C. lindemuthianum*-bean compatible reactions, and its absence in incompatible reactions (O'Connell *et al.*, 1985).

After colonizing the cortex, the pathogen growth switches to a classical necrotophic behaviour, and the intracellular hyphae subsequently give rise to secondary necrotrophic hyphae (Allard, 1974; Skipp and Deverall, 1972) that grow through and between cells, secrete cell-wall depolymerising enzymes in advance of their spread (O'Connell and Bailey, 1991; O'Connell *et al.*, 1985), and rapidly create expanding necrotic lesions (Perfect *et al.*, 1999). Typical brown-lesions appear on the plant surface (O'Connell *et al.*, 2000) and the hyphae spread extensively throughout host tissues (Bailey *et al.*, 1992).

Among the *Colletotrichum* species using this "Dr Jekyll and Mr Hyde" strategy to establish initial infections are *C. lindemuthianum* on bean (*Phaseolus vulgaris*, (O'Connell, 1987; O'Connell *et al.*, 1985)), *C. destructivum* on cowpea

(*Vigna unguiculata*, (Latunde-Dada *et al.*, 1996)), *C. truncatum* on pea (*Pisum sativum*, (O'Connell *et al.*, 1993)), *C. sublineolum* on sorghum (*Sorghum bicolour*, (Wharton and Julian, 1996)), *C. gleoesporioides* on *Medicago sativa*, *Stylosanthes guianensis*, *S. scabra*, *Populus tremoides* (Bailey *et al.*, 1992), and *C. coccodes* on tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*) (Latunde-Dada, 2001). In the case of *C. coccodes* strain (DAOM 183088) that attacks velvetleaf, most reports have focused on the epidemiology and the biological weed control aspects, and ignored the biology of the infection process. However, the timing of symptom appearance on infected velvetleaf leaves which may be as early as 3 days is a strong indication that this strain exhibits a hemibiotrophic feeding strategy in the same manner as *C. coccodes* strains pathogenic on tomato and potato.

A



B

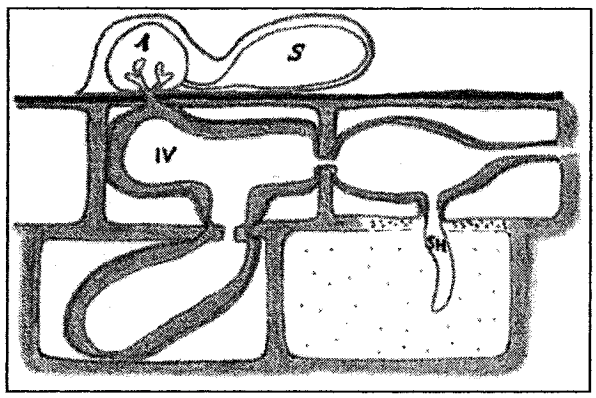


Figure 2.1. *Colletotrichum* plant infection strategies. **A.** *Colletotrichum* necrotrophic infection strategy. Spores (S) germinate to form domed melanized apppressoria (A) from which penetration hyphae develop. The pathogen grows intra- and intercellularly, causing rapid and extensive cell-death. **B.** *Colletotrichum* hemibiotrophic infection strategy. Spores (S) germinate to form domed melanized apppressoria (A) from which penetration hyphae develop. Upon entering host-epidermal cells, penetration hyphae swell to form infection vesicles (IV) and broad primary hyphae, around which the plasma membrane invaginates. Only 48 h after initial penetration, narrow secondary hyphae (SH) are formed. Necrotic lesions appear and spread rapidly. (Adapted from Perfect *et al.*, 1999).

2.5. Plant responses to *Colletotrichum* attack

Many molecular studies dealing with the responses of plants to attack by *Colletotrichum* species have been performed to better understand the basis of disease resistance and susceptibility (Dean *et al.*, 2005; Ferrier-Cana *et al.*, 2003; Fraire-Velazquez and Lozoya-Gloria, 2003; Goodwin, 2001; Goodwin *et al.*, 2004; Hong *et al.*, 2004; Jung *et al.*, 2003; Kim *et al.*, 2004; Lahey *et al.*, 2004; Melotto *et al.*, 2005; Melotto *et al.*, 2004; Narusaka *et al.*, 2004; Pratt *et al.*, 2005; Shan and Goodwin, 2005; Torregrosa *et al.*, 2004; Yu *et al.*, 2005). For instance, a zinc-finger protein encoding gene, *CAZFP1*, was isolated from pepper leaves and shown to be differentially induced in compatible and incompatible interactions with bacteria (*Xanthomonas campestris* races) and with a virulent strain of *Colletotrichum coccodes*. Interestingly, heterologous expression of *CAZFP1* in *Arabidopsis* rendered the plants resistant to bacterial pathogens and more tolerant to drought (Kim *et al.*, 2004).

There are no reports of *Colletotrichum* spp. naturally infecting the model plant *Arabidopsis*, however the latter was found susceptible to *Colletotrichum destructivum* (O'Connell *et al.*, 2004). This species is considered a hemibiotroph that obtains nutrients from living *Arabidopsis* cells (biotrophy), and later switches to necrotrophy during which nutrients are obtained from cells that have been killed by the fungus. Since the genome and transcriptome of *Arabidopsis* are the best characterized among the plant kingdom, and because powerful tools are available for their analysis, the *Colletotrichum-Arabidopsis* pathosystem could greatly help in elucidating key pathogenicity and resistance mechanisms. Using cDNA microarrays, Narusaka and colleagues (2004) investigated the genes

involved in the resistance of *Arabidopsis* to *C. destructivum* and demonstrated that several defence responses were induced in the plant as a result of fungal presence, especially those involved in the jasmonate and the ethylene signalling pathways. By performing crosses between several ecotypes, the authors identified the single and dominant locus (*RCH1*) that confers resistance to *C. destructivum* in one of *Arabidopsis* ecotypes (Narusaka *et al.*, 2004). Whether *RCH1* is involved in the jasmonate and ethylene response pathways remains to be elucidated.

Molecular events involved in the resistance of plants to *Colletotrichum* were also studied in the *C. trifolii–Medicago truncatula* interaction (Torregrosa *et al.*, 2004). Differential macroarray screening of resistant and susceptible interactions allowed the authors to monitor the expression profiles of a set of 92 genes with suspected roles in plant defence and signal transduction, and to highlight qualitative and quantitative differences of gene expression in the two types of interactions (Torregrosa *et al.*, 2004). Most of the genes identified in this study were involved in the phenylpropanoid pathway, cell wall proteins related genes, PR-genes, lipid related signalling, and nodulin genes. Among these genes, a senescence related protein and a lipid transfer protein cDNA were found up-regulated at 48 and 72 h after *C. trifolii* infection in resistant *M. truncatula* line, while an upregulated peroxidase gene was identified at 16 to 24 h after infection of a susceptible *M. truncatula* line.

2.6. Host-specificity

Molecular studies on the process of *Colletotrichum* infection have been undertaken to better understand the basis of virulence and pathogenicity of each fungal strain to its particular plant host. Among such genes, distinction is usually made between those that define the level of disease development (virulence genes *sensu* Agrios or factors of virulence) and others that confer the pathogen the ability to cause disease (pathogenicity genes *sensu* Agrios or factors of host-specificity) (Agrios, 1997).

Genes expressed by plant pathogenic *Colletotrichum* species that condition pathogenicity have been cloned from a few species of *Colletotrichum*, but not from *C. coccodes*. All of the genes presented in Table 2.2 are genes specifically expressed during infection that are likely to play important functions in plant disease. The role of these fungal genes in pathogenicity and disease intensity were confirmed by molecular approaches such as gene disruption and/or eventually heterologous expression. Many pathogenicity genes have been identified in *Colletotrichum*, though not from *C. coccodes*, as playing important roles in the pathogen virulence and include genes related to infection structures, cuticle and cell wall degradation, signal cascade component genes, as well as other genes whose precise function has not yet been identified (refer to Table 2.2).

Table 2.2. Pathogenicity-related genes from *Colletotrichum.*

GENE	*Colletotrichum* SPECIES	Disease caused / plant host	NAME and PUTATIVE FUNCTION	ID[a]	References
INFECTION STRUCTURE					
PKS1	*C. lagenarium*	anthracnose / cucumber	Polyketide synthase; melanin biosynthesis	genomic library	Takano et al., 1995
SCD1	*C. lagenarium*	anthracnose / cucumber	Scytalone dehydrogenase; melanin biosynthesis	cDNA library	Kubo *et al.*, 1996
THR1	*C. lagenarium*	anthracnose / cucumber	Trihydroxynaphtalene reductase; melanin biosynthesis	genomic library	Perpetua *et al.*, 1996
Clap1	*C. lindemuthianum*	anthracnose / bean	Copper transporting ATPase	RIM	Parisot *et al.*, 2002
ClPLS1	*C. lindemuthianum*	anthracnose / bean	Tetraspanin; regulates function of appressoria	deg. PCR	Veneault-Fourrey *et al.*, 2005
Cap20	*C. gloeosporioides*	anthracnose / avocado, tomato	Expressed during appressorium formation	cDNA library	Hwang *et al.*, 1995b
CUTICLE AND CELL-WALL DEGRADATION					
pelB	*C. gloeosporioides*	anthracnose / avocado	Pectate lyase	genomic library	Yakoby *et al.*, 2000; Yakoby *et al.*, 2001
SIGNAL CASCADE COMPONENTS					
Klap1	*C. truncatum*	anthracnose / key lime	Putative transcription factor	REMI	Chen *et al.*, 2005
Cam	*C. trifolii*	anthracnose / alflafa	Calmodulin	deg. PCR	Warwar *et al.*, 2000

ctg-1	*C. trifolii*	anthracnose / alflafa	alpha-subunit of G protein	deg. PCR	Truesdell *et al.*, 2000
Cmk1	*C. lagenarium*	anthracnose / cucumber	MAP kinase	deg. PCR	Takano *et al.*, 2000
Rpk1	*C. lagenarium*	anthracnose / cucumber	PKA (cAMP dependant protein kinase) regulatory sub-unit	deg. PCR	Takano *et al.*, 2001
Cgmek1	*C. gloeosporioides*	anthracnose / avocado	Protein kinase kinase	deg. PCR	Kim *et al.*, 2000
Ct-pkac	*C. trifolii*	anthracnose / alflafa	Catalytic subunit cAMP-dependant protein kinase	deg. PCR	Yang *et al.*, 1999
clk1	*C. lindemuthianum*	anthracnose / bean	Serine/threonine protein kinase	RIM	Dufresne *et al.*, 1998
"Novel" and NONCLASSIFIED PATHOGENICITY GENES					
path-1	*C. magna*	anthracnose / watermelon	Endophyte-pathogen switch?	REMI	Redman *et al.*, 1999
Clta1	*C. lindemuthianum*	anthracnose / bean	Regulator of biotrophic/necrotrophic switch	RIM	Dufresne *et al.*, 2000
CgDN3	*C. gloeosporioides*	anthracnose / *Stylosanthes* spp.	Suppressor "antiavirulence" gene?	cDNA library	Stephenson *et al.*, 2000
-	*C. graminicola*	anthracnose stalk rot and anthracnose leaf blight / maize	Signal peptidase unit	REMI	Thon *et al.*, 2000

Disruption of genes presented in this table results in a reduction or loss of disease symptoms.

[a] ID describes the method of identification: either random insertionnal mutagenesis (RIM), Random Enzyme Mediated Integration (REMI), degenerate PCR (deg. PCR), genomic or cDNA library screening.

For instance, Hwang and co-workers constructed a cDNA library of transcripts expressed during appressorium formation and isolated cDNA corresponding to germinating and non-germinating-stages. Among the cDNAs expressed in the appressoria-forming spores, *Cap22* gene showed limited homology to a variety of surface glycoproteins. The protein CAP22 was immunologically detected at 4 h of exposure to the host wax and remained detectable at high levels on the appressorial cell-wall until 28 h (Hwang and Kolattukudy, 1995). *Cap20*, a gene lacking sequence homology (table 2.2) was also isolated and CAP20 was observed by electron microscopy in the appressorial cell-wall of *C. gloeosporioides*. *Cap20* gene disrupted mutants produced normal appressoria but failed to produce lesions on avocado and tomato fruits (Hwang *et al.*, 1995). The authors used reverse transcription (RT)-PCR to study *Cap20* transcript expression. Interestingly, expression was first confined to the outer segments of the infected tomato fruits and then progressed in the deeper layers of the fruit representing the infecting front.

Gene disruption does not always demonstrate the role of fungal genes in pathogenicity. For example, many fungi pathogenic on tomato and other *Solanaceous* plants, including *C. coccodes*, have the ability to detoxify the toxic steroidal glycoalkaloid alpha-tomatine produced by the plant. Since beta2-tomatinase confers the ability to detoxify tomatine, Sandrock and co-workers investigated whether this gene was responsible for *C. coccodes* pathogenicity on tomato. By disrupting beta2-tomatinase gene in *C. coccodes*, the authors observed a loss of enzyme activity but the mutants retained their abilities to degrade tomatine via other enzyme(s). Moreover, the gene-disrupted mutants were as

pathogenic as wild type isolates on green tomato fruit that is very concentrated in alpha-tomatine (Sandrock and VanEtten, 2001), which rules out not only the possibility that the beta2-tomatinase gene from *C. coccodes* act as a pathogenicity gene but also as a virulence gene. All the above reports clearly demonstrate that knowledge on *Colletotrichum coccodes* pathogenicity remains unelucidated.

2.7. Analytical methods of gene expression

Several recent methods (see below) are currently available not only to analyze gene expression, but also to identify novel genes whose role in plant disease would have remained unsuspected (Alba *et al.*, 2004; Donson *et al.*, 2002; Kahmann and Basse, 2001; Rensink and Buell, 2005; Wan *et al.*, 2002). These methods are divided into (i) non-targeted approaches which include cDNA-AFLP (Amplified Fragment Length Polymorphism), SSH (Suppression Subtractive Hybridization), EST (Expressed Sequence Tags) sequencing, microarrays, differential display, MPSS (Massive Parallel Signature Sequencing), SAGE (Serial Analysis of Gene Expression) and (ii) targeted approaches which include retro-transcription (RT)-PCR and its quantitative derivatives. Both types of approaches are used in combination: non-targeted approaches generally yield a global overview of genes expressed in a particular pathological context and, when combined with targeted approaches, a more precise analysis of individual gene expression is achieved.

Molecular studies dealing with gene expression on *Colletotrichum*-plant interactions are limited to few reports (Goodwin, 2001; Latunde-Dada, 2001;

Perfect *et al.*, 1999), and the application of the above methods to study gene expression in several *Colletotrichum*-plant interaction becomes feasible.

2.7.1. Non-targeted approaches

Among the several transcript profiling techniques available, serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995) and the most recent SuperSAGE (Matsumura *et al.*, 2003) offer advantages of cost effectiveness thanks to the concatenation of expressed sequence tags (ESTs) in short 10 to 45 bp fragments, and are more appropriate for the study of plants and microbes that have well characterized genomes or transcriptomes. Differential display (DD) is one of the earliest gene profiling methods described, allowing the identification of up- and down-regulated genes between several mRNA sources (Liang and Pardee, 1992). For instance, a gene from *Capsicum annuum*, *SAR8.2*, initially identified through DD, was later found to be locally and systemically induced upon infection with *C. coccodes* and in response to biotic and abiotic stresses, suggesting its potential use as a molecular marker of environmental stress (Lee and Hwang, 2003). Adapted from DD, cDNA-amplified fragment length polymorphism (AFLP) (Mathieu-Daude *et al.*, 1999) has been extensively used to study plant-microbe interactions, and is greatly ameliorated with the implementation of fluorescent labelling, capillary based electrophoresis, and with the construction of reference databases in model systems (Donson *et al.*, 2002).

A novel high throughput technique of transcript profiling is Massive Parallel Signature System (MPSS) which involves the massive sequencing of short sequence tags which frequency can be correlated to the expression level of

the corresponding gene (Brenner *et al.*, 2000). As for SAGE, MPSS is currently best suited for well characterized genomes or transcriptomes (Meyers *et al.*, 2004).

2.7.1.1. Expressed Sequenced Tag (EST) sequencing

EST sequencing provides information on the nature (gene discovery) and on the abundance (quantitative estimation) of cDNA sequences in a particular tissue. Massive EST sequencing projects have been carried out for important plant and crop species, and are also one of the most important tools for the discovery of novel genes in orphan species. For example, 840 clones from the *Malva pusilla-Colletotrichum gloeosporioides* f.sp. *malvae* (Collego) interaction were sequenced and their redundancy was compared against 37,000 ESTs, deposited at Genbank, from other host plants interacting with *Colletotrichum* species (Goodwin *et al.*, 2004). The authors found profound differences in EST abundance between different pathogenic interactions. For instance, ESTs encoding cysteine proteinases, heat-shock proteins, pathogenesis-related proteins, and glutathione S-transferase were abundantly present in the pathogenic *Malva pusilla*-Collego interaction. Cysteine proteinases were found more abundant in the resistant *Sorghum bicolor-C. sublineolum* interaction, while heat shock proteins were more abundant in the susceptible interaction between *S. bicolor* and *C. sublineolum* (Goodwin *et al.*, 2004).

Recently, several gene profiling methods have been developed for the study of differential gene expression in order to identify the genes involved in a

given process or treatment. All of these techniques reduce the amount of sequencing associated with large EST projects and are very powerful thanks to their ability to detect changes in expression of mRNAs by selective enrichment. These include representational difference analysis (RDA) (Hubank and Schatz, 1994), subtractive hybridization (Bautz and Reilly, 1966), and suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996).

2.7.1.2. Subtractive hybridization and suppression subtractive hybridization

Standard subtractive hybridization was used to identify putative pathogenicity factors of *Colletotrichum gloeosporioides*, the causal agent of avocado and tomato anthracnose (Hwang and Kolattukudy, 1995). *C. gloeosporioides* germination and appressorium formation are both selectively induced by waxes present only on the avocado plant surface. Waxes from other plants can not induce *C. gloeosporioides* differentiation nor can avocado waxes induce germination and appressorium formation of other *Colletotrichum* (Podila *et al.*, 1993).

Infection-specific sequence tags were generated from the early stages of interaction between maize (*Zea mays*) and *Colletotrichum graminicola* (anthracnose causing agent) (Sugui and Deising, 2002). Sequential subtraction identified transcripts supposedly involved in reprogramming the protein pattern and/or fungal infection structure differentiation, pathogen recognition and mediation of plant defence responses, as well as transcripts that share no similarity with known sequences. Interested in identifying key components of

fungal virulence, fungal pathogenicity and resistance factors of the plant, the authors identified several plant and fungal genes that had already been reported in other pathosystems or when plants were subjected to stress conditions. These genes included maize ubiquitin, leucine-rich repeat protein, lectin and myb-related transcription factor, as well as *C. graminicola* polyketide synthase. Four other transcripts of unknown functions were also identified, which highly suggests that they correspond to new infection specific genes that are important in disease development (Sugui and Deising, 2002).

Suppression subtractive hybridization (SSH), a recently developed PCR-based gene profiling method, is recognised for its ability to detect differentially expressed low-abundance cDNA. SSH can exclude from the analysis the sequences corresponding to mRNA present in equal amounts in two populations (driver and tester) and enrich for the sequences that are more abundant in one of them (Diatchenko *et al.*, 1996; Winstanley, 2002). Its applications to the field of plant-microbe interaction are numerous (Beyer *et al.*, 2001; Beyer *et al.*, 2002; Bittner-Eddy *et al.*, 2003; Cramer and Lawrence, 2004; Gronover *et al.*, 2004; Johnson *et al.*, 2003; Verica *et al.*, 2004; Zuo *et al.*, 2005). The SSH method was used to identify the *CgDN3* gene of *Colletotrichum gloeosporioides*, a "novel" gene in the databases, which appears necessary to avert a hypersensitive response in the tropical pasture legume *Stylosanthes* spp. (refer to Table 2.2 above). Mutants of the isolated *CgDN3* gene could germinate and form appressoria on plant surface but were unable to infect and reproduce on intact host-leaves, on which they initiated a hypersensitive-like response. Mutants lacking *CgDN3* were still able to develop necrotrophically in wounded leaves, suggesting an important

role in the ability of the pathogen to penetrate the host surface (Stephenson *et al.*, 1997; Stephenson *et al.*, 2000). The SSH method also identified a systemic acquired resistance (SAR)-related gene in *Nicothiana benthamiana* plants infected with *Colletotrichum orbiculare* (Shan and Goodwin, 2005). *SAR8.2* gene was found to be induced 8-fold as a result of fungal infection, and silencing of this gene reduced the length of the *C. orbiculare* biotrophic phase of infection by 24 h reinforcing the proposed idea that SAR genes have an important role in the general plant stress response, including the responses of plants susceptible to pathogen attack (Shan and Goodwin, 2005).

SSH has the ability to identify up- and down-regulated genes, but this usually requires further screening using differential screening in order to reduce the amount of sequencing. Traditionally, differential screening is performed by dot-blot macroarrays (Cramer and Lawrence, 2004), Northern hybridization (Gronover *et al.*, 2004), virtual Northern (Bittner-Eddy *et al.*, 2003; Cramer and Lawrence, 2004), colony blot hybridization (Johnson *et al.*, 2003; Mahalingam *et al.*, 2003; Verica *et al.*, 2004), cDNA-AFLP (Birch *et al.*, 1999) and microarrays (van den Berg *et al.*, 2004; Wang *et al.*, 2005). The targets used in the screening can be of various natures ((i) treated and control (tester and driver), (ii) subtracted tester and subtracted driver, (iii) subtracted tester, subtracted driver, unsubtracted tester, and unsubtracted driver, or (iv) subtracted tester, unsubtracted tester, and unsubtracted driver, the choice of which is determined by the number of genes analyzed. Recently van den Berg and colleagues introduced formulae that allow to compile differential screening data that are generated by microarrays (van den Berg *et al.*, 2004) and process the data more efficiently. Two enrichment ratios

(ER1 and ER2) are calculated by incorporating fluorescence data from a simple hybridization design (subtracted tester, unsubtracted driver, and unsubtracted tester). Because the formulae make use of SSH-generated targets, including the subtracted tester, it can be considered more sensitive and more accurate than other screening methods. Not only this method allows to identify and to rule out clones that were not derived from up-regulated transcripts, but it also determines whether a transcript is rare or abundant (van den Berg *et al.*, 2004). In conclusion, the data compilation is greatly simplified and improved which provide the processivity required for the analysis of high-throughput microarray data.

2.7.1.3. Microarray

Microarray analysis of gene expression has emerged in the last few years as a powerful method to analyze large numbers of nucleic acids. The technique involves the immobilization of thousands of DNA sequences (targets) on the surface of glass slides, hybridized with one or more samples (probes) and the hybridization signal is detected and quantitatively analyzed. Examination of the gene expression levels is performed on a global scale with microarrays. Commercial arrays are available for a large variety of plants including *Arabidopsis*, rice, barley, maize, *Medicago*, poplar, soybean, tomato, grape, and wheat through Affymetrix (GeneChip® technology), Agilent, and Illumina. Alternatively, for non-model plants or for more targeted studies, custom arrays can be constructed by synthesis of DNA oligonucleotides or by spotting cDNA fragments directly onto the surface of the glass slides otherwise known as cDNA arrays. Briefly, analysis of microarray-generated data involves six steps (i)

scanning, (ii) spot recognition, (iii) segmentation, (iv) intensity extraction and normalization, (v) detection of differentially expressed genes, and (vi) data mining (Kennedy and Wilson, 2004; Leung and Cavalieri, 2003).

The applications of microarray for global gene expression analyses to the field of plant-microbe interactions are several (Alba *et al.*, 2004; Donson *et al.*, 2002; Kahmann and Basse, 2001; Rensink and Buell, 2005; Wan *et al.*, 2002). For instance, cDNA arrays were used to highlight *Medicago truncatula* genes over-expressed during symbiotic interaction with both *Sinorhizobium meliloti* and *Glomus intraradices*, most of them representing new genes whose role in the symbiosis had not been reported earlier (Manthey *et al.*, 2004). In an attempt to better understand the mechanisms involved in the resistance of peanut to the leaf spot disease, Luo and colleagues (2005) performed a comparative microarray analysis of gene expression between two genotypes of peanut. The authors reported that more than 56 genes were clearly up-regulated in the resistant cultivar (Luo *et al.*, 2005) compared to the susceptible one. Similarly, cDNA microarrays allowed the identification of one thousand genes which originated from a subtracted cDNA library constructed during potato- *Phytophthora infestans* interaction (Wang *et al.*, 2005). The authors monitored the temporal expression of these genes over 72 hours post-infection, and illustrated the diverse patterns of gene expression through hierarchical clustering (Wang *et al.*, 2005). Recently, microarrays were also used to screen SSH subtracted libraries in a differential screening fashion, and thereby highlighted truly up-regulated genes from two distinct libraries made of genes from pearl millet and banana treated with chitin

elicitor and *Fusarium oxysporum* f.sp. *cubense*, respectively (van den Berg *et al.*, 2004).

2.7.2. Targeted approaches

Most of the previously cited high-throughput techniques generate large amounts of gene expression data that require in-depth characterization. Real-time reverse-transcription (RT)-PCR is currently the most widely adopted tool to confirm the differential gene expression data that are obtained by microarray, and to elucidate gene function.

2.7.2.1. Reverse-transcription (RT)-PCR

Retro-transcription, also known as reverse-transcription or retro-transcriptase PCR involves the transformation of an RNA template to a complementary DNA (cDNA) and its amplification by PCR. It is a sensitive method for the analysis of gene expression, and has recently been extensively used in plant pathology.

2.7.2.2. Quantitative real-time RT-PCR

Real-time RT-PCR (as in real-time PCR) reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. SYBR® Green dye which specifically binds to the minor groove of every double-stranded DNA generates an increase in fluorescence emission that is used as a reporter to follow the increase in DNA amounts (Bustin, 2000). Starting

cDNA (or DNA) quantities can be quantified after a few cycles of amplification in which the amount of DNA grows logarithmically from the background to the plateau (exponential phase) (Bustin, 2002; Wilhelm and Pingoud, 2003). Therefore, the higher the starting transcript copy number, the sooner a significant increase in fluorescence is observed. Real-time RT-PCR analysis of gene expression can be performed following several ways: (i) absolute quantification of target transcripts or (ii) relative quantification *i.e.* quantification of target transcripts relative to an additional parameter.

Absolute quantification of gene expression requires the establishment of standard curves which consist in amplifying different known amounts of each cDNA transcript, in the same run as the unknown samples but in separate reactions. Threshold cycles (C_T) numbers are then determined, for the standards and the unknown samples of the run, as fractional numbers at which the fluorescence rises above a fixed baseline. The log of starting cDNA amounts, for a set of standards, is plotted versus their recorded C_T numbers and gives a straight line: the standard curve. This line is used to quantify the amount of each of the cDNA targets in the unknown samples: C_T values are measured for each of the samples, and plotted on the standard curves to determine starting target transcript quantities (Bustin, 2002).

Relative quantification involves the normalisation of target transcript quantities to a reference parameter: (i) one or several house-keeping gene transcripts, (ii) starting total RNA amounts, or (iii) weight of tissue from which RNA material was initially extracted. Normalisation with one or several house-keeping genes is currently considered the most precise and accurate method to

account for (i) the variations in starting RNA amounts occurring between samples and (ii) the differences in reverse-transcription efficiency during the RT step. Several tools are available for the choice of the best house-keeping gene(s) including the software Bestkeeper which determines minimal variation across treatments (Pfaffl *et al.*, 2004) http://www.wzw.tum.de/gene-quantification/beskeeper.html). Relative quantification of gene expression is usually expressed as a ratio of target gene abundance in a treated sample by reference to its abundance in a control sample. Several formulae (Livak and Schmittgen, 2001; Pfaffl, 2001) have been developed to calculate gene expression ratios obtained from QRT-PCR experiments. The recent REST (Relative Expression Software Tool) software integrates both data compilation and statistical analysis thanks to a newly developed pair wise fixed reallocation randomization test (Pfaffl *et al.*, 2002).

2.7.2.3. Applications of RT-PCR to the analysis of *Colletotrichum*-plant interaction

Relative RT-PCR was recently used to study the expression of several plant genes in response to *Colletotrichum* species infections. Dean *et al.* (2005) showed that expression ratios of two different tobacco genes, glutathione S-transferase (GST) and ß-1,3-glucanase, relative to the house keeping gene α-elongation factor were substantially increased following infection by *C. destructivum* and *C. orbiculare*. GST are important enzymes in the course of plant defence to pathogen attack; some act as carriers and transporters of defence

molecules, while others have been shown to sequester pathogen toxins and free radicals in the vacuole (Dean *et al.*, 2005).

Cools and Ishii (2002) applied RT-PCR to monitor the expression of three well-established defense related genes, phenylalanine ammonia lyase (PAL), acidic peroxidase and chitinase induced in cucumber following a pre-treatment with the SAR activator acibenzolar-S-methyl, a chemical that protects cucumber against infections caused by *C. orbiculare* (Cools and Ishii, 2002). There was increase in expression ratio of all the three genes relative to the house keeping gene β-actin cucumber gene (Cools and Ishii, 2002).

The expression of endopolygalacturonase (*cgmpg2*) and pectin lyase (*pnl-1* and *pnl-2*) genes from *C. gloeosporioides* f. sp *malvae* were also monitored by relative RT-PCR. *Cgmpg2* expression was highest relative to the actin gene of *C. gloeosporioides* f. sp *malvae* (*actA*), in culture and during its necrotrophic phase with *Malva pusilla* (Li and Goodwin, 2002). In the case of pectin lyase, there was a differential expression of both genes; *pnl-1* gene expression increased at the onset of the necrotrophic phase, while *pnl-2* was detected in necrotrphic as well as in the biotrophic infection phases of *Malva pusilla* (Wei *et al.*, 2002).

2.8. Conclusion

Colletotrichum coccodes was discovered in 1985 on velvetleaf leaves in Vermont. Since that time, several studies have reinforced our understanding and our ability to efficiently use *C. coccodes* as a mycoherbicide for velvetleaf. However, diagnostic tools to survey the dissemination and the persistence are lacking and the spectrum of our knowledge on the *Abutilon theophrasti-*

Colletotrichum coccodes interaction is still limited. Work is required to understand the pathogenicity mechanisms involved in this pathosystem.

CONNECTING STATEMENT BETWEEN CHAPTERS II AND III

Chapter III describes the development of a strain-specific marker for *Colletotrichum coccodes* (DAOM 183088). Random amplified polymorphic DNA (RAPD) screening was performed against a large collection of organisms to identify a DNA sequence unique to the strain 183088. PCR primers designed on that sequence were then tested for their specificity and sensitivity against pure genomic DNA from various organisms including several other *C. coccodes* strains, heterogeneous fungi, plant and bacteria, as well as more complex DNA sources such as plant and soils spiked or not with the target *C. coccodes* 183088.

The results of this section are the subject of a manuscript that has been published in the Journal of Microbiological Methods (2003, vol. 55 (1), p. 51-64). I have designed the experimental set-up, conducted all of the experiments, and wrote the manuscript. The contributions of Professors S. Jabaji-Hare and Alan Watson who appear as co-authors were as follows: Professor S. Jabaji-Hare provided supervision, and funding throughout the study. She provided valuable suggestions and corrected the manuscript. Professor A. Watson provided funding and revised the manuscript.

CHAPTER III

Detection of the biocontrol agent *Colletotrichum coccodes* (183088) from the target weed velvetleaf and from soil by strain-specific PCR markers

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3.1. Abstract

Diagnostic molecular markers, generated from random amplified polymorphic DNA (RAPD) and used in PCR, were developed to selectively recognize and detect the presence of a single strain of the biocontrol fungus *Colletotrichum coccodes* (183088) on the target weed species *Abutilon theophrasti* and from soil samples. Several strains of *C. coccodes,* 15 species of *Colletotrichum*, a variety of heterogeneous organisms and various plant species were first screened by RAPD-PCR, and a strain specific marker was identified for *C. coccodes* (183088). No significant sequence similarity was found between this marker and any other sequences in the databases. The marker was converted into a sequence-characterised amplified region (SCAR), and specific primer sets (N5F/N5R; N5Fi/N5Ri) were designed for use in PCR detection assays. The primer sets N5F/N5R and N5Fi/N5Ri each amplified a single product of 617 bp and 380 bp, respectively, with DNA isolated from strain 183088. The specificity of the primers was confirmed by the absence of amplified products with DNA from other *C. coccodes* strains, other species representing 15 phylogenetic groups of the genus *Colletotrichum* and 11 other organisms. The SCAR primers (N5F/N5R) were successfully used to detect strain 183088 from infected velvetleaf plants but not from seeded greenhouse soil substrate or from soil samples originating from deliberate-released field experiments. The sensitivity of the assay was substantially increased 1000 fold when nested primers (N5Fi/N5Ri) were used in a second PCR run. N5Fi/N5Ri selectively detected strain 183088 from seeded greenhouse soils as well as from deliberate-released field soil samples without any cross-amplification with other soil microorganisms. This

rapid PCR assay allows an accurate detection of *C. coccodes* strain 183088 among a background of soil micro-organisms, and will be useful for monitoring the biocontrol when released into natural field soils.

3.2. Introduction

Abutilon theophrasti Medik (velveltleaf) is a major annual weed in soybean and corn (*Zea mays* L.) cropping systems in several regions of the USA, Canada, Europe and the Mediterranean (Andersen *et al.*, 1985; Sattin *et al.*, 1992; Spencer, 1984). Velvetleaf is a very difficult weed to control because of its rapid growth rate, capacity to establish a height differential with the crop and prolific seed production (Spencer, 1984). This robust weed has evolved resistance to photosystem II inhibitors such as atrazine (Gray *et al.*, 1996).

Colletotrichum coccodes (Wallr.) Hughes has been reported worldwide on many different hosts. It is primarily found on the Solanaceae and Cucurbitaceae and is particularly destructive on potato and tomato and on several Solanaceous weed species (Andersen and Walker, 1985; Raid and Pennypacker, 1987), including velvetleaf (Watson *et al.*, 2000). The strain *C. coccodes* (Wallr.) Hughes (DAOM 183088) was recovered from velvetleaf leaves in Vermont (Gotlieb *et al.*, 1987), and was introduced as a host-specific pathogen to infect *A. theophrasti* in Canada (Watson *et al.*, 2000). Typically, this strain causes grey-brown foliar lesions on infected *A. theophrasti* plants and areas surrounding lesions become desiccated and diseased leaves are shed pre-maturely. In general, velvetleaf is killed only when inoculated at the cotyledon stage (Wymore *et al.*, 1988). Because of its restricted host range, *C. coccodes* has been considered a potential bioherbicide of velvetleaf, and continuing research in the last decade has focused on laboratory and field studies to optimize *C. coccodes* (183088) inoculum production, formulation, and application methodologies to enhance

weed control efficacy (Hodgson *et al.*, 1988; Wymore and Watson, 1989; Yu *et al.*, 1997).

The causal relationship between the applied bioherbicide and the development of disease symptoms in the host is generally best confirmed by re-isolation of the applied biological control agent exclusively from symptomatic plants. To establish that the disease symptoms resulted from the specific *C. coccodes* strain deployed and not from colonization of the velvetleaf surface by other opportunistic pathogens, *C. coccodes* (183088) must be re-isolated and identified. This procedure requires collection and identification of a large number of isolated cultures. The current method for isolation and enumeration of *C. coccodes* from infected plants and soil mainly relies on axenic culture on selective media (Farley, 1976) and on microscopic examination of morphological traits including conidial color and morphology and the formation of appressoria and sclerotia (Sutton, 1980). Although these classical methods are labor-intensive and time-consuming, they still provide the only means of identification and detection, despite the fact that they are not able to distinguish *C. coccodes* (183088) from *C. coccodes* strains pathogenic on other crops, particularly potato or tomato. In addition, risk assessment studies involving the release of *C. coccodes* (183088) into the environment have created a demand for an accurate tool not only to monitor this strain of *C. coccodes*, but to distinguish it from indigenous background population of microorganisms in the field, including other pathogenic strains of *C. coccodes*.

Molecular tools based on the extraction of nucleic acids from biological samples and amplification by polymerase chain reaction (PCR) of various

sequences has been developed to overcome these limitations. Several of these molecular tools are currently used to detect and monitor soil fungi in natural environments (Bridge and Spooner, 2001). Compared to other diagnostic methods, PCR based-techniques have the advantages that they do not require the target organism to be cultured prior to detection and they can be very sensitive, rapid and specific. The PCR diagnostic assays can be developed by exploiting DNA-sequence polymorphisms within internal transcribed spacer (ITS), unique sequences of mitochondrial DNA, cloned restriction fragments of genomic DNA, the use of sequenced fragments derived from randomly amplified polymorphic DNA (RAPD) markers (Edel, 1998), or universally-primed (UP) markers (Bulat and Mironenko, 1996).

Recently, Cullen et al. (Cullen *et al.*, 2002) designed a nested primer set, Cc1NF1/Cc2NR1, which is specific to sequences unique to pathogenic strains of *C. coccodes* on potato. Although these primers were tested on a large collection of *C. coccodes* strains isolated from potato tubers from different geographical regions, strains of *C. coccodes* pathogenic on other hosts including tomatoes and velvetleaf were not included in their study. Before launching our study, we conducted preliminary tests to check whether the Cc1NF1/Cc2NR1 markers (Cullen *et al.*, 2002) can be used to detect strain 183088 of *C. coccodes* and other strains pathogenic on cucurbits and *Solanaceous* crops. Our results (unpublished) showed that Cc1NF1/Cc2NR1 amplified not only DNA of *C. coccodes* (183088) but DNA from strains of *C. coccodes* pathogenic on tomato, making their application as strain-specific markers to detect *C. coccodes* 183088 in bioherbicide-released experiments impractical, since they will be incapable of

differentiating the target strain 183088 from background field strains pathogenic on potato or tomato.

When a resolution higher than the species level is desired and/or when DNA sequence knowledge is not available for the design of PCR primers, the search of anonymous target DNA sequences has generally proven to be successful. Random markers as products of the PCR-based randomly amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990), also known as arbitrary primed PCR (AP-PCR; (Welsh and McClelland, 1990) is one of the techniques that have been developed to differentiate numerous fungal isolates (Backman *et al.*, 1999; Guthrie *et al.*, 1992; Kelemu *et al.*, 1999; Mesquita *et al.*, 1998; Rodriguez and Owen, 1992; Vaillancourt and Hanau, 1992). This technique, which utilizes a single short (10 nucleotide bases) primer of arbitrary sequence to amplify DNA fragments, requires no prior knowledge of the target site sequence. Since the genome of *Colletotrichum coccodes* is poorly understood, RAPD analysis may prove to be an ideal method for DNA fingerprinting. Sequenced characterized amplified region (SCAR) markers derived from RAPD markers (Paran and Michelmore, 1993) have gained popularity in diagnostics of several biological control agents (Abbasi *et al.*, 1999; Becker *et al.*, 1999; Bulat *et al.*, 2000; Hermosa *et al.*, 2001; Leclerc-Potvin *et al.*, 1999; Schena *et al.*, 2002; Taylor *et al.*, 2003), because they are organism-specific and may represent a single locus in the genome, two advantages that RAPD markers lack.

Our objectives in this study were to develop a PCR detection method that selectively recognizes a single *C. coccodes* strain (183088) from experimental and natural field soils. By converting a RAPD-PCR derived amplicon into a

Sequence Characterized Amplified Region (SCAR), we developed a simple PCR procedure using two primer pairs (outer and nested PCR primers) for identification and detection of *C. coccodes* strain (183088) from pure cultures and from environmental samples originating from bioherbicide-released field trials. This molecular approach to the sensitive identification and detection of *C. coccodes* (183088) opens up new pathways for monitoring the epidemiology of the bioherbicide in field experiments.

3.3. Materials and Methods

3.3.1. Biological material and growth conditions

Thirty-seven heterogeneous organisms including eight strains of *Colletotrichum coccodes,* different genera of fungi and bacteria including some that are common inhabitants of velvetleaf phylloplane and several plants were obtained from commercial sources, culture collections and collaborating researchers (Table 3.1.). All fungi were grown on potato dextrose agar (PDA, Becton Dickinson Microbiology Systems, Sparks, MD) for one week at 28°C in the dark. Five agar plugs (5-mm diameter) were transferred into 75 ml of potato dextrose broth (PDB, Becton Dickinson Microbiology Systems, Sparks, MD) and incubated at room temperature for 12 days under low agitation (100 rpm). Mycelial mats were collected by filtration using Whatman no.1 filter paper. Bacteria were grown in 20 ml of nutrient broth (NB, Becton Dickinson Microbiology Systems, Sparks, MD) media supplemented with 1% D-glucose at 30°C, under low agitation for 48 h. One ml of bacterial culture was

microcentrifuged (12,000 x g) for 2 min. and the supernatant was discarded. The bacterial pellet was directly used for DNA extraction. Plants were grown either under in vitro conditions on Murashige and Skoog medium (Murashige and Skoog, 1962) or grown in pots containing pasteurized greenhouse soil and placed in a greenhouse (adjusted to 23°C day/ 20°C night; 300 μmoles/m^2/s) until sufficient biomass was obtained.

A total of seven soil samples were collected from different regions of Québec (Table 3.2.). Soil A originated from experimental field plots cultivated with corn and soybeans and treated annually with *C. coccodes* (183088) with a background population level of $1x10^4$ CFU of *C. coccodes* per gram of soil as determined by 10 x serial dilution plating on semi-selective medium that promote the growth of *Colletotrichum* species (Farley, 1976). Soils B and C were sampled from cultivated fields located at Macdonald Campus of McGill University, while soil D originated from the Morgan Arboretum of McGill University with poplar and pine vegetation. Soils, E, F and G were sampled from roadside habitats. To avoid large soil aggregates and to obtain homogeneous material, the soils were sieved through a 4-mm sieve prior to DNA extraction. All biological material, except for bacteria, was flash-frozen in liquid nitrogen, freeze-dried for 2-3 days, ground in liquid nitrogen to produce a fine powder, and stored at –80°C prior to genomic DNA extraction.

3.3.2. Preparation of *C. coccodes* inoculum

Conidial production for velvetleaf and soil-seeding was initiated by transferring five agar plugs from a one-week old *C. coccodes* (183088) PDA culture into 250 ml PDB and incubating it for 2 weeks under 100 rpm agitation. Liquid cultures were filtered through Whatman paper no.1, centrifuged for 15 min. at 700 rpm, and the conidial pellet was resuspended in 5 ml of distilled water. Conidial suspension was adjusted with distilled water to a final concentration of $8.3x10^7$spores/ml, using a haemacytometer.

3.3.3. Velvetleaf inoculation

Two-leaf stage velvetleaf seedlings were grown in 8-cm diameter plastic pots (2 plants per pot) containing 125 g Pro-mix, a standard greenhouse soil substrate consisting of sphagnum peat moss:perlite:vermiculite (50:25:25; Premier Horticulture Ltée, Rivière du Loup, QC., Canada) for two weeks in a growth chamber adjusted to 23°C day/20°C night, 300 μmoles/m^2/sec, and 80% humidity. Plants were sprayed with a conidial suspension of *C. coccodes* ($8.3x10^7$ conidia/ml of water) at a rate of 12 ml/m^2 and spread uniformly on the leaf surface using a spray chamber. Control treatments consisted of spraying plants with water only. The experiment consisted of 4 replications per treatment. Leaves from both treatments were harvested five days after incubation and divided into two equal portions. One portion was used for preparation of DNA template and the second was verified for the presence of *C. coccodes* by plating small pieces of leaves onto semi-selective medium, and incubating them for one week at 28°C in the dark.

3.3.4. Greenhouse soil inoculation

Non-sterile and sterile Pro-mix, (autoclaved at 120°C for 1 h. on three consecutive times) was placed into 8-cm diameter pots with holding capacity of 125 g. The treatments consisted of (i) seeded sterile Pro-mix; (ii) seeded non-sterile Pro-mix, and their respective controls. Seeding of Pro-mix consisted of incorporating 5 ml of *C. coccodes* (183088) conidial suspension ($8.3x10^7$ conidia/ml of water) into the upper 2 cm of the soil substrate. Control treatments consisted of sterile and non-sterile Pro-mix mock-treated with 5 ml of water. All pots (three replications per treatment) were incubated for three weeks at 28°C in the dark. The soil from each replication of each treatment was transferred into a Ziploc bag (S.C. Johnson and Son, Brantford, ON), mixed thoroughly to insure uniform distribution, sieved through 4-mm diameter sieve, freeze-dried and kept at –80°C for DNA extraction. To check for the presence of *C. coccodes*, a small portion (0.5 g) of non-freeze-dried soil from each replication was plated on semi-selective medium and incubated at 28°C in the dark for one month. Colony identification of *C. coccodes* (183088) was later confirmed by PCR amplifying DNA extracted from these colonies with N5F/N5R primers.

3.3.5. Preparation of DNA templates

Fungal genomic DNA was isolated from 20 mg freeze-dried mycelium according to the protocol of Lee and Taylor (Lee and Taylor, 1990). For plant material, DNA was isolated using the cethyltrimethylammonium bromide method of Doyle and Doyle (Doyle and Doyle, 1987) with the following modifications: 2

µl of RNAse (10 µg/ml) were added to the sample after the chloroform-isoamyl alcohol extraction step, incubated 30 min. at 37°C, and subjected to a second chloroform-isoamyl alcohol extraction step. Bacterial genomic DNA was isolated according to the methods of Fisher (Fisher, 1985) and Ausubel *et al.* (Ausubel *et al.*, 1987). DNA isolated from fungi, bacteria and plants were quantified by spectrophotometry and adjusted to 5 ng/µl with 10 mM Tris-HCl (pH 7.4). To determine the PCR threshold detection limit of *C. coccodes* (183088), genomic DNA was 10 x serially diluted from 5 ng/µl to 5 fg/µl with 10 mM Tris-HCl (pH 7.4) and amplified with SCAR and nested primers. DNA extraction from seeded and non-seeded greenhouse soil substrate and from natural soils was performed with the UltraClean™ Soil DNA Isolation kit (MoBio Laboratories, Inc., Solana Beach, CA) with the following modifications: (i) the DNA was extracted from 200 mg of freeze-dried soil instead of 250 mg of soil, and (ii) a FastPrep apparatus (Thermo Savant, Holbrook, NY) instead of vortexing the soil was used in order to dislodge DNA from the substrate, at speed level 4 for 20 sec. To verify that the soil DNA protocol has no bearing on *C. coccodes* DNA extractability, samples from all six natural soils (Table 3.2.) were spiked with conidial suspension (8.3 x 10^7 conidia/ml) of *C. coccodes*, mixed well, freeze dried and subjected to soil DNA extraction as described above. All soil DNA was resuspended in 50 µl of solution S5 (MoBio Laboratories, Inc.) and kept at –20°C until PCR amplification.

3.3.6. Random amplified polymorphic DNA (RAPD) amplifications

Thirty-seven Operon decamer primers (kits B and N of Qiagen Inc., Mississauga, ON) were used for initial RAPD screening. The PCR protocol was performed as follows: the reaction mix (25 µl) consisted of 2 µl DNA template (10 ng), 1 µM of RAPD primer, 2.5 µl of a 10x buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 0.5 U of *Taq* polymerase (GibcoBRL® Life Technologies) and 200 nM each of the dNTP (Amersham Pharmacia Biotech, Inc.). Reactions were prepared on ice and overlaid with one drop mineral oil. The amplification was carried out in a PTC®-100 (MJ Research, Watertown, MA). After a denaturation step (94°C for 1 min.), 40 cycles of amplification (94°C for 1 min., 37°C for 1 min., and 72°C for 2 min.), and 10 min. at 72°C as a final extension step were performed. A PCR negative control, consisting of 2 µl dH_20 instead of 2 µl DNA, was included in each PCR run. Only primers that generated polymorphic patterns of amplified products between *C. coccodes* (183088) and the other strains were retained for a second-round screening. OPN-05 was retained for its ability to amplify a 622 bp DNA fragment from *C. coccodes* (183088) DNA, but not from the other fungal strains, bacterial and plant DNA.

Dendrograms were constructed based on the RAPD banding patterns obtained from 20 RAPD primer amplifications. Results for the 20 primers were combined and Pearson correlation was used to cluster analyze the combined data using UPGMA. All calculations were made by the BioNumerics software (Applied Maths, Kortijk, Belgium).

3.3.7. Elution of PCR products and sequencing

The 622 bp amplicon generated by OPN-05 primer, specific to *C. coccodes* (183088), was excised from the gel and cleaned with the Qiagen Gel Extraction kit (Qiagen Inc., Mississauga, ON). Prior to cloning, DNA concentration was evaluated by electrophoresing the pure DNA fragment along with a mass ladder standard (Gene Ruler™ 100 bp DNA Ladder; MBI Fermentas, Burlington, ON) and by spectrophotometry measurements. The 622 bp amplicon was cloned using the Zero Blunt® PCR cloning kit (Invitrogen Corporation, Carlsbad, CA). Ligated plasmids were used to transform *E. coli* competent cells (One Shot™ TOP10, Invitrogen), according to the manufacturer's protocol. Plasmids were extracted from positive clones according to Sambrook et al. (Sambrook *et al.*, 1989). The cloned fragment was cycle-sequenced with the Perkin Elmer 373 automated DNA sequencer (ABI Prism™ 310 Genetic Analyzer, PE Applied Biosystems) using the DYEnamic ET chemistry (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, QC).

3.3.8. Sequence analysis and design of primers

The 622 bp fragment sequence, (OPN-05_{622}, AF 448480) was analyzed with Chroma (Griffith University, Queensland, Australia) and submitted to BLASTN/BLASTX and PROSITE to check potential homologies with sequences or domains. Based on full-length sequence of the 622 bp amplicon, two sets of 20-mer primers, SCAR (N5F/N5R) and nested (N5Fi/N5Ri) were designed using the DNAMAN computer software (Lynnon Biosoft®, Vaudreuil-Dorion, QC), to

amplify diagnostic products of 617 and 380 bp, respectively (Table 3.3.). All primer sequences were submitted to Nucleotide Blast (search for short nearly exact matches). The primers were custom synthesized by AlphaDNA (Montréal, QC).

3.3.9. Sequence Characterized amplified region (SCAR) and nested PCR amplifications

The specificity of the SCAR (N5F/N5R) primer pair to *C. coccodes* (183088) was tested in conventional PCR against all strains of *C. coccodes*, *Colletotrichum* species, a variety of heterogeneous microorganisms and plants (Table 3.1.), and against DNA extracted from velvetleaf and seeded Pro-mix substrate. The nested primer set N5Fi/N5Ri was used in a second-round PCR assay to detect *C. coccodes* (183088) in seeded Pro-mix substrate and field soils. The reaction mix was the same as that of RAPD amplification, except that 2 µl of DNA template (using the SCAR primers) or 2 µl of a 1/20 dilution of first-round PCR product from soil samples (using the nested primers) was used as template. The optimal PCR amplification conditions consisted of an initial denaturation at 94°C for 3 min. followed by 30 cycles at 94°C for 1 min., 62°C for 1 min., 72°C for 1 min. and a final extension step at 72°C for 10 min. A PCR negative control, consisting of 2 µl distilled water (dH_2O) instead of 2 µl DNA, was included in each PCR run.

As a positive control, DNA samples extracted from soil were amplified with primers (ITS-1F/ITS4) that recognize the internal transcribed spacers (ITS1-

ITS2) of fungal nuclear ribosomal DNA (White *et al.*, 1990). This control was performed to insure the quality and integrity of the extracted DNA, to test for the presence of fungal DNA in the soil, and to optimize the DNA concentrations to minimize the risk of obtaining a false-negative result.

3.3.10. Post-PCR analysis

Amplification products from RAPD screening experiments were resolved on 1.4% electrophoresis agarose gels in 0.5 x TBE buffer, PCR products using SCAR, nested and ITS primers were resolved on 0.9% electrophoresis agarose gels in 1 x TAE buffer. A Gene Ruler™ 100 bp DNA Ladder (MBI Fermentas, Burlington, ON) was used as a molecular weight marker. Gels were stained with ethidium bromide and images were recorded by the gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON).

3.4. Results

All of the 37 primers used in this study were capable of amplifying multiple polymorphic DNA fragments from all of the organisms tested. Band patterns were consistent and reproducible. Phylogenetic analyses, conducted on RAPD banding patterns, indicated that the target *C. coccodes* strain 183088 does cluster with a group comprising of the *C. coccodes* strains. However, it shared a 59% genetic similarity with other strains of this group of which it was the most divergent (data not shown).

Among the thirty-seven Operon primers used in PCR screening of *Colletotrichum* species and heterogeneous organisms (Table 3.1), OPN-05 generated a RAPD polymorphic profile that distinguished *C. coccodes* (183088) from the other *C. coccodes* isolates (Fig. 3.1A), as well as from other organisms (Fig. 3.1A, B). The primer amplified a unique 622 bp DNA fragment from *C. coccodes* (183088) (Fig. 3.1). Complete sequencing of the OPN-05_{622} bp amplicon (accession number: AF 448480) and comparison with DNA database sequences (BLASTN program) of other organisms revealed 20.4% similarity in a 127 bp overlap to the sequence of the 5S-rDNA gene of *Neurospora crassa.* Sequence characterized amplified region (SCAR) N5F/N5R and nested primers N5Fi/N5Ri were designed to flank DNA fragments of 617 bp and 380 bp, respectively (Table 3.3). The specificity of the SCAR and nested primers for *C. coccodes* (183088) was tested against all the organisms used during the RAPD screening (Table 3.1). Under the outlined PCR conditions, N5F/N5R primer pair amplified a single fragment of 617 bp from *C. coccodes* (183088) genomic DNA (Figs. 3.2A, B). No PCR product was observed in other strains of *C. coccodes* or in plants, bacteria and other fungal strains (Fig. 3.2). Similarly, the nested N5Fi/N5Ri primers amplified a single amplicon of expected size of 380 bp only from *C. coccodes* (183088) genomic DNA (data not shown), indicating that both sets of primers are strain-specific. The ability of N5F/N5R to detect target DNA in *C. coccodes*-inoculated velvetleaf was confirmed by the presence of a single PCR product of the expected size of 617 bp (Fig. 3.3A; lane 2). No product was detected from non-inoculated velvetleaf (Fig. 3.3A; lane 3), as well as from non-seeded sterile and non-sterile Pro-mix (Fig. 3.3A, lanes 5 and 7). N5F /NFR

primers failed to detect *C. coccodes* (183088) from seeded sterile and non-sterile Pro-mix (Fig. 3.3A, lanes 4 and 6), although the presence and identity of *C. coccodes* (183088) in these soil samples was confirmed by plating a portion of the soil on selective culture medium, and by PCR amplification of DNA, extracted from growing colonies, using N5F/N5R (Fig. 3.3B; lanes 4 and 6). However, a second-round amplification using the nested primer set N5Fi/N5Ri detected the expected 380 bp DNA fragment from *C. coccodes* (183088) grown in seeded sterile and non-sterile greenhouse soils (Fig. 3.3C, lanes 4 and 6). No amplification product was detected in unseeded sterile and non-sterile Pro-mix (Fig. 3.3C, lanes 5 and 7). The lowest amount of *C. coccodes* genomic DNA detectable with N5F/N5R primers was 1 ng of DNA, while the sensitivity of the assay was increased by 10^3 fold using N5Fi/N5Ri in nested PCR (data not shown).

When tested on various types of natural soils, the nested-PCR assay successfully detected *C. coccodes* in soil samples originating from the bioherbicide-released experimental field plots (Fig. 3.4, lane 2). The specificity and sensitivity of both SCAR primer set (N5F/N5R; data not shown) and nested primer set (N5Fi/N5Ri) was confirmed when no signal was generated from DNA extracts of six ecologically different soil samples (Fig. 3.4, lanes 3-7), indicating that both primer sets did not-cross react with non-target DNA. The presence of non-target fungal DNA in all soils, as revealed by several amplified products, was confirmed using the universal primer set ITS4 and ITS-1F (data not shown). Similarly, *C. coccodes* (183088) DNA was PCR amplifiable in all spiked natural

soils, validating that the soil DNA extraction method used in this study was suitable for *C. coccodes* DNA extraction (data not shown).

Table 3.1. Details of *Colletotrichum* species, heterogeneous organisms and plant species used in this study.

Species	Strain electrophoresis designation	Original designation	Origin	Host or Substrate	Region
***Colletotrichum* species**					
C. acutatum	1	5.7.52	A	NA[a]	NA[a]
C. coccodes	2	145740	B	*Solanum tuberosum* cv. *Kennebec*	New Brunswick/Canada
	3	147612	B	*Curcubita* spp.	Ontario/Canada
	4	182572	B	*Lycopersicon esculentum*	British Columbia/Canada
	5	182826	B	roots of rutabaga	Manitoba/Canada
	6[b]	183088	B	*Abutilon theophrasti*	Vermont/USA
	7	184034	B	*Convulvulus arvensis*	Québec/Canada
	8	189075	B	*Solanum tuberosum*	Prince Edward Island/Canada
	9	197041	B	*Solanum tuberosum*	Prince Edward Island/Canada
C. dematium	11	24488	C	*Beta vulgaris*	New Zealand
	12	38107	C	*Lycopersicon esculentum*	Indiana/USA
C destructivum	14	172381	B	seeds of *Medicago sativa*	British Columbia/Canada
	15	179749	B	seeds of *Medicago sativa*	Manitoba/Canada
C gloeosporioides	16	183087	B	isolated from rutabaga root	Manitoba/Canada
	17	44313	C	french bean	NA[a]
C. graminicola	18	2.1.33	A	NA[a]	NA[a]
C. lindemuthianum	19	196853	B	pods of *Phaseolus vulgaris*	Ontario/Canada
C. lini	20	7.2.51	A	NA[a]	NA[a]
C. musae	21	182828	B	dead leaves of palm tree	Alberta/Canada
C. pisi	22	196850	B	*Pisum sativum*	Ontario/Canada
C. trifolii	23	197037	B	stems of *Medicago sativa*	Alberta/Canada
C. viciae	27	196822	B	*Vicia* spp.	Ontario/Canada
C. brassicae	28	116226	B	pods of *Eruca sativa*	Ontario/Canada
C. capscici	29	212661	B	*Portulaca*	Saskatchewan/Canada
C. fuscum	33	216112	B	*Sonchus* spp.	
C. sublineolum	35	212374	A	*Agropyron repens*	
Glomerella cingulata	37		A		

Heterogeneous organisms					
Alternaria alternata	38	F005	D	NA[a]	NA[a]
Botrytis cinerea	39	F014	D	NA[a]	NA[a]
Fusarium solani	40	205	E	soil	Ontario/Canada
Verticillium albo-atrum	41	VaaAT3	E	*Solanum tuberosum*	Ontario/Canada
Pseudomonas fluorescens	42		D	NA[a]	NA[a]
Streptomyces scabies	43	201	D	NA[a]	NA[a]
Plant species					
Lycopersicon esculentum	44	Tomato - F1 Hybrid Affirm	D	NA[a]	NA[a]
Solanum tuberosum	45	Potato - Black Burban	D	NA[a]	NA[a]
Abutilon theophrasti	46	Velvetleaf	D	NA[a]	NA[a]
Zea mays	47	Corn - Pionneer Hybrid 3921	D	NA[a]	NA[a]
Glycine max	48	Soybean - Bayfield	D	NA[a]	NA[a]

[a]not available.
[b]*C. coccodes* strain 183088 is the bioherbicide target organism.
A= D. TeBeest, University of Arkansas, USA.
B= Canadian Collection Fungal Cultures (CCFC), On., Canada.
C= American Type Culture Collection (ATTC), VA., USA.
D= S.J.-Hare, McGill University, Qc., Canada.
E= G. Lazarovitz, Agriculture Agrifood Canada , On. Canada.

Table 3.2. Characteristics and location of soil samples used in this study.

Sample	Soil type	Organic Matter (%)	pH	History / Location	Field Location
A	Sandy loam	5.5	7.1	cultivated with corn and soybean, *C. coccodes* 183088 experimental release	Macdonald farm, Ste-Anne de Bellevue, Qc, Canada
B	Loam	32.7	6.5	cultivated with vegetables	Horticultural soil, Ste-Anne de Bellevue, Qc, Canada
C	Sandy clay loam	22.1	7.2	cultivated with oats/barley	Seed farm, Ste-Anne de Bellevue, Qc, Canada
D	Sandy loam	41.4	5.1	poplar/pine forest	Arboretum, Ste-Anne de Bellevue, Qc, Canada
E	Sandy	0.5	7.7	highway roadside	Laval, Qc, Canada
F	Sandy loam	5.7	7.9	highway roadside	Mascouche, Qc, Canada
G	Clay loam	4.1	8.2	highway roadside	Pointe-Claire, Qc, Canada

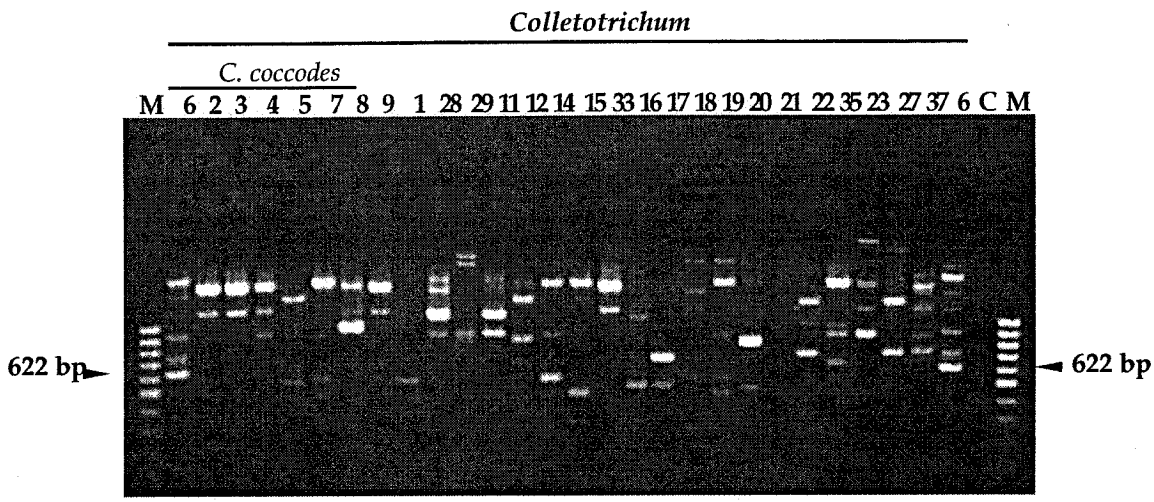
Table 3.3. RAPD, SCAR, nested and ITS PCR primers used in this study.

	Primer	Sequence (5'-3')	Primer length (bp)	Tm (°C)	Amplicon size (bp)
RAPD	OPN-05	ACTGAACGCC	10	37	622
SCAR	N5F	ACGCCACTAACACCTCTCAC	20	58	617
	N5R	ACTGAACGCCCTCGCAAGAT	20	58	
Nested	N5Fi	AATGGCGTTCATGAAGGTAG	20	62	380
	N5Ri	ATCTCAATGAAGTACGTCGC	20	62	
ITS[a]	ITS-1F	CTTGGTCATTTAGAGGAAGTAA	22	60	~530
	ITS4	TCCTCCGCTTATTGATATGC	20	58	

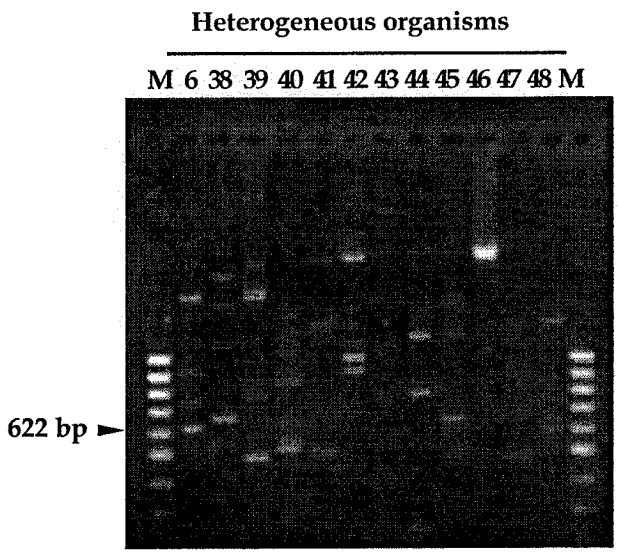
[a]White *et al.*, 1990

Figure 3.1. RAPD profiles obtained with the OPN-05 primer. **A,** on DNA from *Colletotrichum coccodes* and *Colletotrichum* species, and **B,** on DNA from heterogeneous organisms (fungi, bacteria and plant species). Electrophoresis numbers refers to original strain or organism designations (Table 3.1). M = 100 bp molecular weight marker. C = PCR negative control (sterile distilled water). Electrophoresis number 6 is strain 183088 of *C. coccodes*. Arrow markers indicate the 622 bp amplified product that is unique to strain 183088.

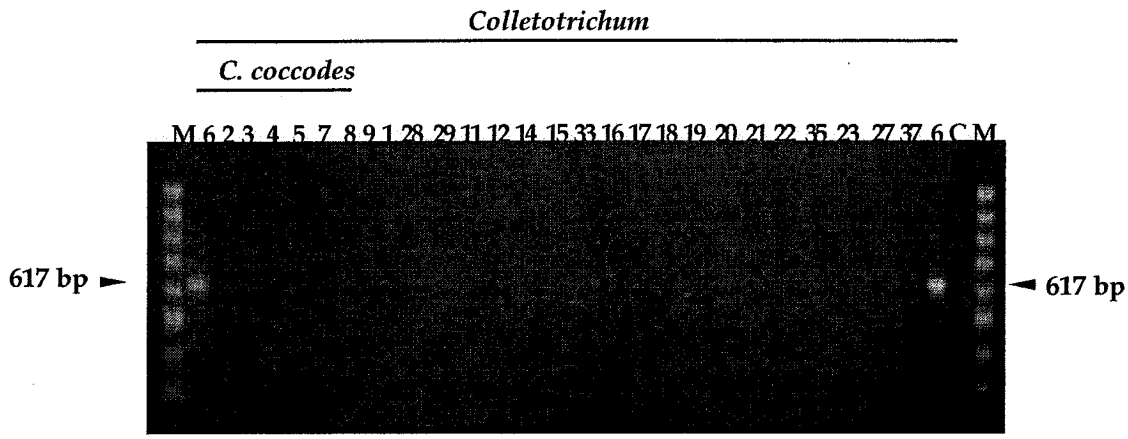
A.


Colletotrichum
C. coccodes
M 6 2 3 4 5 7 8 9 1 28 29 11 12 14 15 33 16 17 18 19 20 21 22 35 23 27 37 6 C M
622 bp
622 bp

B.


Heterogeneous organisms
M 6 38 39 40 41 42 43 44 45 46 47 48 M
622 bp

A.



B.

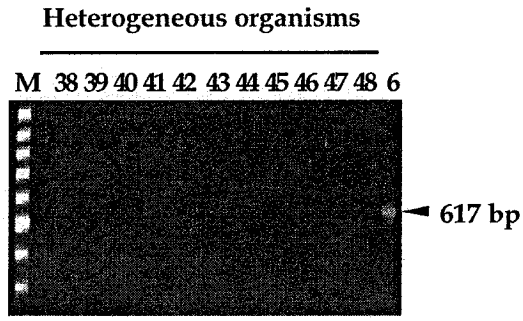
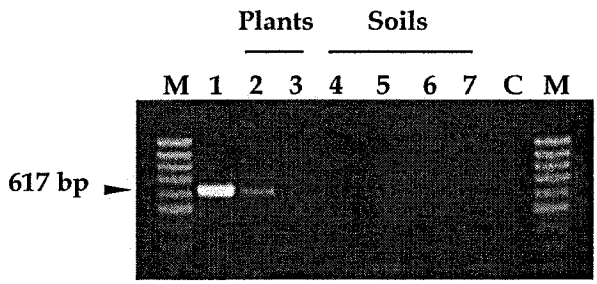


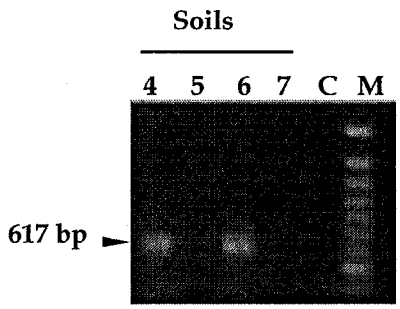
Figure 3.2. Amplicon generated with SCAR primer (N5F/N5R) and amplified under optimal PCR conditions. **A,** *Colletotrichum coccodes* and *Colletotrichum* species, and **B,** heterogeneous organisms (fungi, bacteria and plant species). Refer to Table 3.1 for details on the original strain designation. M = 100 bp molecular weight marker. C = PCR negative control (sterile distilled water). Electrophoresis number 6 is strain 183088 of *C. coccodes*. Arrow markers indicate the 617 bp amplified product that is unique to strain 183088.

Figure 3.3. PCR detection of *C. coccodes* strain 183088 from velvetleaf plants, seeded greenhouse soil substrate and individual *C. coccodes* colonies originated from soil dilution platings. **A,** velvetleaf plants and seeded greenhouse soil substrate and **B,** individual *C. coccodes* colonies originated from soil dilution platings. A and B show the desired amplicon of 617 bp generated with the SCAR primer (N5F/N5R) set after first-round amplification. **C,** Amplification results of nested PCR (primers N5Fi/N5Ri) following the amplification of (2 μl) first-round products shown in Fig. 3.3A. Lane 1 = *C. coccodes* 183088 genomic DNA. Lane 2 = velvetleaf plants infected with *C. coccodes* 183088. Lane 3 = non-infected velvetleaf plants. Lane 4 = sterile soil inoculated with *C. coccodes* 183088. Lane 5 = non-inoculated sterile soil. Lane 6 = non-sterile soil inoculated with *C. coccodes* 183088. Lane 7 = non-inoculated non-sterile soil. M = 100 bp molecular weight marker. C = PCR negative control (sterile distilled water).

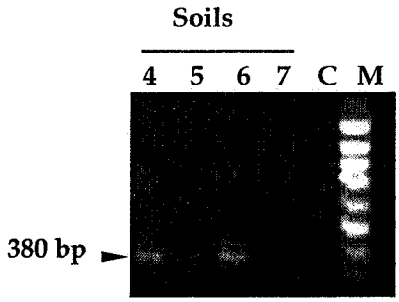
A.


Plants
Soils
M 1 2 3 4 5 6 7 C M
617 bp

B.


Soils
4 5 6 7 C M
617 bp

C.


Soils
4 5 6 7 C M
380 bp

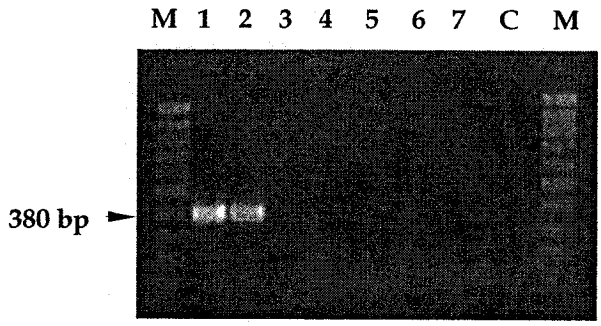


Figure 3.4. Nested PCR detection of *C. coccodes* strain 183088 from natural soil samples. Arrow marker on the left indicates the 380 bp (N5Fi/N5Ri) product. Lane 1 = *C. coccodes* 183088 genomic DNA. Lane 2, 3, 4, 5, 6, 7, 8 corresponds to DNA isolated from natural soils (refer to Table 3.2). A = cultivated soil with background *C. coccodes* 183088 population. B = poplar/pine forest soil. C and D = agricultural field soils. E, F and G = soils collected from Quebec roadsides. M = 100 bp molecular weight marker. C = PCR negative control (sterile distilled water).

3.5. Discussion

Public concern about chemical herbicide residues on crops has led to a progressive increase of interest in alternative strategies for the control of weeds (Gressel, 2000). Biological control by means of microorganisms that naturally occur on weeds looks promising provided that the biocontrol agent is carefully selected and characterized. The potential of strain 183088 of *C. coccodes* in biocontrol strategies of velvetleaf is a case in point, raising the need for feasible and reliable diagnostic tests that can discriminate between this particular strain and other related *C. coccodes* strains. Since identification based on morphology in this case is no longer possible, we pursued the development of molecular diagnostic tests for the specific identification of the strain 183088 of *C. coccodes.*

Various strategies and techniques have been employed to develop PCR assays for the genetic identification and diagnosis of fungal plant pathogens, their use generally reflecting the desired levels of sensitivity and taxonomic specificity achievements. Detection and identification of fungi using primers generated from the internal transcribed spacer (ITS) regions (Bell *et al.*, 1999; Lee *et al.*, 2001; Moricca *et al.*, 1998) or from the intergenic spacer (IGS) region (Chen *et al.*, 2000), are commonly applied in PCR-diagnostic assays. Sreenivasaprasad and co-workers (Sreenivasaprasad *et al.*, 1996) evaluated the potential of the ITS1 region of rDNA as genetic marker for species delimitation in the genus *Colletotrichum.* Their results indicated that species such as *C. coccodes* did not show any intraspecific variability of the ITS1 region. This finding was corroborated by a preliminary investigation conducted earlier in our study where alignment of sequences from several strains of *C. coccodes* (accession numbers: Z32933;

Z32931; Z32930, AJ301984, AJ301957, AJ301953) including strain 183088 (accession number: AY 211498) revealed a 100% similarity (unpublished data), making the *C. coccodes* ITS region not suitable for generating strain-specific markers. Recently Cullen et al. (Cullen *et al.*, 2002) succeeded in detecting potato strains of *C. coccodes* using primers flanking their ITS1/ITS2 regions. Although the designed primers (Cc1NF1/Cc2NR2) exploited the most variable region of the ITS1 (their study), we were able to amplify DNA from pathogenic strains of *C. coccodes* other than potato using their primers (this study, data not shown). Taken together the above findings, suggest that the ITS1 region can not resolve intraspecific variability in *C. coccodes.*

A valid procedure to generate diagnostic fragments for pathogenic and non-pathogenic fungi (Hermosa *et al.*, 2001; Leclerc-Potvin *et al.*, 1999) is the use of arbitrarily primed PCR. Depending on the primers used and on the reaction conditions, random amplification of fungal genomes produces genetic polymorphisms specific at the genus, species or strain levels. In our study, the analysis of several strains of *C. coccodes* and species of *Colletotrichum* by RAPD-PCR and cluster analysis of generated RAPD banding patterns highlighted intra-specific variations between strain 183088 and other related *C. coccodes* strains. This finding opened up the possibility to exploit RAPD profiles as starting information to generate more specific probes such as SCAR primers. RAPD-PCR with the Operon primer OPN-05 enabled the identification of a 622 bp fragment only present in the polymorphic pattern of strain 183088 of *C. coccodes*. This fragment was used as template to generate a set of SCAR primers (N5F/N5R) that consistently amplified a SCAR marker of 617 bp from the genome of strain

183088 up to the limit of 1 ng/µl. The specificity of the N5F/N5R primer set was confirmed when no amplicons were generated with DNA isolated from a large collection of taxonomically diverse organisms. This finding was also strengthened by the lack of detectable cross-reaction with the total microbial community DNA from various natural soils, even after two successive rounds of PCR.

In this study, the SCAR primer specificity was retained when strain 183088 was searched for in greenhouse infected velvetleaf plants. However, the sensitivity was lost when attempts were made to detect the biocontrol in seeded pro-mix soil samples and in deliberate-released field soil samples. The fact that the presence of strain 183088 in these samples was confirmed by dilution plating on semi-selective medium suggests that the absence of amplification could be due to the low amount of strain 183088 in soil or to the presence of PCR inhibitory compounds or both (Bridge and Spooner, 2001). Similar trends were reported by other studies using simple PCR techniques to detect fungal pathogens from soil substrate (Grote *et al.*, 2002).

To avoid false negatives detection, a method with higher sensitivity was required, and so nested PCR assay was developed using the designed nested primer set N5Fi/N5Ri. This additional PCR run managed in detecting strain 183088 in all situations where SCAR primers previously failed, leading to a 1000 fold increase in sensitivity. These results are in agreement with results of other authors who were able to detect less than 25 pg or 1 fg in media such as soil or plant material, respectively (Cullen *et al.*, 2002; Grote *et al.*, 2002).

Humic acids, tannins and lignin-associated products occurring in the soil are known to inhibit PCR amplifications (Bridge and Spooner, 2001). Interference of these PCR inhibitors can be reduced during DNA isolation by the use of various cations, PVPP or hydroxyapatite columns (Braid *et al.*, 2003). Alternatively, the degree of inhibition during PCR could be evaluated by the addition of a fixed amount of a control DNA molecule in each PCR reaction (Weissensteiner and Lanchbury, 1996). In our study, diluting the first round PCR products and re-amplifying them in nested PCR may have partly or completely circumvented the interference of inhibitors by diluting them to a point where PCR amplification is not affected. Under our conditions, the combination of the MoBio DNA extraction kit that used a DNA-binding column, and nested PCR using the universal ITS primers proved efficient in amplifying good quality DNA from seven different soil types varying in the percentage of organic matter. Hence, the lack of amplification of strain 183088 SCAR fragment is a true reflection of the absence of the target organism in these soils.

In summary, strain specific markers were identified and converted into SCAR markers. Both RAPD and SCAR markers were successfully used to discriminate *C. coccodes* 183088 from other strains of *C. coccodes*. These markers were also useful to specifically detect and monitor strain 180388 into deliberately released field soil even in the presence of DNA originating from multiple types of microorganisms. Markers such as the ones we have developed may be important for detection of other biocontrol agents or specific pathogenic microorganisms or for the protection of commercial or patent strains. These diagnostic tools will permit new insights on the ecology of strain 183088

including, mode of spread, survival during unfavourable conditions, ability to colonise weeds, and influence of herbicides on the population of strain 183088 of *C. coccodes.* Finally, as shown, this assay could ultimately be used as a routine field-monitoring tool for the biological management of velvetleaf in efficacy and risk assessment studies. However, the assay as described in this study is based on end-point PCR detection and is, thereby, qualitative. The application of N5Fi/N5Ri primers in real-time PCR assays is currently underway to accurately quantify strain 183088 of *C. coccodes* from diverse environmental samples.

CONNECTING STATEMENT BETWEEN CHAPTERS III AND IV

Strain-specific primers, N5F/N5R and N5Fi/N5Ri, have been developed in chapter III and are now available for the detection of *C. coccodes* 183088 in complex mixtures such as plants and soil. This chapter describes the quantification of *C. coccodes* in deliberate release field soils samples using the primer set N5Fi/N5Ri. In addition, this chapter describes the use of an external control in PCR assays that takes into account the effects of soil PCR inhibitors and allows for the normalization of real-time PCR data. Attempts to quantify *C. coccodes* from various soil samples seeded or spiked with known quantities of *C. coccodes* highlighted the presence of PCR inhibitors that led to biases in the quantification of the target organism. A PCR efficiency compensation control, external control, was spiked in the DNA extracts and quantified in parallel to the target *C. coccodes* DNA to evaluate the PCR efficiency on a sample per sample basis. This allowed us to quantify *C. coccodes* from field soils in which it had previously been released by taking into consideration the effects of PCR inhibitors. The bioherbicide was quantified from fifteen out of eighteen analyzed soil samples including from the control plots that had not been sprayed for several years.

The results of this section are the subject of a manuscript that has been published in the Canadian Journal of Plant Pathology (vol. 28, p. 42-51). Permission has been granted (Email received on January 19th, 2006) by Dr. Zamir Punja, editor-in-chief of the Canadian Journal of Plant Pathology, to use the

content of this manuscript in the present thesis. I have designed the experimental set-up, conducted all of the experiments, and wrote the manuscript. The contribution of Professors S. Jabaji-Hare and Alan Watson who appear as co-authors were as follows: Professor S. Jabaji-Hare provided supervision and funding throughout the study, and provided technical assistance during all of this study. She provided valuable suggestions and corrected the manuscript. Professor A. Watson provided funding, plant and soil samples for PCR analysis, and revised the manuscript. Dr. P. Seguin gave invaluable advice on the choice of the most suitable statistical approach, helped in running the statistical analyses, and critically revised the manuscript.

CHAPTER IV

Real-time PCR quantification of *Colletotrichum coccodes* DNA in soils from bioherbicide field-release assays, with normalization for PCR inhibition

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4.1. Abstract

A real-time polymerase chain reaction (PCR) assay was developed to quantify simultaneously the biocontrol agent *Colletotrichum coccodes* (DAOM183088) and soil compounds inhibitory to PCR. The external control used in this assay was spiked at known concentrations in extracts of soil DNA and amplified in real-time PCR with its own primer set. A comparison between the estimated quantities of the external control and the known quantities added to the extracts allowed an estimation of the PCR efficiency on a sample per sample basis in 18 soil DNA extracts analyzed, originating from bioherbicide field-release trials. All 18 extracts tested positive for the presence of inhibitory compounds, but with substantial variability in the magnitude of PCR efficiency (from 12 to 82%) from 1 g of soil sample to another, even when soil DNA extracts had been diluted. This variability demonstrates quantitatively the heterogeneity of soil with regards to content in PCR-inhibitory compounds. The differences in amplification efficiency were used to normalize the amounts of target *C. coccodes* DNA previously quantified from the same samples.

4.2. Introduction

Polymerase chain reaction (PCR) analysis provides a sensitive and specific method to detect and monitor phytopathogenic fungi in complex environmental samples such as soil. Successful detection and characterization of fungal DNA in soil requires efficient extraction of the DNA and adequate purification from the coextracted contaminants that inhibit PCR. Generally, soils vary greatly in chemical and organic composition and contain abundant humic and fulvic acids, lignin-associated and phenolic compounds, polysaccharides, tannins, and heavy metals (Bridge and Spooner, 2001; Wilson, 1997). The majority of these compounds have been reported to inhibit *Taq* DNA polymerases and other enzymes by denaturation or by binding to them (McGregor *et al.*, 1996; Young *et al.*, 1993), by chelating cofactors like Mg^{++} required for polymerase to process (Tsai and Olson, 1992), and by binding to target DNA (Steffan and Atlas, 1988) in the PCR mix. Thus, soils are one of the most challenging environmental matrices from which to obtain microbial DNA that will withstand accurate PCR quantification.

So far, attempts to circumvent the problem of PCR inhibition have focused on implementing existing DNA purification methods to remove as much inhibitors as possible. These attempts include the use of additives like polyvinylpolypyrrolidone and polyvinylpyrrolidone (Young *et al.*, 1993), or the use of hexadecyltrimethylammonium bromide (Zolan and Pukkila, 1986), hydroxyapatite (Torsvik, 1980), size exclusion (Moreira, 1998; Young *et al.*, 1993) and ion-exchange chromatography (Tebbe and Vahjen, 1993; Torsvik, 1980), CsCl density gradient centrifugation, columns and filters (Tsai and Olson,

1991), glass-bead extraction, or more recently, chemical flocculation (Braid *et al.*, 2003). However, it is possible that these methods also affect the yield of DNA extraction. It is extremely difficult to find a suitable method of DNA isolation in terms of efficiency and reliability especially because the success of a given method is often dependent on the type of soil from which DNA is isolated (Frostegard *et al.*, 1999; Martin-Laurent *et al.*, 2001). Another attempt to partially overcome the problem of PCR inhibitors is to dilute the DNA extracts, prior to PCR, to a point at which PCR inhibitors can no longer interfere with PCR but at which target DNA is still detectable. Two problems inherent to this practice are of concern for real-time PCR users: different soil samples contain different amounts of PCR inhibitors, and performing the same dilution for all samples is likely to result in different PCR efficiencies. So far, there is no standard approach to monitor the extent of PCR inhibition on a sample per sample basis, therfore the ideal dilution (zero inhibitor) at which accurate target-DNA quantification is made for a given soil sample remains undeterminable.

In the medical field, several protocols have been developed to compensate for different PCR efficiencies between patient samples, regardless of DNA quality or the presence of PCR-inhibiting compounds (Broccolo *et al.*, 2002; Meijerink *et al.*, 2001; Stocher and Berg, 2002). These assays are target specific and require multiplex detection of the amplified products. Although the above methods will be difficult to adapt to detect pathogens from environmental sources such as soil, they hold great promise in medicine.

For calibration purposes, DNA quantification by real-time PCR requires the use of external DNA standards. Generally, DNA standards consist of serially

diluted amounts of the organism's genomic DNA (gDNA) or target-DNA sequence, which are amplified in the same run as the unknown samples but in separate reactions. Threshold cycle (C_T) numbers are determined, for the set of external standards, as fractional cycle numbers at which the reporter fluorescence passes a fix threshold above the fluorescence baseline. The log of starting DNA amounts for the standards versus their C_T gives the standard curve a straight line that can be used for direct comparison and determination of unknown target-DNA concentrations. This method is currently the only approach to quantify DNA by real-time PCR (Wilhelm and Pingoud, 2003) and has been successfully employed to monitor DNA from several pathogenic and symbiotic fungi (Atkins *et al.*, 2003a; Bates *et al.*, 2001; Bohm *et al.*, 1999; Cullen *et al.*, 2001, 2002; Filion *et al.*, 2002; Hietala *et al.*, 2003; Lees *et al.*, 2002; Weller *et al.*, 2000). However, it implies one drawback: it relies on the assumption that PCR efficiencies are all identical between standards and unknown sample reactions, and biases due to PCR-inhibitory compounds in the unknown samples are not monitored.

We propose an alternative method in which biases in PCR efficiency caused by PCR inhibitors are monitored on a sample per sample basis. The method is based on the construction of an external control (EC) that consists of a DNA molecule introduced at a specific concentration to soil DNA extracts from experimental samples suspected to contain PCR inhibitors and also to Tris-HCl (control). The method relies on the idea that if identical amounts of the EC are introduced into different soil DNA extracts and in Tris-HCl, the quantities of the control DNA calculated after real-time PCR amplification should be similar in all samples. Any deviation in the quantities of EC quantified from the soil DNA

extract is a reflection of decreased PCR efficiency in that sample. If PCR-inhibitory compounds are highlighted in a sample, their effect on PCR efficiency will have to be taken into account when the amounts of target DNA will be calculated in that same sample. The EC is run in parallel with the target-DNA quantification assay and has its own standard curve that corrects for differences in amplification efficiencies between EC and target DNA. Several requirements have to be met to achieve this method: (i) the EC should not be indigenous to the soil's DNA microbial community and (ii) it should be amplified with a specific PCR primer set that does not cross-hybridize with other DNA molecules isolated from the soil.

In this study, we describe the development of a quantitative real-time PCR assay with an EC DNA molecule that is used for normalization of potentially varying amplification efficiencies between the standards and the samples. We apply this approach to the quantification of *Colletotrichum coccodes* (Wallr.) Hughes DAOM 183088, a bioherbicide strain targeted against the noxious weed velvetleaf (*Abutilon theophrasti* Medik.), in soil samples collected from bioherbicide field-release trials.

4.3. Materials and methods

4.3.1. External control construction

The EC (Table 4.1) was generated by PCR amplification of a 103-bp fragment internal to the polylinker region (base 6203 to 6305, GenBank accession number X02513) of the plasmid M13mp18 (Amersham Biosciences, Piscataway,

NJ, USA). Conventional PCR was conducted in a PTC®-100 (MJ Research, Watertown, MA, USA) as follows. The reaction mix (25 µl) consisted of: M13mp18 DNA template (20 ng/µl), 2 µl; primer M13 universal primers M13F/M13R, 0.5 µmol/l (Table 4.1); 10x buffer (Tris-HCl, 200 mmol/l (pH 8.4), KCl, 500 mmol/l), 2.5 µl; *Taq* polymerase, 0.5 U (Invitrogen, Burlington, Ont., Canada); dNTPs (deoxyribonucleoside triphosphates), each at 200 nmol/l (Amersham Biosciences). Reactions were prepared on ice and overlaid with one drop of mineral oil. A denaturation step at 94°C (3 min.) was followed by 40 cycles at 94°C (1 min.), an annealing temperature of 58°C (1 min.), 72°C (30 sec.) and a final extension step at 72°C (10 min.). A negative control was included with 2 µl of distilled water dH_2O replacing the plasmid template. Following amplification, the EC DNA fragment was cleaned with the QIAGEN PCR purification kit (QIAGEN, Mississauga, ON, Canada) and quantified by spectrophotometry.

4.3.2. Preparation of DNA standards

A range of standards containing different DNA amounts of the EC and the target *C. coccodes* were prepared and included in every real-time PCR quantification run to construct a standard curve and measure the amounts of EC and *C. coccodes* DNA, respectively, in soil DNA extracts.

4.3.2.1. EC DNA standards

Five EC DNA standard suspensions (0.5 ng/µl, 50, 5, 0.5 pg/µl; and 50 fg/µl) were prepared by serial dilutions in Tris-HCl at a concentration of 10 mmol/l (pH 7.4).

4.3.2.2. *C. coccodes* gDNA standards

A stock culture of *Colletotrichum coccodes*, deposited at the Biosystematics Research Institute, Ottawa, ON, Canada, was established from diseased *A. theophrasti* individuals collected in Vermont, USA, and maintained in a sterile, ground oats-soil (2:3) substrate at 4°C. Mycelium from the stock culture was prepared as previously described (Dauch *et al.*, 2003). *Colleotrichum coccodes* gDNA was isolated from 20 mg of freeze-dried mycelium (Dauch *et al.*, 2003; Lee and Taylor, 1990) and resuspended in 50 µl Tris-HCl at a concentration of 10 mmol/l (pH 7.4). The DNA extract was quantified by spectrophotometry, and five standard *C. coccodes* DNA suspensions (5 and 0.5 ng/µl and 50, 5, and 0.5 pg/µl) were prepared by serial dilutions in Tris-HCl at a concentration of 10 mmol/l (pH 7.4).

4.3.3. Real-time PCR

Real-time PCR experiments were carried out in glass capillaries (Roche Diagnostics, Laval, QC, Canada) in a total volume of 20 µl, using a LightCycler™ (Roche Diagnostics). The M13 universal (M13F/M13R) and the *C. coccodes* strain-specific (N5Fi/N5Ri) primers (Table 4.1) were used to quantify

EC and *C. coccodes* DNA, respectively. Each PCR mix consisted of 10 µl of QIAGEN SYBR Green master mix, 1 µl of each primer (5 µmol/l), 4 µl sterile of dH_2O, and 4 µl of DNA template. Thermocycling was performed with an initial denaturation at 95°C (20 min.), followed by 55 cycles at 94°C (15 sec.), 56°C (8 sec.), or 61°C (15 sec.) (for EC or *C. coccodes* DNA amplification, respectively) and 72°C (10 sec.) (slope, 20°C/sec.). Finally, a melting curve was generated by programming the LightCycler to reach 95°C (0 sec.), 65°C (15 sec.) (slope, 20°C/sec.) and 95°C (0 sec.) (slope, 0.1°C/sec.).

Amplification products from all PCR runs were resolved on 1% electrophoresis agarose gels in 1x TAE buffer. A Gene Ruler™ 100-bp DNA (Invitrogen) was used as a molecular-weight ladder. Gels were stained with ethidium bromide, and images were recorded by the gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada).

4.3.4. Preparation of DNA templates

4.3.4.1. Collection of soil samples

Soil samples were collected from bioherbicide *C. coccodes* field-release trials established on two research fields located at the Macdonald Campus of McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada. Historically, these fields have been under cultivation with either corn (field C) or soybean (field S); they are now heavily infested with velvetleaf and are annually treated with foliar sprays of the bioherbicide *C. coccodes* DAOM 183088. Both field soils have a predominant loamy texture, with 2.9% and 2.7% of organic matter and pHs of 5.8

and 5.7, respectively. Three weeks before sampling in June 2003, plots of fields C and S were either (*i*) not treated (controls: C1 and S1), (*ii*) treated with a foliar spray of *C. coccodes* (10^9 conidia/m^2) (C2 and S2), or (*iii*) treated with a tank-mix spray of *C. coccodes* and the herbicide bentazon (Basagran™, BASF Canada, Toronto, ON, Canada) at 0.24 kg active ingredient/ha (C3 and S3). These treatments were replicated 3 times, and plots were assigned to a randomized complete block design.

Detection and real-time PCR quantification were conducted on soil samples from each plot of each field (1 kg of soil per replicate). In the case of the conidia-seeding experiment, 1 kg of soil was collected in the vicinity of each of the C and S fields (referred to as soil samples A and B, respectively), but away from the treated plots, and subsampled into series of 1 g. All soil samples were transferred into a Ziploc bag, mixed thoroughly to insure uniform distribution, and sieved through sieves 4-mm in diameter. Samples were immediately flash-frozen in liquid nitrogen (N), freeze-dried for 2-3 days, and stored at –20°C.

4.3.4.2. Total DNA purification from soil

DNA purification from field soil samples was performed with the UltraClean™ soil DNA isolation kit (MO BIO Laboratories, Solana Beach, CA, USA), with modification as previously established (Dauch *et al.*, 2003), except that 1 g of soil instead of 250 mg was used as a starting material. All soil DNA extracts were diluted to $1/20^{th}$, a least level required to obtain positive signals from all samples, prior to conventional and real-time PCR amplifications.

To insure the quality and integrity of the isolated DNA, confirm the presence of amplifiable DNA, and rule out the risk of obtaining false-negative diagnostic results, universal primers (ITS1-F/ITS4) (Gardes and Bruns, 1993; White *et al.*, 1990) were used in conventional PCR against all DNA samples isolated from soil. The primers (Table 4.1.) are designed to amplify the internal transcribed spacers (ITS) of fungal nuclear ribosomal DNA. Conventional PCR conditions for the ITS amplifications were similar to those described for the construction of EC DNA fragment, except that an annealing temperature of 55°C instead of 58°C was employed.

4.3.5. Efficiency of soil DNA purification and DNA quantification

4.3.5.1. Effect of the soil-extract matrix on *C. coccodes* DNA quantification

The effects of PCR inhibitors present in crude soil extract were assessed by seeding a series of 1 g of dry soil samples (A and B) with 500 µl of a suspension of *C. coccodes* conidia corresponding to 2 x 10^6, 2 x 10^5, 2 x 10^4, and 2 x 10^0 conidia/ml in dH_2O. Conidial production for soil seeding was initiated by transferring five agar plugs from a 1-week-old PDA culture into 250 ml modified Richard's liquid medium (DiTommaso and Watson, 1995). Cultures were incubated for 1 week on a rotary shaker (200 rpm), and conidia were then harvested after washing, filtering through three layers of cheesecloth, and centrifuging for 10 min. at 6,000 x *g*. The conidial pellet was resuspended in dH_2O. The inoculum concentration was adjusted to 2 x 10^6 conidia/ml in dH_2O, using a haemocytometer, and serial dilutions were prepared. To compare the

detection threshold of DNA extracted from seeded soils to that of DNA extracted from pure spore suspensions, 500 µl of each of the serially diluted spore concentration was placed in empty, sterile 2.0 ml snap-capped eppendorf tubes (control treatment). All samples (four replicates per inoculum concentration per treatment) were immediately vortexed 5 sec., flash-frozen in liquid N, freeze-dried for 2 days, and subjected to soil DNA purification as described above. The amounts of *C. coccodes* DNA present in all DNA extracts were then quantified by real-time PCR, using *C. coccodes* strain-specific primers (Table 4.1.).

4.3.5.2. Spiking of DNA extracts with the external control (EC) to monitor PCR inhibition

To monitor the presence of PCR inhibitors, the EC was added at the same concentration to all nonamplified DNA aliquots of soil A and B prior and after dilution to $1/20^{th}$. The aliquots of DNA extracts originated from 1 g of soil seeded with *C. coccodes* conidia (2×10^5/ml). As control treatment, the EC was added at concentrations corresponding to 6.6 pg/µl to (*i*) a Tris-HCl solution (10 mM, pH 7.4) and (*ii*) a 30 ng/µl *C. coccodes* gDNA suspension (four replicates each). These two controls were included to (*i*) determine the amounts of EC quantified by real-time PCR in the absence of PCR inhibitors and (*ii*) rule out that the presence of DNA could inhibit the PCR reaction (30 ng/µl corresponds to the gDNA concentration routinely obtained in our laboratory when extracting DNA from soil). The amounts of EC DNA amplifiable from these soil DNA extracts and controls were determined by real-time PCR, using M13 primers as previously

described. To ensure that EC DNA is not indigenous to the soil microflora, the M13F/M13R primer set was tested in conventional PCR runs against DNA templates extracted from all soils that were not spiked with the EC.

4.3.6. Real-time quantification of *C. coccodes* from field samples

The occurrence of PCR inhibitors was monitored on three biological replicates by the addition of EC DNA at 6.6 pg/µl, before dilution to $1/20^{th}$, to nonamplified soil DNA extracts originating from the fields of the deliberate *C. coccodes* release. As a control, the EC DNA was added to Tris-HCl (10 mM, pH 7.4) at the same final concentration of 6.6 pg/µl. All samples were amplified in real-time PCR, using M13 universal primers to quantify the amounts of EC DNA. The calculated mean of control EC DNA was used as a reference value in which optimal amplification efficiency (100%) was achieved. The percent amplification efficiencies in field soil DNA extracts were estimated by calculating the ratios of EC DNA amounts quantified in each sample divided by the control EC DNA amounts and multiplied by 100. Normalization of *C. coccodes* DNA quantification data was achieved by multiplying *C. coccodes* DNA amounts by 100 and dividing by the percentage of PCR efficiency for a given sample.

4.3.7. Statistical analyses

The EC and *C. coccodes* DNA quantification data were analyzed by the Statistical Analysis System (SAS, 1999), using the analysis of variance procedure, and treatments were separated with *t*-tests ($P < 0.05$). Data from the

effect of the soil-extract matrix on the accuracy of *C. coccodes* DNA quantification experiment were $\log_{10}$ transformed to ensure homogeneity of variances and separated with *t*-tests ($P < 0.05$).

4.4. Results

4.4.1. Construction of Standards

Serially diluted DNA standards containing at least 2 pg of *C. coccodes* or 0.2 pg of EC DNA amplified product showed single expected amplicons of 380 bp (melting point temperature (*T*m) = 84.5°C) and 103 bp (*T*m = 82.7°C), respectively, as determined by either melting-curve analysis or agarose gel electrophoresis (Fig. 4.1A-D). In this study, standard curves were constructed using different concentrations of *C. coccodes* gDNA ranging from 5 ng/µl to 0.5 pg/µl and of EC amplified product ranging from 0.5 ng/µl to 50 fg/µl to calculate the starting concentration of templates in unknown samples. Quantification showed a linear correlation ($r > 0.9959$) between log values of DNA and real-time PCR threshold cycles over the range of DNA concentration examined (Fig. 4.1E-F), demonstrating the high reproducibility among the three replicates tested and the accuracy of the PCR quantification assay. The lowest amount of *C. coccodes* gDNA that was reliably quantified is 2 pg per capillary (Fig. 4.1B).

4.4.2. Effect of the soil-extract matrix on the accuracy of real-time PCR quantification

To determine whether soil types could have a bearing on the accuracy of *C. coccodes* DNA quantification by real-time PCR, comparison of the values of amplified DNA extracted from seeded soils with those obtained from serially diluted pure suspensions of *C. coccodes* conidia were made. A linear relationship (Fig. 4.2A) was obtained between *C. coccodes* inoculum concentrations and the amounts of DNA quantified from each of the soils (soil A, $r = 0.998$ and soil B, $r = 0.993$) as well as from pure fungal spores in water ($r = 0.998$). The assay reliably and accurately quantified as low as 10^4 conidia/g of dry soil, which corresponds to 40 conidia per capillary (Fig. 4.2A). However, significantly ($P = 0.011$) less *C. coccodes* DNA was estimated by PCR, from DNA extract of soil B compared with that from soil A or from pure fungal spores (Fig. 4.2A). Regardless of the conidia concentration, the amounts of *C. coccodes* DNA back-calculated from soil B were consistently 0.7 fold lower than those calculated from soil A, suggesting that the soil B extract matrix had a bearing on the precision of real-time PCR quantification (Fig. 4.2A).

4.4.3. Recovery of added external control

No signal was generated from soil DNA extracts that had not been spiked with the EC (data not shown) when using the M13 primer set, indicating that the primers did not cross-react with DNA from the soil microflora. To estimate whether PCR inhibitors found in soil DNA extracts affect real-time PCR

quantification of naked DNA, the EC was added at 6.6 pg/□l to undiluted and diluted aliquots of soil DNA extracts seeded with *C. coccodes* at 10^5 conidia/ml, and these treatments were compared with those in which the EC DNA fragment was added either to Tris-HCl or to gDNA suspension of *C. coccodes*. When the EC was added to pure soil DNA extracts before dilution, significantly (P = 0.0043) lower EC DNA amounts were detected in soil A and B compared with the control treatment; soil B showed the lowest recovery (25.5%; Fig. 4.2B). However, no significant difference (P = 0.06) in the amounts of EC DNA was found when the EC was added after dilution of the DNA extract (Fig. 4.2B).

4.4.4. *C. coccodes* DNA quantification in bioherbicide field-release trials

Colletotrichum coccodes was detected and quantified in 15 out of 18 soil samples originating from *C. coccodes* bioherbicide field-release trials (Table 4.2; Fig. 4.3). To measure the occurrence of PCR inhibitors in these soil DNA extracts, the EC was spiked in triplicates in (*i*) pure soil DNA extract and (*ii*) Tris-HCl (control), and samples were diluted to maintain the conditions previously decribed for *C. coccodes* DNA quantification ($1/20^{th}$ diluted DNA extract). The amounts of EC calculated in both samples and controls showed decreased PCR efficiencies in all soil DNA extracts from the field (Table 4.2), clearly suggesting that the amounts of *C. coccodes* DNA previously quantified from the same samples were under-estimated. The extent of PCR efficiency in the 18 individual soil samples ranged from 12.21% to 82.19% (Table 4.2), indicating that there was substantial variability in the extent of PCR inhibition from 1 g of field soil to another at a $1/20^{th}$ dilution level. We used these differences in amplification

efficiencies to normalize the amounts of target *C. coccodes* DNA previously obtained. In addition to inhibitory factors, the amounts of *C. coccodes* were substantially variable among replicates of the same field plot (Table 4.2). Nevertheless, samples originating from corn-cultivated plots had consistently more *C. coccodes* DNA than soybean-cultivated plots. Of interest, the amounts of *C. coccodes* DNA quantified in the herbicide-bioherbicide tank-mix treatment were lower than those estimated in soil plots that received *C. coccodes* only.

Table 4.1. Characteristics of polymerase chain reaction primers used, in this study, to quantify an external control and *Colletotrichum coccodes* DNA from soil samples in bioherbicide field-release trials.

DNA Target	Primer	Sequence (5'-› 3')	*Tm* (°C)	Amplicon size (bp)
EC	M13F	GTAAAACGACGGCCAG	50	103
	M13R	CAGGAAACAGCTATGAC	50	
C. coccodes	N5Fi[a]	AATGGCGTTCATGAAGGTAG	62	380
	N5Ri[a]	ATCTCAATGAAGTACGTCGC	62	
Fungal ITS	ITS1-F[b]	CTTGGTCATTTAGAGGAAGTAA	60	~530
	ITS4[c]	TCCTCCGCTTATTGATATGC	58	

Note: ITS, internal transcribed spacer; *Tm*, melting point temperature.

[a]Dauch *et al.*, 2003.

[b]Gardes *et al.*, 1993.

[c]White *et al.*, 1990.

Table 4.2. Quantification by real-time polymerase chain reaction before and after normalization with an external control, of *C. coccodes* genomic DNA (gDNA) in soil originating from bioherbicide field-release trials.

Sample[a]		*C. coccodes* gDNA (pg/g dry soil)[b]	EC DNA (pg)[b]	% PCR efficiency[c]	Normalized *C. coccodes* gDNA (pg/g dry soil)
Soil	Replicate				
C1	**1**	47.70	14.56	54.89	86.90
	2	68.23	12.60	47.50	143.62
	3	23.40	15.94	60.09	38.94
	Mean +/- SE	46.44 +/- 12.96	14.37 +/- 0.97	54.16 +/- 3.65	89.82 +/- 30.26
C2	**1**	0.00	20.60	77.66	0.00
	2	799.75	22.51	84.86	942.40
	3	72.23	15.63	58.93	122.57
	Mean +/- SE	290.66 +/- 255.40	19.58 +/- 2.05	73.82 +/- 7.73	354.99 +/- 295.83
C3	**1**	232.70	17.65	66.54	349.71
	2	136.35	15.46	58.28	233.94
	3	0.00	20.78	78.34	0.00
	Mean +/- SE	123.02 +/- 67.50	17.96 +/- 1.54	67.72 +/- 5.82	194.55 +/- 102.86
S1	**1**	173.55	21.40	80.68	215.11
	2	89.10	21.80	82.19	108.41
	3	7.92	3.24	12.21	64.86
	Mean +/- SE	90.19 +/- 47.82	15.48 +/- 6.12	58.36 +/- 23.08	129.46 +/- 44.63
S2	**1**	26.23	9.92	37.40	70.12
	2	74.90	11.64	43.88	170.68
	3	23.91	3.65	13.76	173.76
	Mean +/- SE	41.68 +/- 16.62	8.40 +/- 2.43	31.68 +/- 9.15	138.19 +/- 34.04
S3	**1**	61.70	13.14	49.54	124.55
	2	0.00	12.96	48.86	0.00
	3	19.24	22.20	83.69	22.99
	Mean +/- SE	26.98 +/- 18.23	16.10 +/- 3.05	60.70 +/- 11.50	49.18 +/- 38.27
Control Tris[d]					
	Mean +/- SE	n.a.[e]	26.53 +/- 0.20	100	na

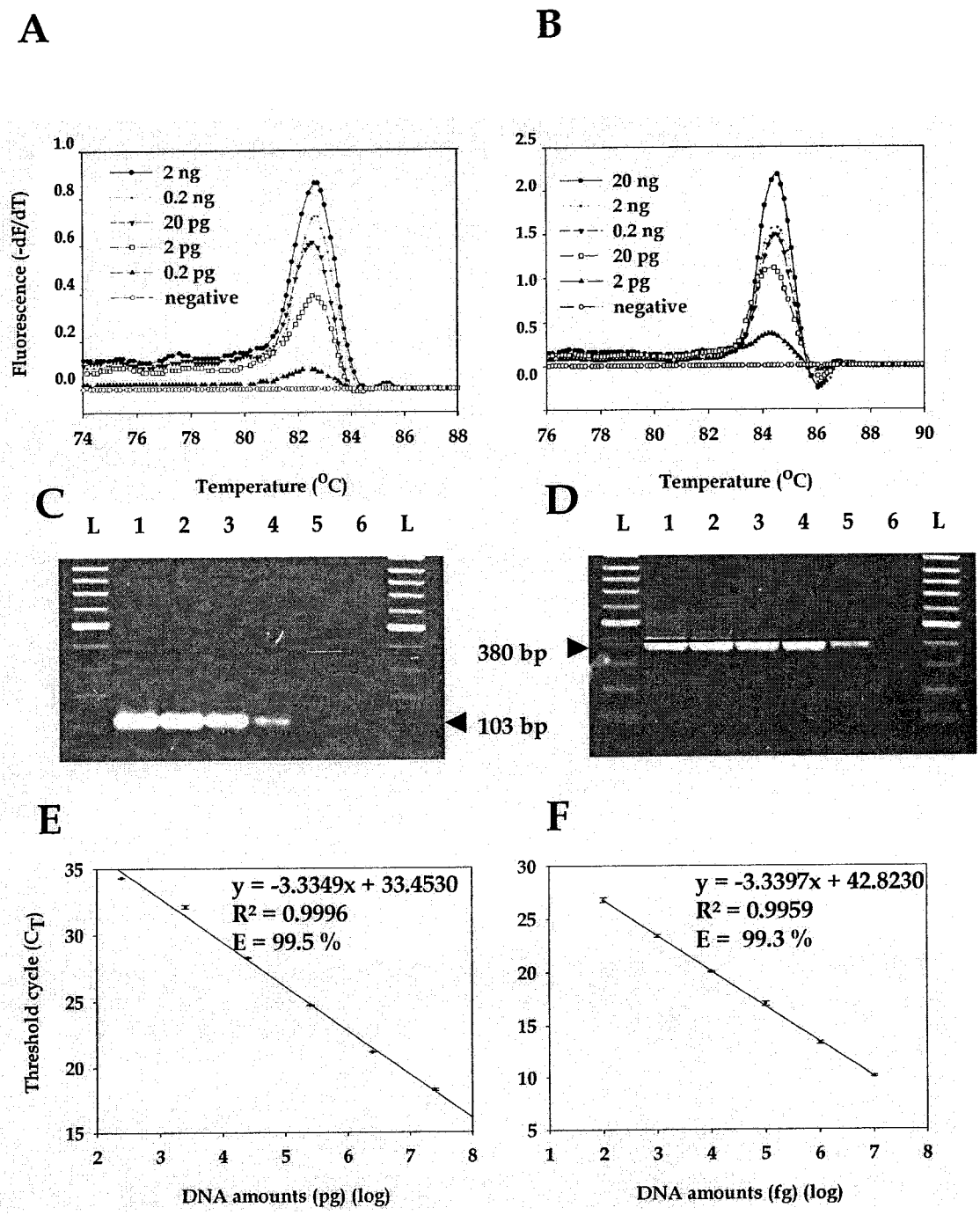
Note: Values presented in the table are means +/- SE of biological replicates. na, not applicable.

[a]Soils originating from corn-cultivated (C) and soybean-cultivated (S) field plots. C1 and S1, not treated; C2 and S2, treated with *C. coccodes*; C3 and S3, treated with *C. coccodes*-bentazon tank mix; control, mean of the external control (EC) DNA amounts quantified from three replicates of Tris-HCl spiked with the EC.

[b]As quantified by real-time PCR.

[c]Calculated as a percentage of the amounts of EC quantified in each sample divided by the amounts of EC quantified in the control Tris-HCl.

Figure 4.1. Real-time polymerase chain reaction (PCR) amplifications of pure DNA standards from an external control (EC) (**A**, **C**, **E**) and *Colletotrichum coccodes* (**B**, **D**, **F**) with M13F/M13R and N5Fi/N5Ri primers, respectively. Melting-peak profiles show single peaks at *T*m = 82.7°C (**A**) and *T*m = 84.5°C (**B**), corresponding, respectively, to 103-bp (**C**) and 380 bp (**D**) PCR products on electrophoresis gels, for amplified DNA standards ranging from 0.5 ng/µl to 50 fg/µl (EC DNA, lanes 1-5, **C**), and from 5 ng/µl to 0.5 pg/µl (*C. coccodes* genomic DNA (gDNA), lanes 1-5, **D**). (**C-D**) Lane 6, negative control (distilled water); L, molecular-weight ladder. (**E-F**) Standard curves were constructed with each range of standards in triplicates (mean +/- standard error), using the fit-point method. E, amplification efficiency.


A
B
Fluorescence (-dF/dT)
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0.2 ng
20 pg
2 pg
0.2 pg
negative
Temperature (°C)
20 ng
2 ng
0.2 ng
20 pg
2 pg
negative
Temperature (°C)
C
L 1 2 3 4 5 6 L
380 bp
103 bp
D
L 1 2 3 4 5 6 L
E
Threshold cycle (C_T)
y = -3.3349x + 33.4530
R² = 0.9996
E = 99.5 %
DNA amounts (pg) (log)
F
y = -3.3397x + 42.8230
R² = 0.9959
E = 99.3 %
DNA amounts (fg) (log)

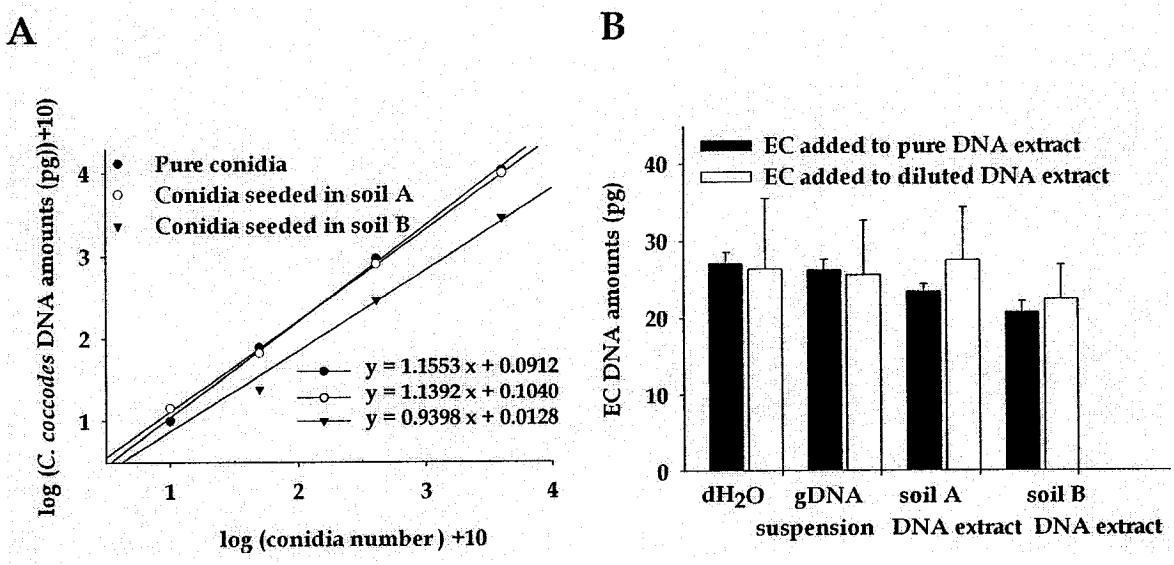

Figure 4.2. Effect of the soil matrix on the accuracy of *Colletotrichum coccodes* DNA quantification by real-time polymerase chain reaction (PCR) (4 µl of DNA template per reaction). (**A**). Soil samples A and B and distilled water control (dH_2O) were seeded with 0, 10^4, 10^5, and 10^6 *C. coccodes* conidia (four replicates), and their *C. coccodes* DNA content were quantified by PCR. (**B**) DNA extracts from soil A and B containing 10^5 *C. coccodes* spores were spiked with the external control (EC; final concentration of 6.6 pg/µl), either before or after dilution of the DNA extract to $1/20^{th}$. Controls consisting of Tris-HCl and genomic DNA (gDNA)-containing Tris-HCl were also spiked with the EC (final concentration of 6.6 pg/µl) and diluted. The amounts of EC DNA from treatments and controls were quantified by real-time PCR. Vertical bars represent standard errors.

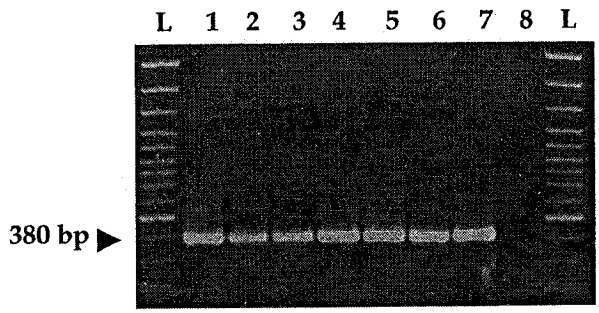

Figure 4.3. Detection of *Colletotrichum coccodes* genomic DNA (gDNA) in field soil samples. N5Fi/N5Ri primers were used to amplify target-DNA template isolated from a *C. coccodes* pure culture (lane 1), soil C (lanes 2-4), soil S (lanes 5-7). Lane 8, negative, distilled water control; L, molecular-weight ladder.

4.5. Discussion

In this study, real-time PCR provided a sensitive method for the quantification of *C. coccodes* from experimental soil samples, allowing to accurately detect 100 spores per gram of soil, corresponding to 1.8 pg, which is a 10^3-fold increase in sensitivity compared with that detected by conventional PCR (Dauch *et al.*, 2003). Although real-time PCR provides a quantitative estimate, enabling comparison of the fungal population at two sites, it was not possible to directly relate the value of DNA amounts to fungal propagule numbers. Because fungi are multinucleate eukaryotic organisms, no direct relationship can be drawn between the amounts of target DNA and the abundance and/or infectivity of the "quantified" fungus.

Polymerase chain reaction (PCR) amplification will be from both nonviable as well as viable propagules, and therefore, quantification may be overestimated for the latter. The extraction of RNA from soil (Burgmann *et al.*, 2003; Mendum *et al.*, 1998), combined with real-time reverse-transcription PCR (RT-PCR) quantification would provide a more accurate method of quantification for this process.

To check for the reliability of our soil DNA purification method, *C. coccodes* conidia seeded into two soil matrices were traced. Although such approach will not reproduce the situation in the field, it can still be justified because it can demonstrate the fate of the organism that was introduced into soils during deliberate release. In addition, if no reliable quantitative results were obtained using this approach, there would be a good reason to believe that serious

methodological problems exist. In our experiments, the linearity obtained between the inoculum concentration and the amounts of DNA quantified from each soil type indicated that the DNA extraction method was robust. The quantities of DNA recovered from soil increased with the number of seeded conidia of *C. coccodes*, but the recoveries were substantially different between the two soil types investigated. These differences were found to be independent of the amounts of seeded conidia present in the analyzed samples, but rather related to the type of soil matrix in which the organism was seeded. This led us to suspect, as in similar published studies, that other factors such as PCR-inhibitory compounds present in the purified fraction of the soil matrix interfere in the amplification process and result in biased PCR efficiency (Bell *et al.*, 1999; Cullen *et al.*, 2001; Dauch *et al.*, 2003; Lees *et al.*, 2002; Martin-Laurent *et al.*, 2001; van de Graaf *et al.*, 2003).

In quantitative PCR studies, target DNA is monitored by comparison to a standard curve consisting of serially diluted amounts of either target gDNA or target amplicon (Roche, 2000). All real-time quantitative PCR studies performed so far on soilborne plant pathogens are based on the assumption that PCR efficiencies are the same for the soil samples and the standards (Filion *et al.*, 2002; Lees *et al.*, 2002; Stubner, 2002; Taylor *et al.*, 2003). However, many factors affect PCR efficiency and, consequently, the outcome of quantitative PCR analyses. It is commonly believed that soil contaminating compounds coextracted with the DNA can inhibit the amplification reaction and, therefore, modify the reaction efficiency. The most commonly adopted practice in detection and quantification of DNA from soil consists in diluting the soil purified DNA

template so that the target DNA can still be detectable while the effects of inhibitors is attenuated (Cullen *et al.*, 2001, 2002; Tsai and Olson, 1992). Although, this is a commonly adopted practice, the use of the quantitative EC of this study shows that even at $1/20^{th}$ dilution factor, varying degrees of inhibition in PCR efficiency still occur in soil DNA extracts. These findings suggest that systematically performing the same dilution for all soil extracts results in different PCR efficiencies, and is not sufficient to achieve the precision level that one would expect to benefit from real-time PCR assays.

In the present report, we addressed this issue and described a novel approach for the accurate normalized quantification of target DNA in soil samples. We spiked the total soil DNA extract with the EC DNA fragment. This DNA was chosen because large amounts can easily be amplified and because the absence of background signals in soils permitted quantification of recoveries. We could have introduced the naked EC directly to the soil prior to sample grinding to assess both DNA extraction recovery and PCR efficiency, but grinding had been shown to strongly modify soil structure and to result in adsorption conditions under which recovery of naked DNA is substantially low, possibly because of the shearing imposed on the soil matrix and (or) the release of nucleases during the early steps of the extraction process (Frostegard *et al.*, 1999; Lee *et al.*, 1996). We found that the EC was capable of highlighting PCR inhibition only when it was added before dilution of the soil DNA extract, but not when added after diluting the soil DNA extract. We are not able to explain this observation, but it could hypothetically be due to the binding ability of PCR inhibiting compound(s) to target or nontarget DNA (Steffan and Atlas, 1988).

The utility of the designed EC in routine soil DNA quantification was investigated using the *C. coccodes* DNA quantification case study. The assay consisted of spiking DNA extracts from 18 different soil samples taken from two locations of bioherbicide field-release trials with known amounts of EC. The EC amplification efficiency of each sample is measured and compared with the one obtained from the amplification of the control sample in which the EC is spiked in Tris-HCl. We also carefully optimized the PCR conditions for each of the target and the EC to achieve the best amplification efficiencies (Fig. 4.1.E-F, 99.5% and 99.5%, respectively). Since absolute quantities of *C. coccodes* and EC DNA in the unknown samples were calculated by comparison with their respective standard curves, we circumvented the biases due to differences in amplicon length, primer Tm and annealing temperatures between *C. coccodes* and EC DNA amplifications. This approach allowed us to normalize the biases created by PCR-inhibitory compounds in every soil sample and to compensate for sample-specific PCR efficiency variations. Furthermore, we showed that this inhibition was not due to the presence of gDNA at the concentrations we routinely obtain when extracting DNA from soil. In this context, it rules out the possibility that DNA from other soil-inhabiting organisms could have interfered with the amplification efficiency. At similar gDNA concentration, other studies have also shown that nontarget DNA did not inhibit the PCR reaction (Bruce *et al.*, 1992; Stubner, 2002).

In biocontrol approaches, monitoring the persistence of field-released biological control agents (BCA) in soil is a prerequisite for the development of effective, long-lasting biocontrol strategies. In this study, *C. coccodes* gDNA was

detectable in soil samples originating from bioherbicide-release field experiments that were not treated that year with *C. coccodes*. This finding was not unexpected because the experimental field had received *C. coccodes* applications in previous years and because *C. coccodes* sclerotia are known to commonly survive from 2 up to 8 years in soil (Dillard and Cobb, 1998). The fact that lower amounts of *C. coccodes* DNA were found in plots treated with the bentazon-bioherbicide mix compared with plots treated only with the bioherbicide could be related to the decrease of velvetleaf population as a result of herbicide application. However, more research is necessary to confirm this observation.

In conclusion, the EC has proved useful in monitoring PCR amplification efficiencies in soil DNA purified samples where PCR inhibitors are problematic. Since this method uses its own PCR primer set, it can virtually be implemented to any other target-DNA quantification assay, provided that the introduced EC is absent from the analyzed samples. Although it requires further investigation, in our opinion the quantitative EC is a robust control that could be applicable to real-time PCR assays by using chemistries other than the SYBR Green I dye, including multiplex amplification to further simplify the process.

CONNECTING STATEMENT BETWEEN CHAPTERS IV AND V

C. coccodes strain-specific primers developed in chapter III were used to evaluate the growth kinetics of the wild-type strain (DAOM 183088) and compare it to that of *C. coccodes* strain T-20a, genetically engineered with the *NEP1* (necrosis and ethylene inducing protein) for hypervirulence on velvetleaf (Amsellem *et al.*, 2002). The genetically transformed strain was made available by Dr. Jonathan Gressel, Weizmann Institute of Science, Rehovot, Israel. Experiments were conducted to optimize and validate the quantification of the wild-type and the genetically transformed strains as well as the host plant DNA by real-time PCR over a period of 14 days after inoculation, a period that spans the biotrophic and the necrotrophic phases of fungal nutrition.

The results of this section are the subject of a manuscript that has been accepted for publication in Plant Disease. I have designed the experimental set-up, conducted all of the experiments, and wrote the manuscript. The contribution of Professors S. Jabaji-Hare and Alan Watson who appear as co-authors were as follows: Professor S. Jabaji-Hare provided supervision and funding throughout the study, and provided technical assistance during all of this study. She provided valuable suggestions and corrected the manuscript. Professor A. Watson provided funding, plant samples for PCR analysis, and revised the manuscript. Dr. P. Seguin gave invaluable advice on the choice of the most suitable statistical approach, helped in running the statistical analyses, and critically revised the

manuscript. Dr. Byeongseok Ahn helped in several inoculation trials from plant growth to disease ratings and reviewed the final version of the manuscript.

CHAPTER V

Molecular monitoring of wild-type and genetically engineered *Colletotrichum coccodes* biocontrol strains *in planta*

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5.1. Abstract

Two strains of *Colletotrichum coccodes*, the wild-type (DAOM 183088) and the T-20a, engineered with *NEP1* gene for hypervirulence on velvetleaf (*Abutilon theophrasti*, Medik.), were monitored *in planta* during the first two weeks of infection. Real-time quantitative PCR (QPCR) was used to assess the extent of colonization of both strains on velvetleaf using SYBR Green chemistry. Quantification of both strains was successful as soon as the conidia were sprayed on the leaves and up to 14 days after inoculation (DAI). The increase in fungal DNA amounts corroborated with the appearance of necrotic lesions on velvetleaf leaves infected with the wild-type strain. Unexpectedly, wild-type *C. coccodes* was even more efficient at infecting velvetleaf than the transgenic T-20a strain. In addition, detection of host DNA allowed us to quantitatively monitor the decrease in plant DNA amounts in response to wild-type strain infection. Expression of the *NEP1* (necrosis and ethylene inducing peptide) transgene by conventional retro-transcription (RT)-PCR was absent in T-20a growing on either V8 agar or *in planta*, suggesting that the gene may be silenced. The application of QPCR to monitor fungal growth development proved invaluable to detect the target organisms in planta prior to the appearance of symptoms.

5.2. Introduction

The genus *Colletotrichum* (teleomorph *Glomerella* (Sutton, 1992)) includes several of the most important worldwide fungal pathogens that are commonly known as anthracnose pathogens. *Colletotrichum coccodes* (Wallr.) Hughes strain DAOM 183088 has been studied as a potential bioherbicide for the control of velvetleaf, *Abutilon theophrasti* Medik (Gotlieb *et al.*, 1987; Watson *et al.*, 2000). It can severely damage or kill velvetleaf when applied to young seedlings bearing only cotyledons or at most one true leaf (Wymore *et al.*, 1988), however when applied at later growth stages, plants continue to grow after shedding their infected leaves. Increased virulence was recently achieved by transferring the *NEP1* gene (necrosis and ethylene inducing peptide) encoding a *Fusarium oxysporum* phytotoxic protein into *C. coccodes* wild-type strain (DAOM 183088) (Bailey *et al.*, 2000; Jennings *et al.*, 2000). The resulting transgenic strain of *C. coccodes,* T-20a, was 9 times more virulent than the wild-type against velvetleaf at the three true-leaf stage when applied as chopped mycelium (Amsellem *et al.*, 2002). Despite this success, T-20a host range was not limited to velvetleaf, as is the case of the wild-type strain, but was extended to include tobacco and tomato plants causing 67 to 100% seedling mortality, respectively (Amsellem *et al.*, 2002). A pre-requisite for the release of wild-type or genetically engineered biological control agents is our ability to monitor them in the environment in order to evaluate their colonization success and to assess environmental risks. With this end in view, we have recently developed strain-specific PCR primers (N5Fi/N5Ri) for the detection of the wild-type *C. coccodes*

(DAOM 183088) *in planta* and in deliberate-release field soils (Dauch *et al.*, 2003).

Some species of *Colletotrichum* are well known to exhibit hemibiotrophic life styles in which they establish themselves in host cells by eluding detection and forming associations with living cells, much like biotrophs. Later during the infection process, they behave as necrotrophs spreading rapidly and actively killing host cells (Perfect *et al.*, 1999). Examination of disease progression by quantifying the amount of fungal biomass especially during the biotrophic phase of a pathogen is not always easy and most traditional methods rely on the appearance of symptoms and/or quantification of fruiting structures (Miller, 1996; Pei *et al.*, 2003), and the quantification of sterols or chitin (Gessner and Schmitt, 1996; Schmitz *et al.*, 1991). Other techniques for assessing fungal biomass include ELISA, monitoring GUS or GFP activity in interactions with micro organisms transformed with the bacterial UidA genes or the green fluorescent protein, respectively, as fungal biomass reporters in transformants (Aly *et al.*, 2001; Chen *et al.*, 2003; Green and Jensen, 1995). The major drawback associated with these methods resides in the fact that plant factors can interfere with the measured parameter (Thomma *et al.*, 1999). Pathogen susceptibility is often monitored through scoring of disease symptoms; however, several studies have shown that development of symptoms does not always correlate with actual pathogen colonization. Therefore it is of utmost importance to assess pathogen growth rather than disease symptoms.

Real-time quantitative PCR (QPCR) is now widely used in the quantitative detection of pathogens due to its sensitivity, specificity, efficiency as well as the

freedom from post-PCR analysis steps (Schena *et al.*, 2004). Recently, it has become the method of choice in plant pathology to monitor the pathogen colonization through DNA quantification in plant tissue (Alkan *et al.*, 2004; Boyle *et al.*, 2005; Gao *et al.*, 2004; Hietala *et al.*, 2003; Vandemark and Barker, 2003). Currently two common approaches of analyzing data from QPCR experiments are used: (i) absolute quantification is achieved by comparison of the unknown sample to a standard curve in which the threshold cycle (C_T) numbers are plotted against starting fungal DNA quantities (Bustin, 2000, 2002) and (ii) relative quantification describes the change in C_T number of an unknown sample in relation to the C_T of a reference such as an untreated control or a sample treated at time zero in a time course study (Livak and Schmittgen, 2001).

Comparisons of the *C. coccodes* wild-type (DAOM 183088) and the transgenic strain T-20a growth kinetics to monitor their infection spanning the biotrophic and necrotrophic growth stages, have not yet been attempted. The objectives of this study was to use QPCR to monitor the molecular growth kinetics of both strains *in planta* when applied as conidia at various times following velvetleaf infections. We used the strain-specific primers N5Fi/N5Ri and primers targeting the *NEP1* gene (Nep1693/1893) to quantify the growth of the wild-type and the transgenic T-20a *C. coccodes* on velvetleaf, respectively. An additional primer set was developed for the quantification of the endogenous HPPD plant gene in response to *C. coccodes* infection.

5.3. Materials and methods

5.3.1. Mycelia and conidia preparation

Colletotrichum coccodes wild type strain (DAOM 183088), pathogenic on velvetleaf, was obtained from the Biosystematics Research Institute (Ottawa, ON, Canada). *C. coccodes* transgenic strain T-20a engineered to express the *NEP1* gene encoding the phytotoxin protein Nep1 (Amsellem *et al.*, 2002) was graciously provided by J. Gressel (Weizmann Institute, Israel). Both strains were grown on potato dextrose agar (PDA, Becton Dickinson Microbiology Systems, Sparks, MD) for one week at 28°C in the dark.

For DNA extraction, five agar plugs (5-mm diameter) were transferred into 75 ml of potato dextrose broth (PDB, Becton Dickinson Microbiology Systems, Sparks, MD) and incubated at room temperature for 12 days under low agitation (100 rpm). Mycelial mats were collected by filtration using Whatman no.1 filter paper, freeze-dried for 2 days and ground into liquid nitrogen. Powdered mycelia were conserved at -20°C until DNA extraction. For RNA extraction, mycelium of *C. coccodes* T-20a was grown in triplicate Petri plates for a week on V8 agar medium (Martinez *et al.*, 2004) overlaid with a nylon membrane (500 PUT, UCB, North Augusta, SC). Fungal mycelium was scraped off the membrane and immediately flash-frozen in liquid N.

Conidia used for velvetleaf inoculation were produced on modified Richard medium (MRM) (DiTommaso and Watson, 1995). Five agar plugs taken from one week-old PDA cultures of either the wild type or T-20a were inoculated in 600 ml of MRM and incubated at 24°C on a rotary shaker (200 rpm) for 7 days. The conidia were harvested through filtration, centrifuged at 4°C for 10 min. at

6000 x *g*, and the conidial pellet was resuspended in ddH_2O and conidial suspensions were adjusted to the desired concentrations in ddH_2O using a haemacytometer.

5.3.2. Plant growth conditions and inoculation

Abutilon theophrasti seeds, collected from fields in Ste-Anne de Bellevue (Qc, Canada), were pre-germinated in 100 x 15 mm Petri dishes on Whatman papers no.1, moistened with 4 ml of distilled water. The Petri dishes were stored for 3 days at room temperature. Two pre-germinated seeds were planted in Cone-tainers™ (Stuewe and Sons, Corvallis, OR) of 164 ml capacity. Prior to use, the Cone-tainers™ were surface sterilized for 45 min. in 3% solution of sodium hypochlorite, rinsed with distilled water and filled with moist non-sterile Promix (Premier Tech, Rivière du Loup, QC, Canada). The Cone-tainers™ were placed on racks, randomized and placed in growth chambers with conditions adjusted to 24/18°C for 10/14 h. (day/night) with a light intensity of 300□M/m^2. One week after seeding, plants were thinned so as to leave one seedling per Cone-tainer™. Plants were watered as needed and were fertilized two weeks after the initial seeding with 20 ml (3g/l) solution of 20:20:20 (N:P:K) until 4 weeks old (5-leaf stage).

Spraying of velvetleaf plants was performed in a spray chamber (Research Instrument MFG. Co. Ltd., Guelph, ON, Canada) to ensure uniform coverage of the leaves. The sprayed plants were placed in a dew chamber (90% humidity, 24°C) for 18 h. to maximize fungal infection. All plants received a constant rate of 10^6 conidia/ml (50 ml/m^2) of the wild type or T-20a *C. coccodes*, while uninfected

plants received ddH_2O (control treatment). Disease scores were established every 24 h. starting from 1 to 14 days after inoculation (DAI). They represent means of 12 replicates/time-point/treatment: 0 = no infection or few hypersensitive reaction; 1 = poor to deficient; hypersensitive reaction on most leaves or necrotic lesions on some leaves; 2 = moderate; advanced necrotic lesions on most leaves; 3 = satisfactory; severe infection on most leaves and some necrotic lesion on the stem, however plants are alive; 4 = complete death of plants.For DNA and RNA extractions, each experimental unit consisted of two leaves from two plants grown in separate Cone-tainers™. There were 4 replicates per treatment. For the study of *C. coccodes* growth kinetics *in planta* (real-time PCR), two 3rd leaves were excised at the base of the petioles at 6 different time points (0, 1, 2, 5, 7, and 14 days after inoculation (DAI)) for DNA extractions and at 12 h. and 7 DAI for RNA extractions. All leaf samples for DNA and RNA extractions were ground in liquid nitrogen (N) with the addition of 0.5 g NaCl and 0.33 g PVPP/replicate to prevent the mucilaginous material from interfering in the nucleic acid extraction processes. RLT buffer from the Qiagen RNeasy Plant Mini-Kit (Qiagen, Mississauga, ON, Canada) was immediately added to the samples used for RNA extraction as recommended by the manufacturer. All samples were kept at -80°C prior to RNA and DNA extractions.

To compare DNA quantification accuracy and detection threshold of DNA extracted from inoculated velvetleaf to that extracted from pure conidia suspensions, 500 µl of each serially diluted concentration of conidia (2×10^3, 2×10^4, 2×10^5, 2×10^6 and 2×10^7 conidia/ml of ddH_2O) of *C. coccodes* wild-type were placed in 2 ml screw-capped Eppendorf tubes (4 replicates). Twenty mg of

Xymotech silica beads (Xymotech Biosystems, Inc., Mont-Royal, QC, Canada) were added to each tube and conidia were violently disrupted in a FastPrep apparatus (Thermo Savant, Holbrook, NY) set at speed level 4 for 20 s. Samples were immediately frozen at –80°C prior to DNA extraction. For the plant DNA detection threshold, serial dilutions of 10^5, 10^6, 10^7 and 10^8 conidia/ml were sprayed on the plants (4 replicates) using the above described conditions. Two 3rd leaves were excised, immediately dipped in liquid N and stored at -80°C prior to DNA extraction. All experiments were repeated at least twice with comparable results.

5.3.3. Nucleic acid extractions and cDNA retro-transcription (RT)

Total fungal genomic DNA (gDNA) was isolated from 20 mg freeze-dried mycelium as previously described (Dauch *et al.*, 2003). Total gDNA from velvetleaf leaves and conidia from the wild type were extracted using the Qiagen DNeasy Plant Mini-Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's protocol except that all pipetting steps were carried out on ice, centrifugation steps at 4°C and an additional washing step was performed. DNA was eluted in 200 µl and 100 µl of Qiagen buffer for velvetleaf leaves and *C. coccodes* conidia, respectively. DNA extracts from all biological material were quantitatively and qualitatively estimated by spectrophotometry. Samples giving A_{260}/A_{280} nm ratios below 1.7 or above 2.0 were rejected. All extracts were run on 1% agarose gels (1 x TAE) to verify the absence of DNA degradation.

Fungal RNA was isolated from mycelia using the Qiagen RNeasy Plant Mini-Kit following the manufacturer's recommendations. To examine the

expression of *NEP1* gene during T-20a interaction with velvetleaf, total RNA was extracted from inoculated velvetleaf leaves using the same Qiagen kit with the following modifications: two lilac columns were loaded with 700 µl RLT buffer re-suspended plant powder, and their precipitate was pooled in a single pink column for each sample unit. RNA extracts were treated with TURBO DNase (Ambion Inc., Austin, TX) to remove any potential DNA contaminant, and checked for the absence of DNA contamination in conventional PCR using conditions described for ITS primer amplification (Table 5.1). The reaction consisted of 2 µl RNA extract as a template and 2 µl of velvetleaf gDNA (10 ng/µl) as a positive control, respectively. Five hundred nanograms DNA-free RNA were loaded on 1.2% agarose denaturing formaldehyde gels to check the integrity of the isolated nucleic acids. cDNAs were generated from 500 ng RNA with the Omniscript RT and QuantiTect Rev. Transcription kits (Qiagen, Mississauga, ON, Canada).

5.3.4. PCR Primer Sets

Primers used for the quantification of *C. coccodes* wild type, T-20a and velvetleaf are presented in Table 5.1. N5Fi/N5Ri (SCAR) was previously developed as a strain-specific primer set for *C. coccodes* (velvetleaf strains) (Dauch *et al.*, 2003). It amplifies part of an anonymous DNA sequence (380 bp; accession number AF448480) and is used in this study to quantify *C. coccodes* wild-type DNA. The Nep1F/R primer set (Table 5.1) flanks the *NEP1* gene (accession number AF036580) and is used to detect *C. coccodes* T-20a DNA and cDNA. It amplifies a putative product of 341 bp in conventional PCR assays.

However since we could not optimize them in real-time PCR assays, new primers (Nep1693/1893) were designed (anneal on base 1693 and 1893 of the *NEP1* gene) for T-20a quantification. Nep1693/1893 should amplify a 200 bp amplicon from both T-20a DNA and cDNA. The primer set HPPDF/R was used to quantify the 4-hydroxyphenylpyruvate dioxygenase gene (HPPD; accession number AY 138969) from velvetleaf. HPPD gene encodes a product that is involved in the degradation of aromatic amino-acids. As a reporter of plant health and similar to other plant health reporter genes, HPPD DNA quantification was used to evaluate the effects of fungal infection on the amounts of quantifiable plant DNA (Gao *et al.*, 2004; Hietala *et al.*, 2003; Winton *et al.*, 2002). Universal primers were used in conventional PCR to check for the presence of amplifiable DNA (ITS4/5 primers flank the Internal Transcribed Spacer (ITS) regions (1 and 2) of plants and fungi; (White *et al.*, 1990)). These primers are expected to amplify DNA from velvetleaf, and wild-type and T-20a *C. coccodes* (Table 5.1). The histone 4 primers, H4-1a/-1b (Table 5.1) target the ascomycete histone 4 gene (Glass and Donaldson, 1995), and were used to check the integrity of the cDNA synthesized from RNA originated from T-20a mycelia and from T-20a infected velvetleaf plants, and to exclude false-negative results obtained with *NEP1* gene specific primers. They amplify a DNA fragment of 250 bp and 200 bp from T-20a *C. coccodes* gDNA and cDNA, respectively.

5.3.5. Conventional PCR conditions

Conventional PCR amplifications were performed in an Applied Biosystems 9600 (Foster City, CA) as follows: the reaction mix (25 µl) consisted

of 2 µl DNA template, 0.2 µM each of the primers, 2.5 µl of 10 x buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.5 U of *Taq* polymerase (Invitrogen, Burlington, ON, Canada) and 200 nM each of dNTP (Invitrogen, Burlington, ON, Canada). Reactions were prepared on ice and cycled for 3 min. at 94°C, 30 cycles of 1 min. at 94°C, 1 min. at 62°C, 59°C, 60°C, 58°C or 68°C for N5Fi/N5Ri, Nep1F/R, HPPDF/R, ITS4/5 and H4-1a/-1b, respectively, and 1 min. at 72°C, and a final extension step at 72°C for 10 min. A negative control containing ddH_2O was included in each run. PCR products were resolved on 1% agarose gels (1xTAE). The absence of satisfactory spectrophotometry readings and electrophoresis of purified DNA, or the absence of amplified products during the ITS-PCR steps, was sufficient to reject the sample and DNA was re-extracted from the original tissue.

5.3.6. Conventional retro-transcription (RT)-PCR conditions

RT-PCR was performed to check (i) the quality of the synthesized cDNA with Histone 4 (H4-1a/-1b) primers (Table 5.1) and (ii) the presence/absence of *NEP1* gene expression with the Nep1F/R primers (Table 5.1) using conditions described earlier. The cDNA template was used as such or diluted in ddH_2O (2, 5, 10, 50 or 100 fold) and the positive control consisted of 2 µl T-20a *C. coccodes* gDNA (10 ng/µl).

5.3.7. Absolute DNA quantification by real-time PCR

Absolute real-time PCR quantification was conducted to determine the quantities of *C. coccodes* strains in velvetleaf samples. External standards for *C. coccodes* DNA quantification consisted of 6 serially diluted DNA of T-20a or wild-type gDNA (5 ng, 0.5 ng; 50 pg; 5 pg; 0.5 pg and 50 fg/µl). Velvetleaf DNA standards consisted of 6 serial dilutions (0.5 pg; 50 fg; 5 fg; 0.5 fg; 50 ag and 5 ag/µl) of the purified HHPD amplicon obtained with the HPPD primer set using Qiagen PCR purification kit (Qiagen, Mississauga, ON, Canada). All standards were prepared in Tris-HCl 10 mM (pH 7.4) and included in triplicates in each real-time PCR quantification run to construct a standard curve and calculate quantities of fungal or plant DNA in the unknown samples. Quantification algorithms used for the calculation of unknown DNA amounts included the amplification threshold, the adaptive baseline and the moving average (Stratagene Mx300P^{TM} real-time PCR system software, version 2.0, La Jolla, CA). Standard curves were constructed from triplicate standards using their C_T numbers, PCR cycle numbers at which the fluorescence of the sample exceeds the background fluorescence, and the known starting DNA amounts. Unknown DNA amounts from the samples were back calculated using the standard curves.

Each real-time PCR reaction mix consisted of 10 µl Stratagene Brilliant SYBR Green master mix (Stratagene, La Jolla, CA), 1.25 µl of each primer (2 µM), 2.2 µl sterile ddH_2O, 0.3 µl ROX reference dye (Stratagene, La Jolla, CA) and 5 µl 1/10th diluted (wild-type *C. coccodes* and velvetleaf DNA quantifications) or 4 µl 1/10th diluted DNA template (T-20a *C. coccodes* DNA

quantification). Thermo cycling was performed with an initial denaturation at 95°C (10 min.) followed by 40 to 50 cycles at 95°C (30 s.), 63°C (N5Fi/N5Ri), 61°C (Nep1693/1893) or 64°C (HPPDF/R) (1 min.), 72°C (30 s) (2.5°C/s). Finally, a melting curve was generated by programming the thermocycler to reach 95°C (60 s), 55°C (30 s) (2.5°C/s) and 95°C (0 s) (0.1°C/s). Each run included a negative control and was repeated twice on two different days (2 technical replicates).

To rule out any interference of PCR inhibitors, a preliminary test was run to quantify the amounts of wild-type *C. coccodes* from 5 randomly selected plant DNA extracts originating from the growth kinetics DNA quantification experiment. DNA extracts were run in real-time PCR with triplicate standards to quantify the amounts of *C. coccodes* DNA starting from either $1/5^{th}$, $1/10^{th}$, $1/20^{th}$, $1/100^{th}$ or non-diluted DNA extracts. By calculating the amounts of target DNA and correcting for the dilution factor, a $1/10^{th}$ dilution was found optimal for reliable quantification from all samples.

5.3.8. Post-PCR analyses

Amplification products from every PCR run were resolved on 1% electrophoresis agarose gels in 1xTAE buffer. A 100 bp DNA Ladder (Invitrogen, Burlington, ON, Canada) was used as a molecular weight ladder. Gels were stained with ethidium bromide and images were recorded by the gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada).

5.3.9. Experimental design and statistical analysis

For the molecular growth kinetics study, there were 3 treatments (wild-type, T-20a, and control), 4 replicates per treatment, and 6 time points. For the DNA extraction test, 5 conidial concentrations of *C. coccodes* conidia were tested in 4 replicates. To assess the DNA detection threshold in planta, 4 *C. coccodes* conidial concentrations were sprayed in 4 replicates on velvetleaf leaves. Rating for disease development was done on twelve plants/fungal strain every 24 h. starting from 1 to 14 days after inoculation (DAI). All pants placed in growth chambers were randomized, per experiment, following a RCBD. HPPD and *C. coccodes* DNA quantification data were analyzed by the Statistical Analysis System (SAS, 1999), using the analysis of variance procedure. Treatment means comparisons in each experiment were made using the least significant difference (LSD) test at $P = 0.05$ level.

5.4. Results

5.4.1. Specificity and sensitivity of DNA amplification

SYBR Green real-time PCR assays were developed for the quantification of two *C. coccodes* strains and their host plant velvetleaf DNA. Using their respective primers sets, genomic DNA (gDNA) from *C. coccodes* wild type and T-20a showed single expected amplicons of 380 bp and 200 bp, respectively, as determined by either melting curve analysis (Tm = 83.8°C and Tm = 81.2°C, respectively, Fig. 5.1A-B) or agarose gel electrophoresis (Fig. 5.2, lanes 2, 9). The

HPPD primers also detected a single DNA fragment of 155 bp (Fig. 5.2, lane 7) corresponding to a specific melting temperature of 83.6°C (Fig. 5.1C) from velvetleaf DNA but not from fungal DNA (Fig. 5.2, lanes 5 and 6). A linear regression relation between logarithm of starting DNA quantities and PCR threshold cycles (C_T) numbers over the range of DNA concentrations was established (data not shown). For all of the three target organisms studied, the regression correlation coefficient (R^2) of detection exceeded 0.996 over 6 orders of magnitude, indicating high linearity. Depending on the target organism, as low as 2.5 x 10^{-4} ng DNA for wild-type *C. coccodes*, 2.0 x 10^{-4} ng DNA *C. coccodes* T-20a, and 2.5 x 10^{-8} ng DNA for velvetleaf could be quantified, the lowest starting DNA quantities examined. Amplification plots were highly reproducible between triplicate samples (data not shown), and fluorescence data from negative controls containing no templates always remained below the detection threshold (data not shown).

5.4.3. DNA extraction accuracy and reproducibility

Experiments were conducted to evaluate the accuracy and reproducibility of the DNA extraction method combined with real-time PCR quantification of wild-type *C. coccodes* using two approaches: DNA was extracted and then quantified from (i) pure serial dilutions of wild-type *C. coccodes* conidia and from (ii) velvetleaf plants sprayed with serial dilutions of wild-type *C. coccodes* conidia. Both approaches resulted in a good correlation between the amounts of *C. coccodes* DNA quantified and the amounts of target organism initially present

whether in pure stand (Fig. 5.3A; R^2 = 0.9363) or detected on the surface of velvetleaf (Fig. 5.3B; R^2 = 0.9891) over 5 and 4 orders of magnitude, respectively. Starting from a pure stand of *C. coccodes* conidia, the assay was able to reproducibly detect as little as 50 conidia/reaction, the lowest conidial concentration examined (Fig. 5.2A). By extrapolation, the amount of *C. coccodes* DNA/conidium could be estimated to be 2.6 x 10^{-6} ng (data not shown).

5.4.4. Direct and indirect assessment of *C. coccodes* invasion of velvetleaf

At each time point and spanning over the first 5 days after inoculation (DAI), the average amount of DNA (6 pg/0.1 g leaf tissue) detected from both wild-type and T-20a *C. coccodes* strains was not significantly different ($P > 0.05$) from each other (Fig. 5.4A). Over the same time period, few necrotic lesions started to develop on wild type-infected leaves only that became clearly discernable at 7 DAI (Fig. 5.4B), the time at which the amount of wild-type DNA compared to T-20a DNA significantly ($P < 0.01$) increased by 7-fold (Fig. 5.4A). No apparent symptoms were observed on velvetleaf infected with T-20a (Fig. 5.4B). By day 14, the amount of wild-type DNA detected increased by 9-fold (653 pg/0.1 g leaf tissue) being statistically different ($P < 0.05$) from the amount of T-20a DNA (74 pg/0.1 g leaf tissue) with extensive necrotic lesions developed on all leaves to such an extent that only two biological replicates could be salvaged for DNA extraction (Fig. 5.4B). At the same day period, only few lesions appeared on the foliage of velvetleaf infected with the transgenic strain (Fig. 5.4B).

The alteration in velvetleaf DNA mass in response to wild-type and T-20a *C. coccodes* infections was monitored using the primer set HPPD. Compared to the control treatment, the first apparent and significant ($P < 0.001$) decrease in velvetleaf DNA in response to the wild-type strain was detected seven days after inoculation, followed by a substantial drop, reaching a low DNA amount 0.36 pg/0.1 g leaf tissue by day 14 as compared to the transgenic strain treatment and the uninfected control. In contrast, velvetleaf DNA amounts in response to T-20a infection remained similar to those estimated from uninfected velvetleaf foliage (control) at 7 days of infection, but significantly ($P < 0.001$) decreased by day 14 (Table 5.2), however this decrease was not as substantial as that observed with the wild strain treatment. The assessment of disease severity performed on whole plants infected with either the wild-type or the T-20a strain corroborated well with the DNA quantification data showing no severe infection when T-20a was inoculated on velvetleaf plants.

5.4.5. Expression of the NEP1 gene *in vitro* and *in planta*

No *NEP1* transcript was detected in RNA isolated from either pure mycelium of T-20a growing on V-8 agar (Fig. 5.5A, lane 2) or from velvetleaf foliage inoculated with T-20a and harvested after 12 h (Fig. 5.5A, lane 3) or 7 days after inoculation (Fig. 5.5A, lane 4). However, the transcript encoding the ascomycete Histone 4 gene was detectable in all of the analyzed samples (Fig. 5.5B, lanes 2-4), not only confirming the good quality of the cDNA, but also its successful amplification.

Table 5.1. PCR primer characteristics.

DNA Target	Primer	Differential PCR amplification			Application[a]	Sequence (5'to 3')	*Tm* (°C)	Amplicon size (bp)
		Colletotrichum coccodes		velvetleaf				
		Wild-type	T-20a					
C. coccodes SCAR (anonymous sequence)	N5Fi[b]	✓	✓	×	C & Q	AATGGCGTTCATGAAGGTAG	62	380
	N5Ri[b]					ATCTCAATGAAGTACGTCGC	62	
C. coccodes *NEP1* gene	Nep1F[c]	×	✓	×	C	CGGCAGCAGCGTAGAGGGTAG	70	341 (DNA and cDNA)
	Nep1R[c]					CCGACGGTTGTCAGCCATACAC	62	
C. coccodes *NEP1* gene	Nep1693	×	✓	×	Q	CCTCTTCTCCACATTGCCGA	62	200 (DNA and cDNA)
	Nep1893					GCGGCACGAGCATATGTCT	60	
velvetleaf HPPD gene	HPPDF[d]	×	×	✓	C & Q	GCTGCCTGTCGTTTCTCGTG	64	155
	HPPDR[d]					TGGGAAAGAGCGATGGAAGG	62	
Internal Transcribed Spacers 1 and 2	ITS4[e]	✓	✓	✓	C	TCCTCCGCTTATTGATATGC	58	~600 (*C. coccodes*)
	ITS5[e]					GGAAGTAAAAGTCGTAACAAGG	63	~800 (velvetleaf)
Histone 4 gene of ascomycetes	H4-1a[f]	✓	✓	×	C	GCTATCCGCCGTCTCGCT	60	~250 (gDNA)
	H4-1b[f]					GGTACGGCCCTGGCGCTT	62	~200 (cDNA)

[a] C: conventional PCR, Q: Quantitative real-time PCR
[b] Dauch *et al.*, 2003.
[c] Amsellem *et al.*, 2002.
[d] Primers amplifying part of the 4-hydroxyphenylpyruvate dioxygenase gene of *Abutilon theophrasti* (accession number: AY 138 969).
[e] White *et al.*, 1990.
[f] Glass *et al.*, 1995.

Table 5.2. Velvetleaf DNA amounts during time-course infection by two strains of *Colletotrichum coccodes*.

Days after inoculation (DAI)	Plant DNA amounts[a]		
	wild-type *C. coccodes* infected plants	T-20a *C. coccodes* infected plants	Control plants
0	2.03 +/ - 0.32	1.77 +/ - 0.44	1.91 +/ - 0.24
1	1.26 +/ - 0.36	1.93 +/ - 0.35	1.76 +/ - 0.11
2	1.25 +/ - 0.18	1.97 +/ - 0.19	1.91 +/ - 0.23
5	1.22 +/ - 0.30	2.11 +/ - 0.31	2.16 +/ - 0.35
7	1.12 +/ - 0.43a	1.80 +/ - 0.18b	1.81 +/ - 0.16b
14	0.36 +/ - 0.02a	1.78 +/ - 0.22b	2.15 +/ - 0.24c

[a] Data presented are mean amounts (pg DNA/0.1 mg leaf tissue) +/- SE as quantified by real-time PCR with HPPD primers from 4 replicates / time-point/ treatment. Means within a row followed by a different letter are significantly different ($P < 0.05$).

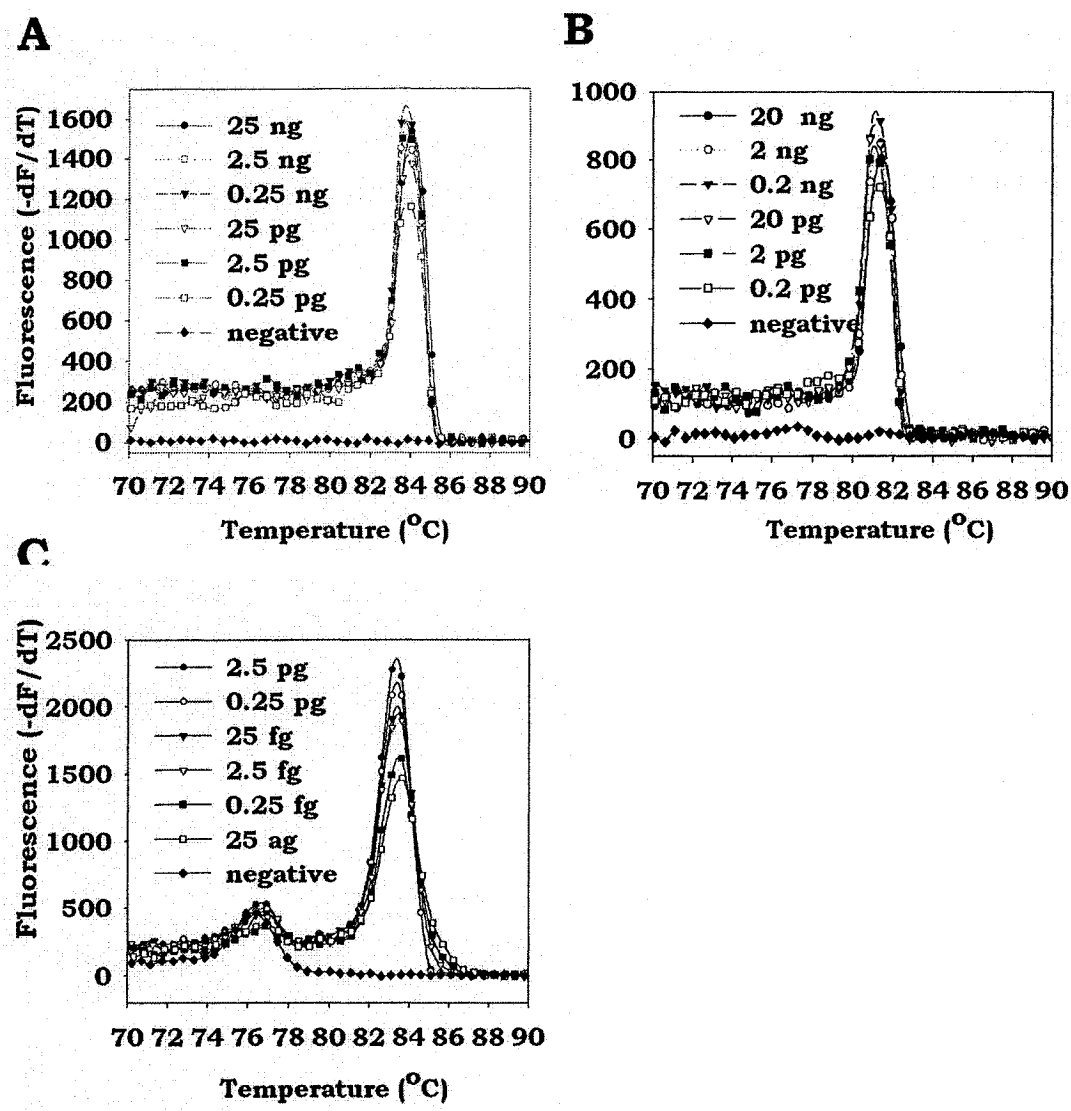


Figure 5.1. Real-time PCR melting peaks for the quantification of wild-type (**A**), T-20a (**B**) *Colletotrichum coccodes* and velvetleaf (**C**) DNA.

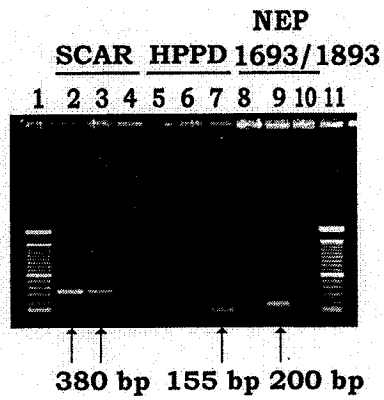


Figure 5.2. PCR gel electrophoresis showing specificities of the primers: SCAR (lanes 2-4); HPPDF/HPPDR (lanes 5-7); Nep1693/1893 (lanes 8-10) on wild-type *Colletotrichum coccodes* gDNA (lanes 2, 5, 8); T-20a *Colletotrichum coccodes* gDNA (lanes 3, 6, 9); velvetleaf gDNA (lanes 4, 7, 10); lanes 1 and 11 contain a 100 bp ladder.

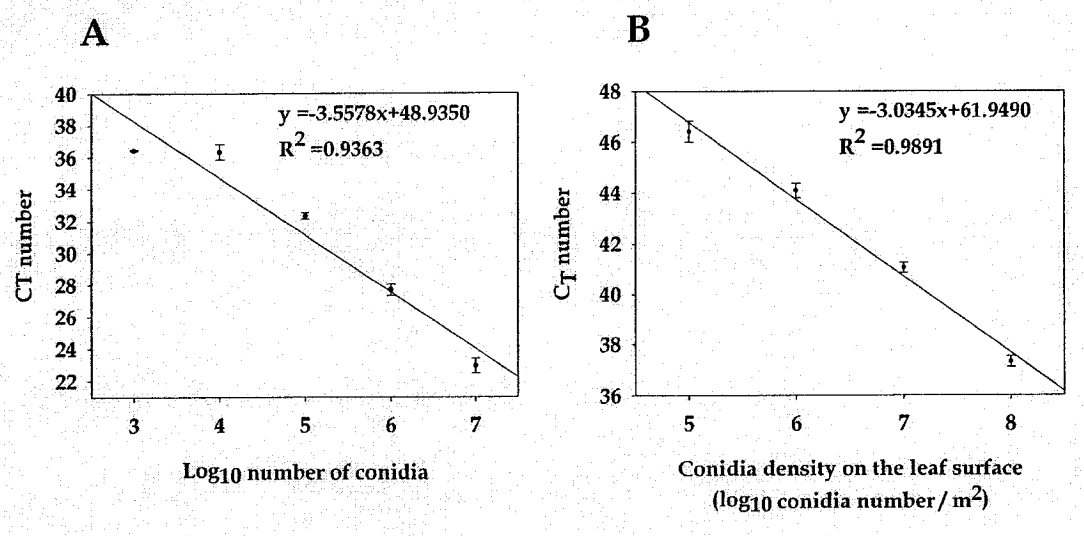
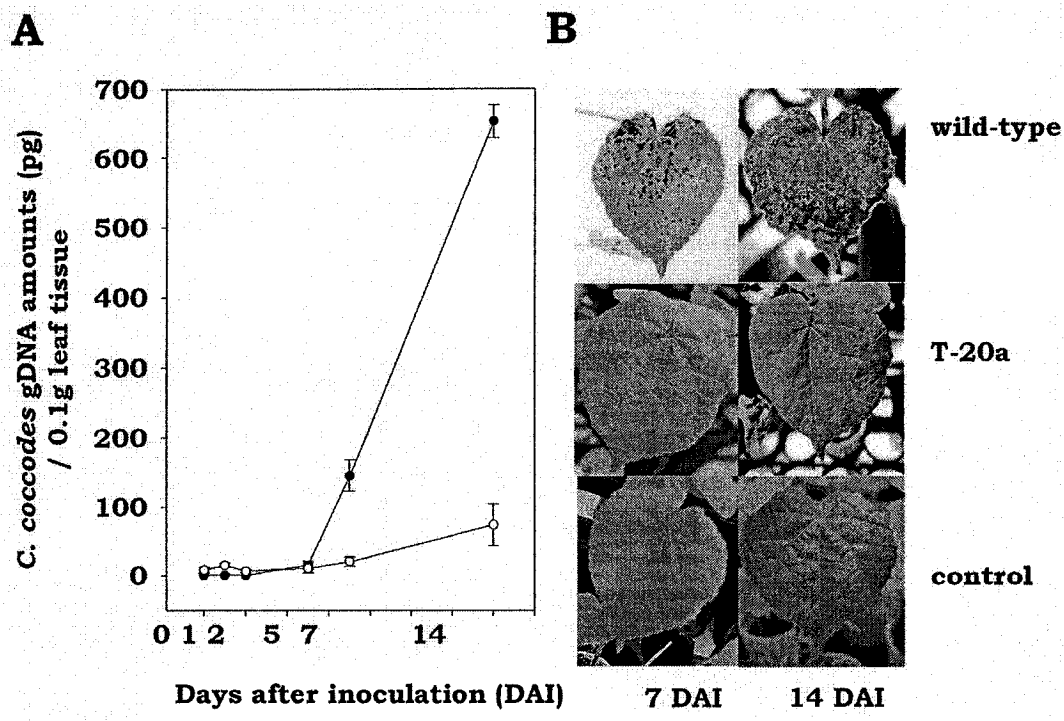
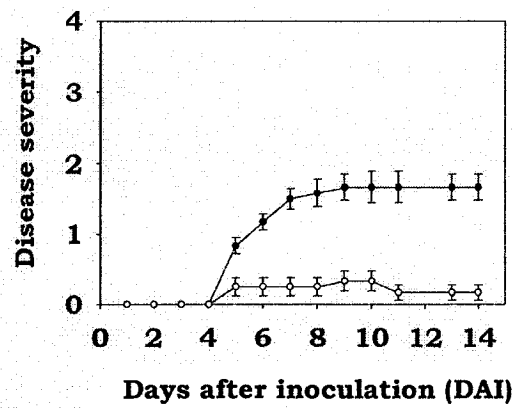


Figure 5.3. Relationship between C_T numbers quantified by real-time PCR and wild-type *Colletotrichum coccodes* conidia numbers. (**A**) in a pure stand, and (**B**) sprayed on velvetleaf leaves. The means (4 replicates /conidial concentration) are C_T numbers $\pm$ standard errors as quantified by real-time PCR with SCAR primers.

Figure 5.4. Wild-type and T-20a *Colletotrichum coccodes* time-course infection on velvetleaf (day 0 to 14 after inoculation (DAI)). (**A**) Fungal DNA quantification. □ = wild-type and □ = T-20a *Colletotrichum coccodes*. The numbers are mean values of 4 replicates/time-point/treatment ± standard errors as quantified by real-time PCR with SCAR and Nep1693/1893 primers, respectively. (**B**) Photographs of velvetleaf leaves infected and mock treated 7 and 14 DAI. (**C**) Disease severity assessment performed on entire plants from 1 to 14 DAI on □ = wild-type and □ = T-20a *Colletotrichum coccodes* infected leaves.


A
B
700
600
500
400
300
200
100
0
C. coccodes gDNA amounts (pg)
/ 0.1g leaf tissue
0 1 2 5 7 14
Days after inoculation (DAI)
wild-type
T-20a
control
7 DAI
14 DAI


C
4
3
2
1
0
Disease severity
0 2 4 6 8 10 12 14
Days after inoculation (DAI)

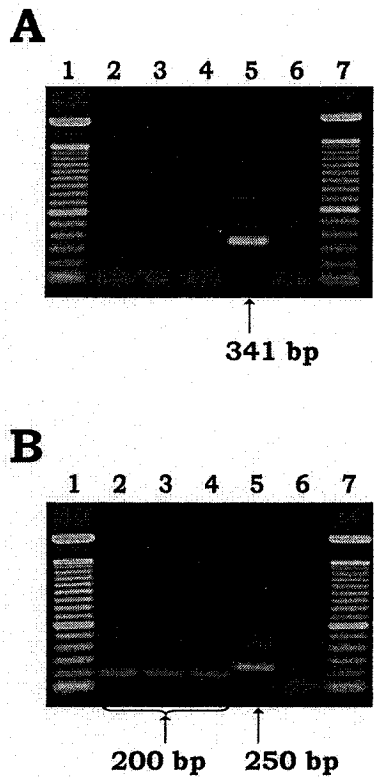


Figure 5.5. Detection of *NEP1* gene expression in T-20a *Colletotrichum coccodes* by RT-PCR. The assay was performed with (**A**) NEP1F/R primers (*NEP1* gene), and (**B**) Histone 4 primers (house-keeping gene). cDNA templates from T-20a mycelium on V8 agar (lane 2) and T-20a infected velvetleaf harvested at 12 h. (lane 3) and 7 days (lane 4) after infection. gDNA template from T-20a *Colletotrichum coccodes* (lane 5, positive control). No template control (lane 6, negative). Lanes 1 and 7 contain a 100 bp ladder.

5.5. Discussion

In this study, we report on the molecular monitoring of two *C. coccodes* biocontrol strains under controlled growth conditions together with the quantification of host plant DNA by real-time QPCR. All of the primer's specificities and efficiencies have been established and rigorously validated for the 3 different targets. Calibration experiments with external standards demonstrated a strong dependency of the C_T numbers on the logarithm of starting DNA quantities over 6 orders of magnitude. Taken all this together, we demonstrate as did other recent studies (Alkan *et al.*, 2004; Boyle *et al.*, 2005; Brouwer *et al.*, 2003), the utility of real-time QPCR for reliable and accurate assessment of pathogen growth on velvetleaf and for monitoring velvetleaf DNA in response to pathogen infections.

When applied on the surface of velvetleaf, the wild type *C. coccodes* does not develop any necrotic lesions before 5 days after inoculation. In this case, real-time QPCR proved to be reliable in assessing the outcome of the infection prior to the appearance of symptoms on the host before 5 days after inoculation, suggesting that during this period, *C. coccodes* has a biotrophic stage of growth. The switch to the necrotrophic stage seemed to occur after 5 days, at which the appearance of necrotic lesions coincided with the onset of an exponential like-growth on the leaf as determined by QPCR. These results clearly demonstrate the effectiveness of QPCR for monitoring the progress of *C. coccodes* strains *in planta* during the invisible disease stage when velvetleaf is symptomless.

Among the aspects we verified are the reliability and accuracy of our methodology for pathogen quantification. We have shown here that dilution series

of pathogen conidia can be accurately quantified and correlated with DNA amounts over a large concentration range in the absence or presence of the host using real-time PCR. We demonstrated that the presence of velvetleaf tissue did not interfere in the quantification of *C. coccodes* conidia, a pre-requisite for *in planta* DNA quantification. The threshold of wild-type *C. coccodes* detection corresponds to 50 conidia/PCR reaction (in the absence of the host) or 10^5 conidia/m^2 of velvetleaf tissue (in the presence of the host), the lowest inoculum concentration examined. Another aspect we evaluated was the potential interference of PCR inhibitors originating from plants which are known to affect the efficiency of PCR amplification (Wilson, 1997). We have shown that $1/10^{th}$ dilution level of extracted DNA was ideal to preclude any bias due to PCR inhibitors.

In this study, there was a slight and constant decrease of velvetleaf DNA over the course of *C. coccodes* infections. This observation can reasonably be accounted for by plant cell collapse during pathogen progression. Some studies dealing with plant pathogen DNA quantification have used an endogenous plant reference in order to normalize for the amount of material loaded in the PCR reaction (Boyle *et al.*, 2005; Gao *et al.*, 2004; Hietala *et al.*, 2003; Winton *et al.*, 2002). This approach is feasible when comparing pathogenic strains of similar aggressiveness or host line resistance (Boyle *et al.*, 2005; Hietala *et al.*, 2003). We have chosen not to normalize the pathogen concentration to an endogenous plant gene because of the difference in aggressiveness between the two strains studied. Furthermore, since tissue necrosis caused by a pathogen infection can lead to plant cell collapse and decreased amounts of detectable host DNA, it can be

anticipated that normalizing pathogen DNA amounts to the amounts of detectable host genes such as HPPD can lead to an overestimation of pathogen biomass (Gachon and Saindrenan, 2004). As a solution to this problem, all leaf samples analyzed in this study were normalized to the tissue weight and the decrease in plant DNA amounts observed across time are considered a consequence of necrotic lesions rather than a consequence of the unequal PCR loading of the sample PCR reactions.

Strain *C. coccodes* T-20a was genetically engineered for increased virulence on velvetleaf by insertion of *NEP1* (Amsellem *et al.*, 2002), a gene that is thought to participate in the natural virulence of many *Fusarium oxysporum* individuals (Bailey *et al.*, 2002) in nature. Amsellem *et al.* (2002) recently showed that the NEP1 genetically transformed *C. coccodes* strain T-20a is more virulent than the wild type and attacks velvetleaf faster causing extensive necrotic lesions and early seedling death. They also demonstrated that the Nep1 protein was expressed *in planta* as early as 24 h. after plant inoculation (Amsellem *et al.*, 2002). In addition to the results of this study, we have conducted several independent inoculation trials, using chopped mycelia (data not shown) as the inoculum source of T-20a instead of conidia on young velvetleaf plants, and found that despite he fact that T-20a grew on the surface of velvetleaf, only few necrotic lesions developed on the foliage of velvetleaf even after 14 days of infection, and their appearance was significantly delayed compared to those produced by the wild-type *C. coccodes* infected plants. These results corroborated well with the molecular monitoring of T-20a on velvetleaf when the fungus was applied as conidia. The discrepancy between our results and those of Amsellem *et*

al. (2002) is difficult to explain. We have used velvetleaf seeds from different sources and conducted several experiments at the cotyledonary and later stages of development under variable growth conditions, and applied both strains as either chopped mycelia or conidia. Irrespective of these conditions, we were not able to obtain good infectivity for the T-20a strain (data not presented).

Studies on the *Phytophthora sojae* - soybean pathosystem have shown that the transcripts naturally encoding a Nep1 like protein in *P. sojae* could only be detected from 12 h. post plant inoculation (Qutob *et al.*, 2002). The fact that in this study we could not detect any expression of the *NEP1* transcript in T-20a mycelia or *in planta* 12 h and 7 days after inoculation, combined with the inability of T-20a to cause disease on velvetleaf led us but to suspect that gene rearrangement events may have silenced the expression of the *NEP1* gene in this strain. Interestingly, we were able to detect the expression of the fungal house-keeping gene H4 in the same tissue samples which clearly indicates that the RT-PCR method was able to detect the activity of fungal genes. One can argue that the failure to detect *NEP1* gene expression in our samples may be due to low levels of *NEP1* RNA copies that are below the detection threshold, although several cDNA dilutions (from 2 to 100 fold) were tested generating similar results. This may hold true for infected velvetleaf samples assayed at 12 h but it is unlikely when one considers the absence of *NEP1* expression 7 days after inoculation, the time at which T-20a population had increased by 3 fold.

In the current work, a powerful tool has been developed to quantify accurately the growth kinetics of *C. coccodes* strains on the host weed velvetleaf and to assess their aggressiveness. The procedure will allow studying the invasion

of the biocontrol agent even at early stages of velvetleaf infection, a prerequisite for the development of effective biocontrol strategies.

CONNECTING STATEMENT BETWEEN CHAPTERS V AND VI

This chapter describes the construction of a subtracted cDNA library containing infection-specific ESTs from velvetleaf-*Colletotrichum coccodes* DAOM 183088 interaction at 12 h and 24 h post-infection. The *C. coccodes* strain is host specific for velvetleaf and the purpose of this chapter was to isolate genes expressed *in planta* that are likely to play a role in the specificity of the interaction. The methodology consisted in isolating cDNAs that are expressed during the pathogenic interaction of *C. coccodes* with its host velvetleaf but not expressed during the non-host interaction of the same *C. coccodes* strain with a related *Malvaceae* plant, okra (*Hibiscus esculentus* cv. Clemson spineless). A total of 139 ESTs, representing genes preferentially expressed in the velvetleaf-*C. coccodes* pathogenic interaction compared to okra-*C. coccodes* interaction, velvetleaf alone or *C. coccodes* alone, have been isolated. Putative functions were assigned to 94 of them in functional categories including transcription, energy, signaling, cell growth and maintenance, oxidative stress and defense.

The results of this section are the subject of a manuscript that has been submitted for publication to Molecular Plant Pathology. I have designed the experimental set-up, conducted all of the experiments, and wrote the manuscript. Professors S. Jabaji-Hare is my supervisor. She is the corresponding author on this manuscript. She supervised and provided funding and valuable suggestions throughout the study, and corrected the manuscript. Danielle Morissette, Ph.D. candidate in Dr. S. Jabaji-Hare's laboratory, helped in technical trials, and final

correction of the manuscript. Drs. Roland Brousseau and Luke Masson, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada, made their microarray printing facility available, provided technical advice and guidance in the microarray section and contributed to the final revision of the manuscript, respectively. Dr. Martina Strömvik helped in the data mining section by providing help and equipment for the construction of stand-alone sequence libraries for blast analyses, and revised the final manuscript version.

CHAPTER VI

Analysis of up-regulated transcripts during velvetleaf-*Colletotrichum coccodes* interaction by SSH and cDNA microarray differential screening

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6.1. Abstract

We used suppression subtractive hybridization (SSH) and microarray differential screening to generate cDNA libraries enriched for *in planta* up-regulated host and pathogen genes 12-24 h after infection of velvetleaf leaves with the mycoherbicide *Colletotrichum coccodes*. A total of 139 ESTs, representing preferentially expressed genes, were analyzed of which 94 were assigned putative functions including transcription, energy, signaling, cell growth and maintenance, and oxidative stress and defense. Eleven ESTs had high similarity scores in the databases but no associated known function, while 34 ESTs showed either no significant homology with sequences deposited in the public databases or no hit, and thus were considered as novel ESTs. Genes related to oxidative stress and defense formed the largest category corresponding to 60% of the ESTs. Among these ESTs, metallothioneins (MT) type 3 proteins were represented by 79%. This is the first report on the expression of plant MT type 3 in response to fungal infection. We also identified several ESTs whose sequences encoded an ethylene response element binding (EREB) protein, WRKY and bZIP proteins that are likely to play roles in transcription, while other ESTs encoded proteins such as ascorbate peroxidase and reticuline oxidase. Seven selected genes identified by microarray analysis were validated by real-time quantitative QRT-PCR. Relative to uninfected velvetleaf leaves, these genes were significantly temporally induced by the presence of *C. coccodes* on velvetleaf leaves.

6.2. Introduction

Colletotrichum coccodes (Wallr.) Hughes is a suspected hemibiotrophic fungal pathogen that causes two distinct diseases, anthracnose and black dot on several cucurbits and *Solanaceaous* crops. It is particularly destructive on potato and tomato and on several weed species (Andersen and Walker, 1985; Raid and Pennypacker, 1987), including velvetleaf (*Abutilon theophrasti* Medik.) (Watson *et al.*, 2000). Velvetleaf is a major weed found in soybean and corn (*Zea mays* L.) cropping systems in several regions of the USA, Canada, Europe and the Mediterranean (Andersen *et al.*, 1985; Sattin *et al.*, 1992; Spencer, 1984). The strain *C. coccodes* (DAOM 183088) was recovered from velvetleaf leaves in Vermont (Gotlieb *et al.*, 1987), and introduced as a host-specific pathogen to infect and limit the spread of velvetleaf in Canada (Watson *et al.*, 2000). Typically, this strain causes anthracnose that is characterized by grey-brown lesions on leaves of velvetleaf that eventually bring about desiccation and premature shedding of the leaves. Because of its restricted host range, *C. coccodes* (183088) is considered a potential mycoherbicide of velvetleaf, and continued research to enhance the weed control strategy had focused in the last decade on studies targeted to optimize inoculum production (Yu *et al.*, 1997), application (Hodgson *et al.*, 1988; Wymore and Watson, 1989), efficiency (Ahn *et al.*, 2005a, 2005b; Amsellem *et al.*, 2002), and detection (Dauch *et al.*, 2003) methodologies for this strain.

Despite that recent studies dealing with *Colletotrichum* species provided excellent models for studying the molecular basis of infection structure, differentiation and fungal-plant interactions, and led to the identification of genes

and their transcriptional activation during the infection process (Goodwin, 2001; Goodwin *et al.*, 2004; Idnurm and Howlet, 2001; Latunde-Dada, 2001; Perfect *et al.*, 1999; Shan and Goodwin, 2005), a full understanding of the molecular factors determining pathogenicity or the molecular mechanisms employed by the hosts to counteract their invaders is lacking or limited to very few *Colletotrichum* model species. One neglected area that merits in-depth knowledge is the molecular understanding of weed-mycoherbicide interaction.

Since expression of disease in velvetleaf-*C. coccodes* interaction is thought to be the result of differential expression of specific gene sets contributing to fungal pathogenicity or to resistance in velvetleaf, we sought to investigate the expression of genes at early stages of velvetleaf infection using a Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.*, 1996) technique and differential screening by cDNA microarrays (van den Berg *et al.*, 2004).

6.3. Materials and methods

6.3.1. Biological material and growth conditions

Colletotrichum coccodes (DAOM 183088) strain was cultured on potato dextrose agar (PDA, Becton Dickinson Microbiology Systems, Sparks, MD) for one week at 28°C in the dark. Conidial production was initiated by transferring five agar plugs into 600 ml of modified Richard medium (MRM) (DiTommaso and Watson, 1995) and incubated at 24°C with agitation (200 rpm) for 7 days. The conidia were harvested through filtration, centrifuged at 4°C for 10 m n.at 6000 x *g*, and the conidial pellet was resuspended in double distilled water

(ddH_2O). Conidial suspension was adjusted with ddH_2O to a final concentration of 10^9 conidia/ml, using a hematocytometer.

Velvetleaf (*Abutilon theophrasti* Medik.) and okra (*Abelmoschus esculentus* (L. Moench) seeds were collected from fields in Ste-Anne de Bellevue (Qc., Canada) and purchased from William Dam Seeds (Dundas, ON, Canada), respectively. They were placed in 100 x 15 mm Petri dishes on Whatman paper no.1, moistened with 4 ml of ddH_2O and pre-germinated in the dark for 3 days. One pre-germinated seed was planted in greenhouse pots (145 x 150 mm) filled with Promix (Premier Tech, Rivière du Loup, QC, Canada). The plants were placed in growth chambers following a randomized complete block design (RCBD) with conditions adjusted to 24/18°C for 10/14 h. (day/night) with a light intensity of 300□M/m^2. Plants were watered as needed and were fertilized two weeks after the initial seeding with 50 ml solution of 20:20:20 (N:P:K) (3%) until 4 weeks old.

6.3.2. Inoculation of leaves with *C. coccodes*

Spraying of velvetleaf (susceptible) and okra (tolerant) plants was performed in a spray chamber (Research Instrument MFG. Co. LTD., Guelph, ON, Canada) to ensure uniform coverage of the leaves. Some plants received a constant rate of 10^9 conidia/ml (50 ml/m^2) of *C. coccodes* conidia, while others received ddH_2O (mock treatment) (Fig. 6.1A). All sprayed plants were placed in a dew chamber (90% humidity, 24°C) for 18 h. to maximize fungal infection. Leaf samples (the third leaf of five plants grown in separate pots) from each treatment were harvested 12 and 24 h. after spraying.

6.3.3. Isolation of genomic DNA and RNA

Genomic DNA (gDNA) from *C. coccodes* was isolated from 20 mg of freeze-dried 12-day-old mycelial mats as previously described (Dauch *et al.*, 2003). Total gDNA from velvetleaf leaves was extracted using the Qiagen DNeasy Plant Mini-Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's protocol except that all pipetting steps were carried on ice, centrifugation steps at 4°C, and an additional washing step was performed. DNA isolated from all biological material was quantitatively and qualitatively estimated by spectrophotometry and run on 1% agarose gels (1xTAE) to verify the absence of degradation.

Fungal RNA was extracted from *C. coccodes* conidial suspensions (10^9 conidia/ml) incubated at room temperature for 12 and 24 h. Conidia were ground to a fine powder in liquid nitrogen with the addition of silica beads (Xymotech Biosystems, Inc., Mont-Royal, QC, Canada). Leaf samples from infected and mock treated plants were ground in liquid nitrogen with the addition of 0.5 g NaCl and 0.33 g PVPP/replicate to prevent the mucilaginous material from interfering in the nucleic acid extraction processes. Total fungal and plant RNA were extracted from 100 mg conidial powder with the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's recommendations and treated with Qiagen DNase (Qiagen, Mississauga, ON, Canada) to remove any potential DNA contaminant. RNA extracts were checked for the absence of DNA contamination in conventional PCR using ITS4/ITS5 primer set targeting the Internal Transcribed Spacer (Gardes and Bruns, 1993; White *et al.*, 1990) (Table 6.1). Five hundred nanograms DNA-free RNA were loaded on 1.2% agarose

denaturing formaldehyde gels to check the integrity of the isolated nucleic acids. Four □g RNA from each of the eight different sources (Fig. 6.1) were precipitated (protocol from the SMART™ cDNA synthesis kit (BD Biosciences-Clontech, Palo Alto, CA). Each of the tester and driver source precipitates were pooled and resuspended in 8 µl DEPC water. cDNAs were individually synthesized from 1 □g driver or tester RNA using the SMART™ cDNA synthesis kit (BD Biosciences-Clontech, Palo Alto, CA).

6.3.4. Suppression subtractive hybridization

Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.*, 1996) was performed between the cDNA populations of the tester (T: susceptible plant infected with *C. coccodes*) and the driver (D1: mock-treated susceptible plant, D2: *C. coccodes* conidia and D3: tolerant okra inoculated with *C. coccodes* (Fig. 6.1). The SSH procedure was performed with the PCR Select cDNA Subtraction kit (BD Biosciences-Clontech, Palo Alto, CA) following the manufacturer's directions. The PCR mixture containing up-regulated gene fragments (subtracted tester, ST) was cloned using the TOPO TA Cloning kit (pCR 4® vector; Invitrogen, Carlsbad, CA, USA) and transformed into TOP10 electro competent cells (Invitrogen, Carlsbad, CA, USA) using a Gene Pulser II (Biorad, Mississauga, ON, Canada) with 2.4 kV, 25□F and 200 Ohms in Gene pulser cuvettes (Biorad, Mississauga, ON, Canada). *E. coli* cells were plated on LB ampicillin (50 □g/ml) plates, and a total of 1487 clones were individually collected after 24 h., transferred in 200 □l liquid LB ampicillin 96-well plates for 4 h.

growth at 37°C. These cultures were used to establish stocks (50% glycerol at stored at -80°C) and to amplify the inserts by PCR.

6.3.5. cDNA microarray differential screening

cDNA microarrays were used to confirm the differential expression of the clones. Briefly, 5 □l of culture were diluted to $1/10^{th}$ in sterile ddH_2O and heated at 94°C for 10 min. to be used as PCR template. The SSH nested adaptor primers (BD Biosciences-Clontech, Palo Alto, CA) that flank each insert in the plasmid were used to amplify the inserts and to insure that multiple inserts are excluded. One hundred µl PCR reactions (5 µl template, 2 µM each of the M13 universal primers, 10.5 µl of 10x buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 U of *Taq* polymerase and 200 nM of each of the dNTPs (Invitrogen, Burlington, ON, Canada) were thermocycled for 94°C, 1min.; 58°C, 1 min.; 72°C, 1 min. 30 s.; 30 cycles; with a final extension of 72°C, 10 min. Five □l aliquots were resolved on 1% (1xTAE) agarose gels. Single PCR products were purified using a vacuum manifold and MultiScreen FB plates (Millipore, Nepean, ON, Canada) using the following protocol: plates were pre-wet with 50 □l binding buffer (200mM MES pH 5.6; 7M Guanidine-HCl; adjusted to pH 5.6 with NaOH) and vacuum applied for 1 min. using a vacuum manifold (Millipore MultiScreen, Millipore, Nepean, ON, Canada). One hundred µl binding buffer was added to individual PCR products mixed by pipetting, transferred to the purification plate and subjected to several washing steps with 50 □l binding buffer and 200 □l 80% ethanol using vacuum. Plates were centrifuged (1,000 x *g*; 5 min. in a CR4-22 (Jouan,

Winchester, VG)) and placed back on the manifold for two successive elutions (75 and 50 □l ddH2O; pH 8.0) (7 and 10 min. vacuum) in UV-transparent 96-well plates (Corning, Big Flats, NY). Optical densities at 260 and 280 nm were recorded using ELx800™ fluorescence reader (Bio-Tek Instruments, Winooski, Vermont, USA). Two □g of DNA were transferred into Corning plates (V plates, Big Flats, New York, USA), lyophilized, re-suspended in 50% DMSO and 50% Ultra pure water to give a final concentration of 0.2 □g/□l and arrayed on glass slides with a Virtek printer (Chipwriter Pro SDD2, Virtek, Ontario, Canada). 1,152 ESTs were spotted and immobilized in triplicates on Corning GAPS II slides (Corning, Big Flats, New York, USA).

6.3.6. Hybridization

Two types of hybridization probes were used (*i*) SSH generated probes: UT, UD and ST (Fig. 6.1B) for differential screening and (*ii*) ribosomal DNA (velvetleaf and *C. coccodes* ITS (DQ287984, AY211498), small (DQ287985, DQ287988) and large (DQ287986, DQ287987 and DQ287989 DQ287990) nuclear ribosomal sub-unit genes) (Table 6.1) to check for rRNA redundancy. For differential screening, a total of eight hybridizations were carried out (4 x 2 replicates) including the ST/UD, the ST/UT hybridizations and their respective dye-swaps. A single hybridization was performed with a mix of ribosomal PCR products (ITS, small and large nuclear ribosomal sub-unit genes) amplified from velvetleaf and *C. coccodes* gDNA (Table 1). All probes were prepared by direct labeling with the BioPrime labeling kit (Invitrogen, Burlington, On, Canada) and Cy3/Cy5 dyes (Amersham Biosciences Piscataway, NJ, USA).

Pre-hybridization buffer (5 x SSC, 0.1% SDS, 0.1% BSA) was sterilized by filtration (0.22 μm) and pre-heated at 42°C. Twenty μl were deposited on the surface of the slides and incubated in Corning hybridization chambers for 1 h. at 42°C. Cover slips were then removed by dipping the array in filtered 0.1 x SSC and arrays were washed twice with 0.1 x SSC, and finally dried under a clean air stream. Hybridization was carried out in a total volume of 20 μl consisting of at least 1/3 volume of Dig Easy Hyb (Hoffmann-La Roche Ltd, Mississauga, ON, Canada) and 15 pmoles each of the Cy3- and Cy5-labelled probes. The labeled cDNAs in the hybridization buffer were heated at 95°C for 5 min. and chilled on ice for 1 min. Probes were placed onto the center of the arrays in sealed hybridization chambers (Corning, Big Flats, New York, USA) and immediately incubated at 42°C in a water bath. After 17 h., cover slips were washed off in 1 x SSC, 0.2% SDS preheated to 42°C, then the slides were transferred in successive 1 x SSC, 0.2% SDS baths with gentle shaking (42°C for 10 min., 37°C for 5 min., room temperature for 5 min.). Slides were finally incubated for 5 min. at room temperature in 0.1 x SSC and dried under an air stream. Each experiment was repeated twice.

Microarrays were scanned with the ScanArray Express HT (Perkin Elmer, Vaudreuil-Dorion, Qc, Canada). Separate images were acquired for separate fluorochromes at a resolution of 10 μm per pixel. Data analysis was performed with ImaGene software (Biodiscovery, El Segundo, CA, U.S.A.). After segmentation, local background correction and global normalization with LOWESS (locally weighted polynomial regression) (Cleveland, 1979), the

median intensities of triplicate spots were $\log_2$ transformed and analyzed using the following formula ER1=1/2[$\log_2$Cy3ST/Cy5UD-($\log_2$Cy3UD/CyST)] and ER2=1/2[$\log_2$Cy3ST/Cy5UT-($\log_2$Cy3UT/CyST)] where ER1 and ER2 are enrichment ratios of ST/UD and ST/UT, respectively, compiled from slides hybridized with ST and UD, and ST and UT, respectively. UT/UD ratio equals the antilog of (ER1-ER2) in the base 2 (van den Berg *et al.*, 2004)

6.3.7. Sequence Analysis

Single pass sequencing of 139 up-regulated cDNA clones was performed at the McGill University and Genome Quebec Innovation Center (Montreal, Qc, Canada) using either the SSH nested adaptor or the M13 universal primers. Sequence analysis was carried out with Chromas 2.3 (http://www.technelysium.com.au/chromas.html) and Biology WorkBench 3.2 (http://workbench.sdsc.edu/). Sequences were then analyzed by BLAST (Altschul *et al.*, 1997) analysis against different blast target databases. Stand-alone blast (blastn and blastx) analyses were first run against local databases (constructed in mid-September 2005) and comprising (i) all fungal DNA and amino acid sequences available (999,129 and 329,935 sequences, respectively) as well as (ii) 3,812,678 EST sequences from the following fourteen plant genera: *Arabidopsis*, *Beta*, *Brassica*, *Glycine*, *Gossypium*, *Lycopersicon*, *Medicago*, *Nicotiana*, *Oryza*, *Phaseolus*, *Solanum*, *Triticum*, *Vitis* and *Zea*, and 163,024 amino acid sequences from *Arabisopsis thaliana*, *Gossypium*, *Oryza sativa*, *Glycine max*, *Nicotiana*, *Lycopersicon*, *Solanum*, *Populus* and *Hordeum*. Standard blast (blastn and blastx) analyses were also conducted through the web interface at NCBI

(http://www.ncbi.nlm.nih.gov/BLAST/) on sequences from Viridiplantae, Fungi or without any organism restriction. Searches were performed using a cut-off E-value of $1e^{-4}$ (corresponding to a normalized E (nE) value of 4).

Additional database searches were performed using the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST Database version 1.5 (http://cbr-rbc.nrc-cnrc.gc.ca/services/cogeme/), the *Magnaporthe grisea* Genome Database release 2.3 (http://www.broad.mit.edu/annotation/fungi/magnaporthe/index.html), as well as the *Neurospora crassa* Genome Database release 7 (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html). Searches were performed with blastx and blastn algorithms available at the respective web sites using a cut-off E-value of $1e^{-5}$ (corresponding to a normalized E (nE) value of 5).

Metallothionein sequence alignments and dendrograms were performed with CLUSTAL W (Thompson *et al.*, 1994), ClustalX (Thompson *et al.*, 1997) and Treeview (Page, 1996) according to the neighbor-joining method and confidence values for the branches were determined using bootstrap analysis where 1000 trees were generated from nucleic sequences corresponding to the open reading frames (ORFs). Nucleic sequences encoding metallothioneins type 3 from other plant species were also included in the analysis (*Actinidia deliciosa* var. *deliciosa* (L27811), *Arabidopsis thaliana* (NM_112401), *Gossypium hirsutum* (AY857933) and *Populus balsamifera* subsp. *trichocarpa* x *Populus deltoides* (AY594300). The protein sequences were predicted using EXPASy

(Expert Protein Analysis System) translate tool (Gasteiger *et al.*, 2003) available at http://ca.expasy.org/tools/dna.html.

6.3.8. Primer design and PCR analysis

Eight primer sets (Table 6.1) flanking seven target genes and the house keeping gene (HKG) actin were designed with Primer 3 (Rozen and Skaletsky, 2000), and tested in conventional PCR against velvetleaf and *C. coccodes* gDNA, the tester and driver cDNA pools, and infected okra cDNAs in order to confirm the plant origin of each amplicon. Real-time PCR experiments were performed to validate the up-regulation of the seven target genes.

All conventional PCR reactions were performed in an Applied Biosystems 9600 (Foster City, CA, USA) with primers synthesized by AlphaDNA (Montreal, QC, Canada). PCR products were all resolved on agarose gels (1%, 1xTAE) with Gene Ruler™ 100bp DNA Ladder (Invitrogen, Burlington, ON, Canada), stained with ethidium bromide and pictures recorded by a gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada).

Real-time PCR was conducted in a Mx3000p thermocycler (Stratagene, La Jolla, CA, USA). Each real-time PCR reaction mix consisted of 10 µl Stratagene Brilliant SYBR Green master mix (Stratagene, La Jolla, CA, USA), 1.25 µl of a mix of the two primers (2 µM each), 7.45 µl sterile ddH_2O, 0.3 µl ROX reference dye (Stratagene, La Jolla, CA) and 1 µl of template (tester or driver cDNA $1/10^{th}$ diluted) or 1 µl ddH_2O (no template control). Thermo cycling was performed with an initial denaturation at 95°C (10 min.) followed by 40 to 50 cycles at 95°C (30

s.), 58°C (30 s.), 72°C (30 s.) (2.5°C/s.). Finally, a melting curve was generated by programming the thermocycler to reach 95°C (60 s.), 55°C (30 s.) (2.5°C/s.) and 95°C (0 s.) (0.1°C/s.). Data generated by real-time RT-PCR were estimated using Stratagene analysis software. Each run included a negative control and was repeated three times on three different days (data are presented as means of three technical replicates).

The relative expression ratios of the seven target genes in the tester cDNA pool (sample) versus the driver (control) were calculated using equation [1] (Pfaffl, 2001). E_{target} and E_{ref} represent the PCR amplification efficiencies for target and reference (HKG) genes, respectively, and were calculated from equation [2] (Pfaffl 2002, http://www.gene-quantification.info/). ΔCP_{target} is the crossing points (CP) deviation of driver – tester for each of the targets; ΔCP_{ref} is the CP deviation of driver – tester of the HKG.

$$\text{Ratio} = (E_{target})^{\Delta CP_{target}\,(\text{control-sample})} / (E_{ref})^{\Delta CP_{ref}\,(\text{control-sample})} \qquad [1]$$

$$E = (RnB/RnA)^{\wedge[\,1\,/\,CPB\,-\,CPA]} \qquad [2]$$

The relative expression ratios of the seven target genes in the *C. coccodes* infected velvetleaf (sample) versus the mock-treated velvetleaf plants (control) were calculated at 12 h and 24 h after treatment using REST-MCS beta software version 2 (Pfaffl *et al.*, 2002) (http://www.gene-quantification.info/) using two technical and at least three biological replicates. REST software calculates the relative expression ratio of the target gene on the basis of the PCR efficiency (E) and crossing point difference (ΔCP), and on a newly developed Pair Wise Fixed Reallocation Randomization Test. The statistical test was calculated at $P = 0.05$

and 2,000 randomizations were performed. Individual real-time PCR efficiencies were estimated with equation [2]. Each target gene was considered differentially expressed compared to the control, if the fold change of expression was over 2 (i.e. $\log_2 > 1$ (Pfaffl *et al.*, 2002) with $P < 0.05$).

6.4. Results

6.4.1. Creation of a subtracted cDNA library

We constructed a suppression subtractive hybridization (SSH) library with RNA induced during velvetleaf-*Colletotrichum coccodes* susceptible interaction at early sequential time points. These time-points were chosen to investigate genes readily induced upon plant recognition and early fungal penetration events. In order to restrict the analysis to pathogen-induced velvetleaf and velvetleaf-induced *C. coccodes* genes, subtraction was performed between the pathogenic interaction cDNAs (tester) and the cDNAs of each of the interacting partners alone (driver). In addition, the driver contained cDNA of the non-pathogenic interaction okra-*C. coccodes* (control) (Fig. 6.1A) in order to enrich for transcripts specifically expressed during the pathogenic interaction, and eliminate transcripts that are expressed by the two *Malvaceaous* plants as a general response to pathogen attack. Gene specific primers (Table 1) were used to monitor the pre- and post-SSH enrichment levels of the housekeeping gene histone 4. This step was necessary to evaluate the efficiency of the subtraction process (Table 6.1). There was a significant reduction in the abundance of histone 4 (4 PCR cycles,

data not shown) in the enriched library, indicating that the subtraction method was successful.

A total of 1487 clones were isolated from the forward subtracted library. The insert sizes ranged from 200 to 1,300 bp. These clones were candidates for transcripts specifically up-regulated in infected leaves of velvetleaf. A total of 1,152 clones (77%), containing single inserts, were spotted and immobilized in triplicate on microarray glass slides and differentially screened with combinations of SSH generated probes.

6.4.2. Differential screening

Enrichment ratios (ER1 and ER2) were calculated for each clone on the array and plotted against each other (Fig. 6.2). Positive ER1 and ER2 values indicate transcript enrichment during SSH relative to their levels in the unsubtracted driver (UD) or UT unsubtracted tester (UT), respectively (Fig. 6.1B). Compilation of the microarray data generated ER1, ER2 values and UT/UD ratios (Fig. 6.2, Table 6.2) confirmed that 143 clones (12%) were up-regulated in the tester (ER1>ER2) compared to the driver, while the rest of the clones had escaped subtraction (ER1<ER2), being as abundant in the driver as in the tester. Among the up-regulated candidates, several clones (72 in total) had negative ER2 values suggesting they correspond to abundant transcripts, while 71 clones had positive ER2 values suggesting they correspond to rare transcripts that had been enriched in the SSH library (Fig. 6.2, Table 6.2).

6.4.3. Sequence analysis and annotation of expressed sequence tags

One hundred and thirty nine clones representing genes preferentially expressed in the velvetleaf-*C. coccodes* interaction were successfully sequenced and screened for putative functions using different blast analyses targeting either nucleic or amino acid sequences from plants, fungi as well as a wide range of other organisms. Putative functions were assigned for 94 sequences (68%) (Fig. 6.3A, Table 6.2), while 11 clone sequences (8%) had high similarity scores in the databases but no associated known function. Another 34 sequences (24%) showed either no significant homology with sequences (17%) in the public databases or no hit (7%), and thus were considered novel ESTs (Table 6.2, Fig. 6.3A). In some instances where the analysis suggested multiple but possibly distinct functional roles as in the case of clones 105 and 1244 (similar to elongation factor 1 alpha and vitronectin adhesion proteins), and 1477 (similar to pathogenicity-related protein 4A, CBP20 precursor and hevein-like precursor proteins), the functional multiplicity was kept and is presented as such in Table 6.2. The 94 clones for which function was identified were categorized into functional classes (transcription, energy, signaling, cell growth and maintenance, and oxidative stress and defense). Genes related to oxidative stress and defense formed the largest category corresponding to 60% of ESTs (Fig. 6.3B).

Among the oxidative stress and defense related genes, 79% were identified as metallothioneins (44/56). Other proteins allocated to the same functional category included ascorbate peroxidase (clone 180) and reticuline oxidase (clone 538), suspected to be involved in alkaloid compound formation. A major latex protein homologue was identified that contains a domain for pathogenesis related-

protein (Bet v I family), as well as RD22- (clone 177) and B12-D- (clone 1353) like proteins which are known to be associated with plant dehydration and senescence responses, respectively (Table 6.2).

We also identified several clones (12 ESTs), which are likely to play roles in transcription (13%). Among these clones, five clones encoding ethylene response element binding (EREB) protein and two clones encoding the WRKY and bZIP proteins, respectively, were found (Table 6.2). EREB and WRKY have been previously shown to be involved in the biotic and abiotic stress ethylene response pathway, in which we also identified the ethylene synthesizing enzyme (ACC oxidase, clone 1254). Sixteen percent of the clones with associated function are predicted to play roles in cell growth and maintenance including mitochondrial ATP synthase, polyubiquitin as well as several ribosomal proteins that were undetected by the ribosomal probes used in the microarray screening. The remaining sequences are distributed between signaling (2%) and energy related proteins (2%). All the transcriptionally enhanced clones, whose functions had been identified, originated from velvetleaf based on the percentage and length of the similarity found, except for three clones. NCBI Blast search tool highlighted the histone 4 gene (clone 1138) while searches against the Phytopathogenic Fungi and Oomycete EST Database identified an ABC transporter (clone 751) and a malonyl-coA carboxylase (clone 1236) (Table 6.2).

One of the most frequently encountered genes in our library was the plant metallothionein type 3 (44 clones) for which we confirmed the presence of two conserved cysteine-rich domains and characteristic motifs associated with this plant gene by virtual translation. Nucleic acid and predicted amino acid sequence

alignments of the metallothionein ESTs for which complete coding regions were available revealed some sequence diversity unlikely to have solely arisen from potential sequencing errors (data not shown). The cladogram (Fig. 6.4) was established based on the alignment of 21 metallothionein ORF non-redundant nucleic sequences. It shows the metallothionein ESTs recovered in the up-regulated velvetleaf-*C. coccodes* interaction library cluster in three main clades (Fig. 6.4).

6.4.4. Validation and QRT-PCR expression analysis of selected clones

To validate conclusions drawn from the ER1 and ER2 comparisons and to confirm that the isolated clones are truly corresponding to differentially expressed transcripts, quantitative real-time PCR (QRT-PCR) was performed on tester and driver cDNA pools for seven selected genes encoding metallothionein type 3, EREB, WRKY and bZIP transcription factors, reticuline oxidase, ascorbate peroxidase and ACC oxidase. All of the QRT-PCR-ratios (Table 6.3) corroborated with UT/UD ratios (Table 6.2) (i.e., clones having UT/UD >1 gave QRT-PCR ratios >1) confirming up-regulation of the transcripts during the pathogenic interaction. The highest expression ratio (2.38) was obtained for the reticuline oxidase gene while the bZIP transcription factor encoding gene had the lowest change in transcript abundance (1.06) (Table 6.3). Because the SSH library was designed to take advantage of the sequence similarity between two evolutionary related *Malvaceaous* plants, velvetleaf and okra, it was important to investigate the abundance of each of the seven selected clones on cDNA from infected

velvetleaf and mock treated velvetleaf. This allowed us to rule out any potential interference of velvetleaf-okra gene relatedness in the QRT-PCR analysis, and validate the clones on true biological replications. Expression ratio levels examined over two sequential time points confirmed that the seven selected plant clones were induced in response to *C. coccodes* as compared to mock-treated velvetleaf leaves (Table 6.3). Depending on the gene and the time point, substantial and significant ($P < 0.05$) fold increases were observed. At 12 h of infection, expression levels of the WRKY transcription factor was the highest (7-fold), while at 24 h of infection, reticuline oxidase was up-regulated by 16-fold (Table 6.3).

Table 6.1. PCR primer characteristics.

Target	Primer	Size (bp)	Sequence (5'- 3')	*Tm* (°C)[a]	Amplicon size (bp)
Plant rRNA small sub-unit[b]	SS23[c]	20	TTGGTTGATCCTGCCAGTAG	60	~1700
	1769R[d]	19	CACCTACGGAAACCTTGTT	56	
Plant rRNA large sub-unit part 1[b]	N-NC26S1[e]	17	CGACCCCAGGTCAGGCG	60	~1600
	1499R[e]	20	ACCCATGTGCAAGTGCCGTT	62	
Plant rRNA large sub-unit part 2[b]	N-NC26S8[e]	18	ACGTTAGGAAGTCCGGAG	56	~1800
	3331R[e]	20	ATCTCAGTGGATCGTGGCAG	62	
Internal Transcribed Spacers 1 and 2[bf]	ITS4[g]	20	TCCTCCGCTTATTGATATGC	58	~ 600 to 800
	ITS5[g]	22	GGAAGTAAAAGTCGTAACAAGG	63	
Fungal rRNA small sub-unit[f]	NS1[h]	19	GTAGTCATATGCTTGTCTC	54	~1800
	NS8[h]	20	TCCGCAGGTTCACCTACGGA	64	
Fungal rRNA large sub-unit part 1[f]	LR0R[h]	17	ACCCGCTGAACTTAAGC	52	~ 1400
	LR7[h]	17	TACTACCACCAAGATCT	48	
Fungal rRNA large sub-unit part 2[cf]	LR7R[h]	19	GCAGATCTTGGTGGTAG	52	~ 1600
	LR12[h]	17	GACTTAGAGGCGTTCAG	52	
Metallothionein type 3[b]	AtMt3F	19	GTGCGTGAAGCAAGGAAAC	58	106
	AtMt3R	20	TCCACACTTGCAATCGTTCT	58	
EREB transcription factor[b]	AtErebF	20	GTGACGCAGTATCAGCAACA	60	113
	AtErebR	22	TGAGGAAGACAACACACAAACA	62	
WRKY transcription factor[b]	AtWrkyF	20	ATCGACCTGCAACGATCATA	58	118
	AtWrkyR	20	TCTGCCTTTCTTGTGCCTTT	58	
bZIP transcription factor[b]	AtbZIPF2	19	GTTGGCTGAGAGATGCTTG	58	129
	AtbZIPR2	20	ATCACCACCAATTCCATCGT	58	
Reticuline oxidase[b]	AtRoxF	21	GATTCGAAATCTCAGGTGGAA	60	100
	AtRoxR	22	GGAACAGTAGATTGTGGCTTGA	64	
Ascorbate Peroxidase[b]	AtAPoxF	27	TTCTATTTCATTCATTTCACACACTCT	70	104
	AtAPoxR	23	TTTTCAACTGCCTTCTGGTATTC	64	
ACC oxidase[b]	AtACCoxF	21	AATTGGGGCTTCTTTGAGGTA	60	102
	AtACCoxR	20	TTGCTCCATGCATTTCTTGT	56	
Actin[b]	VvactF	18	AATGGCCGATGGTGAGGA	56	159

	VvactR	20	TCCTTCTGACCCATCCCAAC	62	
Histone 4[f]	H4-1a[i]	18	GCTATCCGCCGTCTCGCT	60	200
	H4-1b[i]	18	GGTACGGCCCTGGCGCTT	62	

[a] *T*m is provided as *T*m = 2x(A+T)+4x (C+G)
[b] Velvetleaf origin
[c] Nickrent, 2000.
[d] Nickrent *et al.*, 1994.
[e] Kuzoff *et al.*, 1998.
[f] *C. coccodes* origin
[g] White *et al.*, 1990.
[h] Vilgalys, 1996.
[i] Glass *et al.*, 1995.

Table 6.2. Differentially expressed cDNAs detected in the microarray differential screening.

Genbank ID	Clone ID	Putative protein name	Hit Genbank ID	Hit organism name	nE[a] value	ER1[b]	ER2[c]	UT/UD ratio[d]
FUNCTION INFERRED								
Transcription								
DV767725	757	Ras releted GTP binding protein	Q40570	*Nicotiana tabacum*	32	-0.5	-0.8	1.2
DV767726	537	Protein kinase	AAK01950	*Arabidopsis thaliana*	10	0.4	0.1	1.2
DV767727	1291	Histone deacetylase	AAQ24532	*Solanum chacoense*	11	1.0	0.8	1.1
DV767729	31	Ethylene Responsive Element Binding (EREB) protein	AAX68526	*Gossypium hirsutum*	29	0.9	0.2	1.7
DV767730	57	Ethylene Responsive Element Binding (EREB) protein	AAX68526	*Gossypium hirsutum*	12	0.7	0.2	1.5
DV767728	656	Ethylene Responsive Element Binding (EREB) protein	AAX68526	*Gossypium hirsutum*	6	0.5	0.2	1.2
DV767732	867	Ethylene Responsive Element Binding (EREB) protein	AAX07460	*Gossypium hirsutum*	29	0.8	0.2	1.5
DV767731	962	Ethylene Responsive Element Binding (EREB) protein	AAX68526	*Gossypium hirsutum*	15	0.4	0.3	1.0
DV767733	1376	WRKY transcription factor	DAA05072	*Oryza sativa*	33	0.1	0.0	1.1
DV767734	681	WRKY transcription factor	DAA05072	*Oryza sativa*	33	0.2	0.2	1.1
DV767735	1436	bZIP Transcription factor	AAG25728	*Arabidopsis thaliana*	17	0.9	0.4	1.4
DV767736	1317	bZip Transcription factor	AAG25728	*Arabidopsis thaliana*	17	0.8	0.7	1.1
Energy								
DV767737	849	Photosystem II binding protein	NP_054492	*Nicotiana tabacum*	100	0.7	0.3	1.4
DV970106	926	Photosystem I sub-unit N	AAO49652	*Phaseolus vulgaris*	38	0.1	-0.4	1.5

Signalling								
DV767738	1254	1-aminocyclopropane-1-carboxylate (ACC) oxidase	BAA94601	*Populus euramericana*	100	0.5	0.4	1.1
DV767739	776	Zinc finger protein	AAQ84334	*Oryza sativa*	30	0.5	0.4	1.1
		Putative multiple stress-responsive zinc-finger protein	BAD35521	*Oryza sativa*	30			
		Pathogenesis-related protein-like protein	AAO72541	*Oryza sativa*	11			
Cell growth and maintenance								
DV767793	1236	Malonyl-coA carboxylase	BG809651	*Magnaporthe grisea*	15	1.1	0.7	1.3
DV767740	105	Elongation factor EF1 alpha	CAD60652	*Solanum tuberosum*	57	-0.3	-0.3	1.0
		Vitronectin-like adhesion protein	AAA20836	*Nicotiana tabacum*	57			
DV767741	1244	Elongation factor EF1 alpha	AAF42976	*Zea mays*	85	0.2	0.1	1.1
		Vitronectin-like adhesion protein	AAA20836	*Nicotiana tabacum*	84			
DV767742	618	F1 ATPase subunit alpha	BAA32243	*Beta vulgaris*	15	0.6	0.2	1.3
DV970108	619	F1 ATPase subunit alpha	BAA32243	*Beta vulgaris*	15	0.9	0.2	1.6
DV767743	1138	Histone 4	EAA73824	*Gibberella zeae*	22	1.0	0.0	1.9
DV767744	1173	Polyubiquitin	T48345	*Arabidopsis thaliana*	100	-0.1	-0.7	1.5
DV970115	1218	Polyubiquitin	1604470A	*Zea mays*	100	0.1	-0.5	1.6
DV767745	1415	26S proteasome ATPase subunit RPT5a	AAF22525	*Arabidopsis thaliana*	79	0.8	0.3	1.4
DQ296478	1229	Ribosomal L2	1211235BW	*Nicotiana tabacum*	61	1.8	0.2	3.0
DQ296475	661	Ribosomal S30	BAD36047	*Oryza sativa*	23	0.8	0.1	1.7
DQ296479	1480	Ribosomal S30	BAD36047	*Oryza sativa*	23	0.3	0.1	1.1
DQ296480	1481	Ribosomal S30	BAD36047	*Oryza sativa*	23	0.3	0.0	1.2
DQ296477	1181	Ribosomal S16	CAA53567	*Gossypium hirsutum*	14	0.3	0.2	1.1
DQ296476	787	Ribosomal L24	BAD82702	*Oryza sativa*	20	0.0	0.0	1.0

Oxidative stress and defense								
DV767807[e] to DV767846 and DV970112 to DV970114		Metallothionein-like protein	AAW47577	*Gossypium hirsutum*	n.a.[f]	0.6[g]	-0.1[g]	1.7[g]
DV767754	538	Reticuline oxidase (berberine bridge)	AAM98220	*Arabidopsis thaliana*	21	1.0	-0.1	2.1
DV767753	539	Reticuline oxidase (berberine bridge)	AAM98220	*Arabidopsis thaliana*	42	1.4	-0.2	3.2
DV767770	886	Similar to major latex-related protein (domain for pathogenesis related-protein Bet v I family)	CAC83580	*Arabidopsis thaliana*	33	-0.5	-0.9	1.3
DV767746	180	Ascorbate peroxidase (Plant heme peroxidase family (PEROXIDASE_4). active site signature (PEROXIDASE_2).and proximal heme-ligand (PEROXIDASE_1) domains)	AAL08496	*Hordeum vulgare*	100	1.9	-0.2	4.3
DV767747	900	P450 monooxygenase	T02955	*Zea mays*	27	1.6	0.8	1.7
DV767748	628	P450 monooxygenase	T02955	*Zea mays*	23	2.2	0.9	2.6
DV767749	605	P450 monooxygenase	T02955	*Zea mays*	24	2.0	0.7	2.4
DV767750	1282	P450 monooxygenase	T02955	*Zea mays*	23	1.2	0.7	1.5
DV767751	1477	PR protein 4A	S23799	*Nicotiana tabacum*	50	1.0	0.1	1.9
		Pathogen- and wound-inducible antifungal protein CBP20 precursor	AAB29959	*Nicotiana tabacum*	50			
		Hevein-like protein precursor	AAN15365	*Arabidopsis thaliana*	44			
		Chitin-binding protein N	AH003967	*Hordeum vulgare*	6			

DV767752	177	Dehydration-induced protein RD22-like	AAL67991	*Gossypium hirsutum*	62	1.1	0.3	1.7
DV767759	209	Class III chitinase	AAD27874	*Sphenostylis stenocarpa*	6	-0.4	-0.5	1.1
DV970109	1353	Senescence associated protein B12D-like	AAL91215	*Arabidopsis thaliana*	32	0.5	0.2	1.2
Others								
DV767755	1128	Short chain alcohol dehydrogenase (domain for short-chain dehydrogenases/reductases)	T02257	*Nicotiana tabacum*	29	1.7	1.1	1.5
DV767756	1364	IgE-dependent histamine-releasing factor	A38958	*Oryza sativa*	38	0.3	-0.1	1.3
		Translationally controlled tumor protein homolog	P35681	*Oryza sativa*	38			
DV767757	727	14/3/3 protein	BAD10938	*Nicotiana tabacum*	66	-0.1	-0.5	1.3
DV970107	1038	Selenium binding protein	CAC67501	*Medicago sativa*	26	0.1	-0.2	1.2
DV767760	15	Auxin-induced (aldo/keto reductase family) like protein	AAB71960	*Arabidopsis thaliana*	23	0.5	-0.1	1.5
		L-galactose dehydrogenase (L-GalDH)	NP_195093	*Arabidopsis thaliana*	8			
DV767761	58	Auxin induced aldo keto reductase	AAD31332	*Arabidopsis thaliana*	21	0.4	-0.2	1.5
DV767801	751	ATP-binding cassette (ABC transporter)	AJ487848	*Phytophthora infestans*	5	1.1	-0.1	2.4
UNKNOWN FUNCTION								
DV767762	143	ATFP4-like	BAA98186	*Arabidopsis thaliana*	15	1.7	1.0	1.7
		Heavy-metal-associated domain-containing protein	NP_568695	*Arabidopsis thaliana*	15			
DV970110	1368	Unknown	BAD83437	*Nicotiana tabacum*	22	1.2	-0.1	2.4
DV767763	1259	Unknown	AAM62544	*Arabidopsis thaliana*	5	0.3	0.0	1.2

		Flavanone 3-hydroxylase-like protein	AAM62620	*Arabidopsis thaliana*	1			
		Oxidoreductase. 2OG-Fe(II) oxygenase family protein	NP_197841	*Arabidopsis thaliana*	1			
DV767764	798	Unknown	BAD83437	*Nicotiana tabacum*	13	0.4	-0.1	1.4
DV767765	977	Unknown	BAD83437	*Nicotiana tabacum*	31	1.0	-0.2	2.3
		Unknown. NitaMp027-like	YP_173374	*Nicotiana tabacum*	31			
DV767766	1199	Copper chaperone	AY603358	*Populus alba* x *Populus tremula* var. *glandulosa*	4	0.1	-0.2	1.2
		Putative pentatricopeptide (PPR) repeat-containing protein	BAC80084	*Oryza sativa*	0			
DV767767	1300	Ankyrin repeat family protein	NP_200273	*Arabidopsis thaliana*	23	1.0	0.1	1.8
DV767768	1344	Unknown	AAM67347	*Arabidopsis thaliana*	20	0.0	-0.1	1.1
DV767769	1170	Hypothetical protein	BAD83437	*Nicotiana tabacum*	39	1.0	0.2	1.8
		M030_ARATH Hypothetical mitochondrial protein AtMg00030 (ORF107a)	P93276	*Arabidopsis thaliana*	29			
DV767771	1435	Unknown protein; 9323-8826	AAG52577	*Arabidopsis thaliana*	21	0.5	0.3	1.1
		Paired amphipathic helix repeat-containing protein	NP_177163	*Arabidopsis thaliana*	0			
		Disease resistance-like protein	AAZ30328	*Populus tremula*	0			
DV767772	951	Unknown protein; 9323-8826	AAG52577	*Arabidopsis thaliana*	16	0.2	0.1	1.1
NO SIGNIFICANT HOMOLOGY								
DV767758	1194	ADP-ribosylation factor-like protein	T48640	*Arabidopsis thaliana*	2	0.3	0.2	1.1
DV767773	543	Hypothetical protein	AAL16159	*Arabidopsis thaliana*	0	1.1	0.3	1.7
DV767774	1193	Hypothetical protein	AAL16159	*Arabidopsis thaliana*	0	0.4	0.2	1.2
DV767775	1162	Hypothetical protein	AAU93579	*Solanum demissum*	1	-0.1	-0.1	1.0
		Putative PPR-repeat protein	AAP54989	*Oryza sativa*	0			

DV767776	689	Hypothetical protein	AAT69623	*Oryza sativa*	0	0.9	0.2	1.6
DV767777	1070	Hypothetical protein	AAT73683	*Oryza sativa*	0	1.3	0.1	2.3
DV767778	1299	Hypothetical protein	AAP53814	*Oryza sativa*	0	1.3	0.0	2.5
DV970111	749	Hypothetical protein	AAT73683	*Oryza sativa*	0	1.3	0.0	2.5
DV767779	666	Hypothetical protein	AAP53814	*Oryza sativa*	0	1.5	0.0	2.7
DV767780	1196	Unknown protein	AAK97731	*Arabidopsis thaliana*	1	0.6	0.2	1.3
		Putative CLAVATA3/ESR-related 44 precursor	AAT36743	*Arabidopsis thaliana*	1			
DV767781	113	Hypothetical protein	AAT69623	*Oryza sativa*	0	1.5	-0.2	3.4
DV767782	499	Hypothetical protein	AAP53814	*Oryza sativa*	0	2.2	-0.3	5.6
DV767783	630	Ubiquitin carboxyl-terminal hydrolase-related	NP_201001	*Arabidopsis thaliana*	0	1.1	-0.2	2.4
DV767784	1114	Repetitive proline-rich cell wall protein 1 precursor	P08012	*Glycine max*	0	1.8	-0.2	3.8
DV767785	631	Hypothetical protein	AAO39858	*Oryza sativa*	0	1.6	-0.1	3.3
DV767786	1277	Bacterial spot disease resistance protein 4	AAR21295	*Lycopersicon esculentum*	0	0.3	0.1	1.2
		Bacterial spot disease resistance protein 4	AAR21295	*Lycopersicon esculentum*	0			
DV767787	1002	AC020646_15 T32E20.31	AAF79792	*Arabidopsis thaliana*	0	0.3	0.1	1.2
DV767788	1117	Carbamoyl-phosphate synthetase III	CD040515	*Phytophthora sojae*	2	0.3	0.1	1.1
		Helicase domain-containing protein	NP_176103	*Arabidopsis thaliana*	0			
DV767789	215	WRKY transcription factor 27	AAL13041	*Arabidopsis thaliana*	0	-0.7	-1.2	1.5
DV767790	530	Putative latex protein allergen	BAD09208	*Oryza sativa*	0	0.4	0.2	1.1
DV767791	1200	Hypothetical protein	AAC62876	*Arabidopsis thaliana*	0	0.1	0.0	1.1
		GLR35_ARATH Glutamate receptor 3.5 precursor (Ligand-gated ion channel 3.5) (Ionotropic	Q9SW97	*Arabidopsis thaliana*	0			

		glutamate receptor GLR6)						
		Putative ligand-gated ion channel subunit	AAC69939	*Arabidopsis thaliana*	0			
		Glutamate receptor family protein	NP_565743	*Arabidopsis thaliana*	0			
DV767792	261	Putative latex protein allergen	BAD09208	*Oryza sativa*	1	0.4	0.0	1.3
DV767794	881	Unknown protein	XP_479403	*Oryza sativa*	0	0.5	-0.3	1.7
DV767795	864	Hypotherical protein	78708711	*Oryza sativa*	0	1.5	0.1	2.7
NO HIT IN DATABASES								
DV767796	1205	---				0.5	0.2	1.2
DV767797	967	---				0.6	0.1	1.4
DV767798	617	---				0.4	0.1	1.2
DV767799	1041	---				0.7	-0.1	1.8
DV767800	587	---				-0.4	-0.4	1.0
DV767802	1393	---				-0.3	-0.5	1.1
DV767803	1154	---				0.4	0.1	1.3
DV767804	1332	---				1.0	0.4	1.5
DV767805	1129	---				0.2	0.0	1.2
DV767806	179	---				1.0	-0.2	2.2

[a] normalized E value.

[bc] SSH enrichment ratio 1 and 2 (ER1 and ER2) were calculated from the screening by microarrays as $\log_2$(ST/UD) and $\log_2$(ST/UT), respectively. ST, subtracted tester; UD, unsubtracted driver; UT, unsubtracted tester.

[d] UT/UD ratio = antilog of (ER1-ER2) in the base 2.

[e] Genbank accession numbers of the 44 metallothionein sequences.

[f] n.a. not applicable.

[g] the average of 44 values.

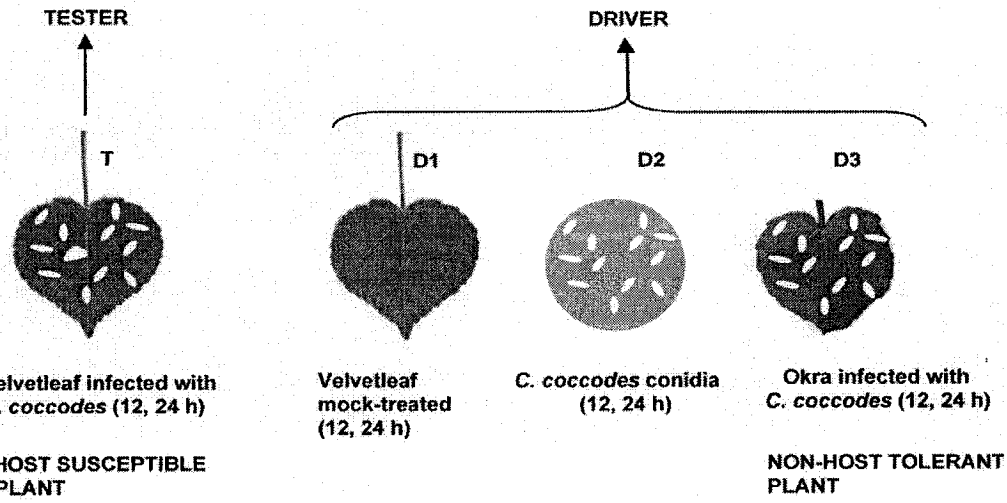
Table 6.3. Gene expression ratios confirmed by quantitative real-time QRT-PCR.

Gene	Putative protein function	Tester/ driver expression ratio	Velvetleaf infected/not infected expression ratio[a]	
			12 h.	24 h.
Metallothionein type 3	Protection against heavy metals and oxidative stress	1.99	1.69 (0.76)	11.22 (3.68)[b]
EREB transcription factor	Ethylene inducible regulation of disease resistance pathways	1.26	6.44 (2.69)[b]	4.93 (1.62)[b]
WRKY transcription factor	DNA binding protein involved in defense and development signalling	1.77	7.19 (2.85)[b]	3.46 (1.13)
bZIP transcription factor	DNA binding protein involved in regulation of transcription	1.06	1.67 (0.74)	9.63 (3.15)[b]
Reticuline oxidase	(S)-reticuline synthesis leading to benzophenanthridine alkaloid compound (phytoalexin) formation	2.38	6.11 (2.61)[b]	16.24 (5.32)[b]
Ascorbate peroxidase	Antioxidant (H_2O_2 reduction)	1.18	2.25 (1.17)[b]	12.73 (4.17)[b]
ACC oxidase	Ethylene synthesis	1.43	0.89 (-0.16)	3.97 (1.30)[b]

[a] Values between brackets are $\log_2$ ratios

[b] Gene is significantly induced by the presence of *C. coccodes* on velvetleaf compared to mock-treated plant (control) ($P < 0.05$).

A.



B.

SSH designation	
ST Subtracted Tester	contains cDNAs unique to the tester population
SD Subtracted Driver	contains cDNAs unique to the driver population
UT Unsubtracted Tester	contains all of the tester cDNAs
UD Unsubtracted Driver	contains all of the driver cDNAs

Figure 6.1. Scheme for the isolation of RNA from infected velvetleaf leaves (tester), uninfected velvetleaf leaves, infected okra leaves and *Colletotrichum coccodes* conidia (driver). (**A**) RNAs were used for the construction of a suppression subtractive hybridization (SSH) cDNA library to isolate ESTs unique to the velvetleaf -*Colletotrichum coccodes* interaction (tester). (**B**) Designation of SSH cDNA populations used in the differential screening analyses.

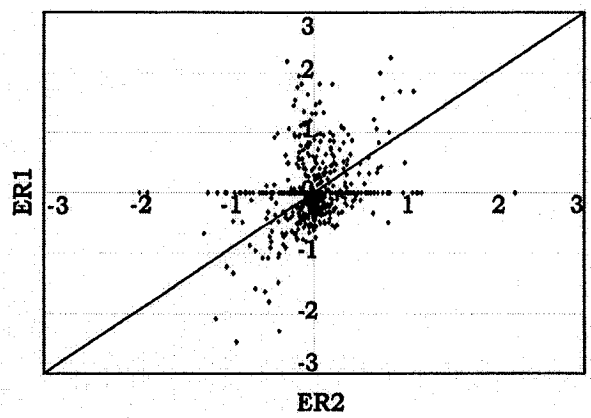


Figure 6.2. Differential screening of velvetleaf-*Colleotrichum coccodes* SSH library by cDNA microarrays. Suppression subtractive hybridization (SSH) scatterplot showing enrichment ratio 1 (ER1) and enrichment ratio 2 (ER2) for each of the clones. ER1 and ER2 were calculated by $\log_2$ transformation of the subtracted tester (ST) median fluorescence divided by the unsubtracted driver (UD) median fluorescence and $\log_2$ transformation of the subtracted tester (ST) median fluorescence divided by the unsubtracted tester (UT) median fluorescence, respectively. Clones lying above the diagonal line correpond to truly up-regulated genes (ER1>ER2) while clones lying below the line escaped subtraction. Positive ER2 values indicate that clones correspond to rare transcripts and have therefore been enriched in the library. Negative ER2 values indicate abundant transcripts which quantity had been normalized in the library.

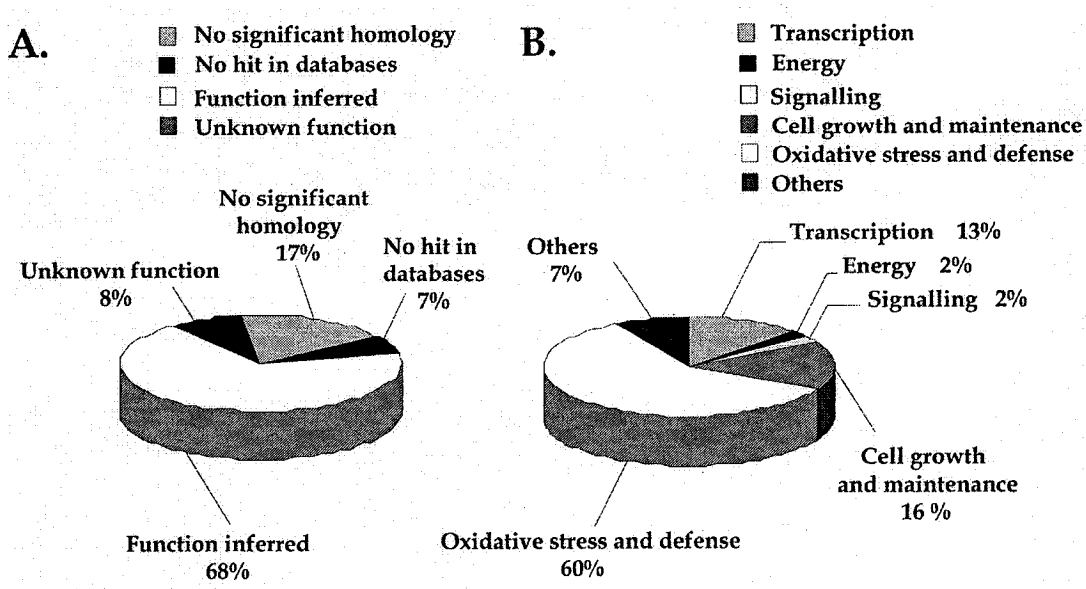


Figure 6.3. Functional distribution of velvetleaf and *Colletotrichum coccodes* up-regulated clones. (**A**) 139 ESTs categorized toward putative identification. (**B**) Putative functional classes for the 94 sequences for which putative function could be inferred. Percentages were calculated from the total 139 up-regulated ESTs (**A**) and from the 94 sequences with assigned functions (**B**).

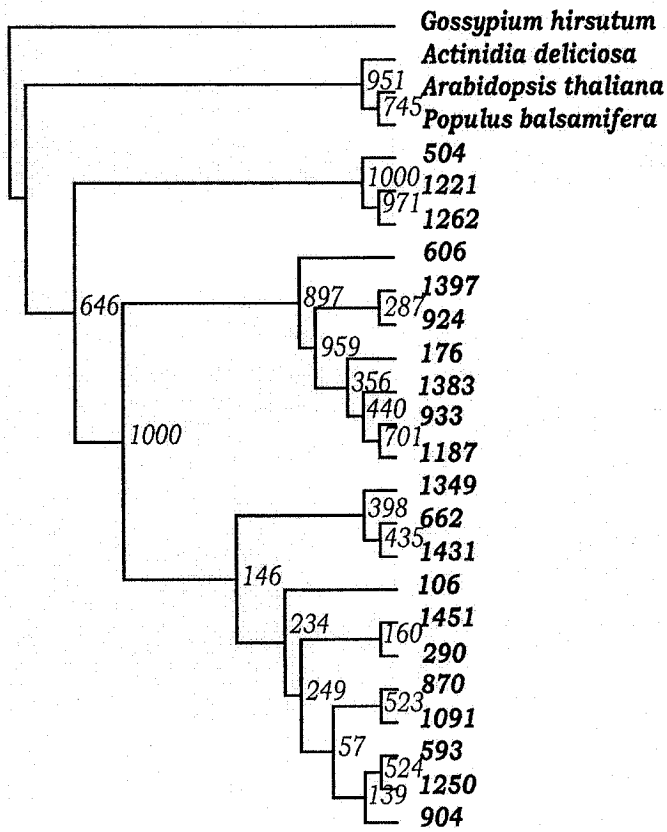


Figure 6.4. Phylogenetic tree based on the analysis of the type 3 metallothionein nucleic (open reading frames) sequences from *Gossypium hirsutum* (AY857933); *Actinidia deliciosa* var. *deliciosa* (L27811); *Arabidopsis thaliana* (NM_112401); *Populus balsamifera* subsp. *trichocarpa x Populus deltoides* (AY594300); and *Abutilon theophrasti* (this study, accession numbers: DV767807 to DV767846 and DV970112 to DV970114; the bold numbers to the right of the tree indicate clone ID). A total of 1,000 bootstrap trees were generated and the number supporting each branch is indicated. The higher the number, the more confident the resolution.

6.5. Discussion

In this study, we report on the isolation of a limited number of ESTs that are up-regulated during early infection of the noxious weed velvetleaf by the biological control agent *C. coccodes*. The SSH strategy was chosen because it (i) allows for the isolation of thousands of genes simultaneously expressed under specific conditions and (ii) includes normalization steps that enrich for low abundance transcripts (Diatchenko *et al.*, 1996; Winstanley, 2002). By careful choice of the driver populations, that not only included velvetleaf and *C. coccodes* cDNAs but also the tolerant okra-*C. coccodes* interaction cDNAs, we concentrated on up-regulated ESTs specific to the susceptible velvetleaf-*C. coccodes* interaction and eliminated those that are induced as a general response to *C. coccodes* in both *Malvaceaous* plants. Traditionally, differentially expressed gene candidates obtained by SSH are further screened by dot blot macro-arrays (Cramer and Lawrence, 2004), colony blot hybridization (Mahalingam *et al.*, 2003) or cDNA-AFLP (Birch *et al.*, 1999). However in this study, we opted to screen the differentially enriched cDNA library using cDNA microarray technology, the most widely and currently used source for functional analysis in biological sciences (Aharoni and Vorst, 2002; Kazan *et al.*, 2001; Wan *et al.*, 2002). Enrichment ratios (ER1 and ER2) calculated with the formulae of van den Berg et al. (van den Berg *et al.*, 2004), allowed us to identify and rule out clones that were not derived from up-regulated transcripts and to determine whether transcripts were rare or abundant. However *in silico* prediction of genes using cDNA microarrays is not sufficient for determining significant alterations in gene expression, thus it is essential to combine the cDNA microarray analysis with

existing validation techniques. In this study, we validated the microarray data with real-time quantitative RT-PCR (QRT-PCR) and clearly confirmed the expression profile of a number of significantly up-regulated genes by QRT-PCR quantification of gene abundance in the tester and driver cDNA pools and in velvetleaf leaves at two early *C. coccodes* infection time points. The fact that the selected genes showed substantial induction kinetics that clearly differ from those of the housekeeping gene, i.e., the actin gene of velvetleaf, demonstrates that the selected EST corresponded to infection specific genes. Similar to what others had recently observed, gene expression ratios determined by microarrays generally underestimate those determined by QRT-PCR (Dowd *et al.*, 2004; Luo *et al.*, 2005).

Overall, 12% of the transcripts in our library were identified as up-regulated, which lies in the 5-16% range reported for plant-microbe and plant-inducer interaction studies (Cramer and Lawrence, 2004; Verica *et al.*, 2004). Bioinformatic searches of several databases including the non-redundant GenBank database, local databases built from NCBI plant and fungal sequence data, the COGEME Phytopathogenic Fungi and Oomycete EST Database, the *Magnaporthe grisea* and *Neurospora crassa* Genome Databases, revealed that 76% (105) of the sequences had at least one significant hit in one of the databases. Furthermore, the majority (102 sequences) had a highest similarity in plant databases, while only three ESTs had reasonably good similarities in fungal databases. Not surprisingly, given the lack of available fungal DNA sequences, the remaining 24% of sequences resulted either in non-significant homologies or

in no homology at all. These clones could belong to *C. coccodes* and/or represent novel plant genes with specialized functions expressed during the infection process. The under-representation of fungal genes in our library could be also attributed to the low amounts of *C. coccodes* biomass in the infected tissue compared to the host biomass. This observation is not inconsistent with what others have noted in their enriched libraries of different plant-microbe interaction (Beyer *et al.*, 2001; Bittner-Eddy *et al.*, 2003).

Among the fungal candidate genes, clone 751 was identified as an ATP-binding cassette (ABC) transporter, based on its homology with *Phytophthora infestans* ABC transporter (accession number AJ487848). ABC transporters are postulated to be involved in the extrusion of hydrophobic molecules over various membranes against a concentration gradient (Del Sorbo *et al.*, 2000; Schoonbeek *et al.*, 2002). In plant pathogens, their function has been associated with secretion of pathogenicity and virulence factors (Schoonbeek *et al.*, 2002), or protection against exogenous plant defense compounds (Fleissner *et al.*, 2002; Schoonbeek *et al.*, 2001; Urban *et al.*, 1999). Since transporters are required during infection for the active secretion of antibiotics, host-specific and non-host-specific toxins as well as plant defense compounds, it is likely that the induction of an ABC transporter in response to velvetleaf could play a role in fungal pathogenesis. Efforts are currently underway to evaluate the importance of this gene in velvetleaf-*C. coccodes* interaction.

The majority of genes identified in this study were categorized as genes involved in the plant's oxidative stress and defense. For instance, reticuline oxidase (EST 538 and 539) and ascorbate peroxidase (EST 180) were identified

by homology to *Arabidopsis thaliana* and wheat genes, respectively. Both types of enzymes are components of the reactive oxygen species (ROS)-scavenging and producing pathways of plants (Mittler *et al.*, 2004). In plants, reticuline oxidase is responsible for the formation of hydrogen peroxide (H_2O_2) and (S)-scoulerine, a precursor of benzophenanthridine alkaloids (Dittrich and Kutchan, 1991). On the other hand, the activity of antioxidant responsive enzymes such as ascorbate peroxidase whose role in plant defense had been well documented (Grimaud *et al.*, 2001) could be a response to ROS generated by the oxidative stress effect of infection. As previously reported, ROS play different roles in the response of plants to pathogen attack by both signalling the activation of plant defense response and amplifying the damage (Mittler *et al.*, 2004). Our findings of reticuline oxidase and ascorbate peroxidase in the velvetleaf-*C. coccodes* subtractive library are in agreement with the previously reported dual actions of H_2O_2 (Dat *et al.*, 2000). The fact that the real-time QRT-PCR results clearly indicate an increased expression of reticuline oxidase and ascorbate peroxidase by more than 16- and 12-fold, respectively in velvetleaf leaves relative to the control 24 h. after *C. coccodes* infection, is a strong indication that both play a role in plant defense.

Several genes representing 13% of the up-regulated sequences were predicted to encode a variety of transcription factors including five ESTs for ethylene responsive element binding (EREB) proteins (31, 57, 656, 867, 962), four for WRKY and bZIP proteins (1376, 681,1436 and 1317), one for a Ras-related GTP binding protein (EST 757), and one for a protein kinase (537). Collectively these proteins are linked with plant stress responses, and have been

implicated in defense gene regulation (Eulgem *et al.*, 2000; Singh *et al.*, 2002). Increased transcript levels of WRKY and EREB transcription factors, upon pathogen infection, have been reported in different plant pathogen systems (Eulgem *et al.*, 2000) strongly suggesting a role in the activation of plant defense mechanisms. The fact that our library was enriched with genes encoding transcription factors together with the significant increases in mRNA expression ratios of WRKY, EREB as early as 12 h. and bZIP 24 h. after *C. coccodes* infection strongly indicates that these transcription factors are linked to the stress imposed on velvetleaf by the pathogen attack.

Two percent of the predicted up-regulated genes were involved in signalling processes. Notably, EST 727 matched a 14-3-3 protein from *Nicotiana tabacum*, a protein known to play roles in a variety of cellular processes (Yaffe, 2002) including the regulation of defense pathways as a response to stress (Finnie *et al.*, 1999). Several 14-3-3 proteins have been identified as members or transcription complexes (Lu *et al.*, 1992), and there is growing evidence that they constitute phosphorylation-independent signal transduction systems (Ferl, 2004). Recently, two 14-3-3 protein members were identified as a regulator of salicylic acid signalling (Lu *et al.*, 2003) and a linker in the interaction between R gene product and *Tobacco mosaic virus* elicitor (Konagaya *et al.*, 2004) in *Arabidopsis* and *Nicotiana*, respectively. The implication of our 14-3-3 gene in velvetleaf-*C. coccodes* interaction requires further examination.

By far the largest group of genes (44 clones) categorized under oxidative stress and defense encoded metallothionein type 3 (MTs) proteins with the majority having the best hit in databases against *Gossypium hirsutum*

metallothionein (accession number AAW47577). MTs are heavy metal binding ligands that are ubiquitous in Eukaryotes (Cobbett and Goldsbrough, 2002), however their function in plants is not straightforward. Metallothioneins contain cysteine residues that bind a variety of metals through mercaptide bonds, and their arrangement together with the primary amino acid sequence forms the basis of their classification into four types. MTs type 3 were found in high levels in ripening fleshy fruits, in plants producing non-fleshy fruits, like *Arabidopsis* and in senescing leaves (Cobbett and Goldsbrough, 2002), but there are no reports on the presence of MTs type 3 as a result of pathogen infection. Plant MT gene expression is regulated by different stresses including pathogen attack (Butt *et al.*, 1998; Choi *et al.*, 1996) and symbiotic interaction (Laplaze *et al.*, 2002), however, most of the expressed plant MT genes reported belonged to type 2. This is the first report of an over-expression of an MT type 3 as a response to pathogen attack. Studies in mammalian systems have proposed an antioxidant function for metallothionein through protection of DNA from oxidative damage caused by free radicals (Chubatsu and Meneghini, 1993; Miura *et al.*, 1997). As the attack of fungal pathogens is followed by an important oxidative stress for the plant, the metallothioneins isolated in this study could play a role in scavenging deleterious oxygen radicals. The significance of the number of ESTs as well as the diversity observed in their coding sequences remains unclear. MTs are known to be encoded by a family of genes, often in multiple copies, and the fact that many different sequences were isolated when velvetleaf was challenged with *C. coccodes* suggests that they have important functions. Whether these functions are redundant or divergent will require further investigation.

In conclusion, SSH has allowed us to create a cDNA library enriched in sequences specifically expressed during the interaction between a weed and a mycoherbicide. Future work targeted at gene knock-out experiments will provide more insight into the function of these genes in pathogenicity.

CONNECTING STATEMENT BETWEEN CHAPTERS VI AND VII

This chapter describes the temporal expression analysis of three plant genes identified, in chapter VI, as type 3 metallothionein and bZIP transcription factor, as well as another gene corresponding to a type 2 metallothionein, during the infection of velvetleaf leaves with *C. coccodes* over a period of 14 days. Expression analysis of the genes was performed using quantitative real-time reverse transcription (RT) PCR.

The results of this section are the subject of a manuscript that has been accepted for publication in Phytopathology. I have designed the experimental set-up, conducted all of the experiments, and wrote the manuscript. Professors S. Jabaji-Hare is my supervisor and the corresponding author on this manuscript. She supervised the work, provided funding, technical assistance, valuable suggestions throughout the study, and corrected the manuscript.

CHAPTER VII

Metallothionein and bZIP transcription factor genes from velvetleaf and their differential expression following *Colletotrichum coccodes* infection

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7.1. Abstract

Colletotrichum coccodes is a potential biocontrol agent of velvetleaf (*Abutilon theophrasti*), a noxious weed of corn and soybeans. Previous investigation of the determinants involved in velvetleaf-*C. coccodes* interaction had shed light on particular plant and fungal genes expressed in this pathosystem. Here, we report on the temporal expression patterns of two distinct types (type 2 and 3) of metallothionein (MT) and a bZIP transcription factor genes in velvetleaf leaves following infection with *C. coccodes* using quantitative reverse transcription-polymerase chain reaction (QRT-PCR). Gene expression ratios were significantly upregulated 1 day after inoculation (DAI), time at which velvetleaf leaves appeared symptomless. Depending on the gene, transcript levels as high as 16-fold were attained. Two DAI, bZIP and type 3 MT expression ratios dropped to levels substantially and significantly lower than those estimated for non-infected plants. Necrotic symptoms appeared 5 DAI and increased with time, during which gene expression levels were maintained either below or at levels to those observed in the control. These findings indicate that *C. coccodes* altered the expression of type 2 and 3 MT and bZIP genes. In addition, this is the first report on induction of a type 3 MT in plants in response to a pathogen attack.

7.2. Introduction

Plant metallothioneins (MTs) are small highly conserved cysteine-rich heavy metal binding proteins, typically classified into four categories (types 1-4) based on their amino acid sequence and cysteine residue arrangement (Cobbett and Goldsbrough, 2002). Plant MT gene expression is regulated by various factors including metal ions, developmental stages, symbiotic interaction and various stress responses (Cobbett and Goldsbrough, 2002; Palmiter, 1998). For instance, zinc induced MT gene expression in poplar (Kohler *et al.*, 2004) while copper reduced the levels of tobacco MT (Thomas *et al.*, 2005). MT gene expression was also induced in senescing tissues of *Quercus* (Mir *et al.*, 2004), *Populus* (Bhalerao *et al.*, 2003), *Ipomoea* (Huang *et al.*, 2001) and during actinorhizal symbiosis (Laplaze *et al.*, 2002). Stress such as wounding and virus infection were shown to induce the expression of MT in tobacco (Choi *et al.*, 1996) while differential expression of MT was detected in *Arabidopsis* following infection of compatible and incompatible strains *Peronospora* (Butt *et al.*, 1998). In this respect, plant MTs have been proposed to function as reactive oxygen species (ROS) scavengers (Akashi *et al.*, 2004; Wong *et al.*, 2004) that possibly protect plant DNA from oxidative damage caused by deleterious free radicals liberated during the oxidative burst (Chubatsu and Meneghini, 1993; Miura *et al.*, 1997).

Although the majority of the MT genes expressed belong to type 2 (Cobbett and Goldsbrough, 2002), types 1, 3 and 4 MT genes have also been reported to be involved in various plant processes. For example, transcript accumulation of type 1 MT was shown in various organs, and in response to pathogen attack (Butt *et al.*, 1998). High levels of type 3 MT transcripts were

found in ripening fruits (Clendennen and May, 1997; Cobbett and Goldsbrough, 2002; Reid and Ross, 1997), and in senescing plant leaves (Breeze *et al.*, 2004), while the expression of type 4 MT genes seems to remain confined in maturing seeds (Guo *et al.*, 2003; White and Rivin, 1995; Zhou *et al.*, 2005).

In plants, bZIP (basic region/leucine zipper motif) transcription factors are members of a large family of signaling components that regulate gene expression of several biological processes including pathogen defence (Jakoby *et al.*, 2002). As with metallothioneins, members of the bZIP transcription factor family have been induced in senescing leaves of tobacco (Yang *et al.*, 2002) and *Arabidopsis* (Chen *et al.*, 2000; Lin and Wu, 2004), as well as in plants responding to pathogen attack (Kim and Delaney, 2002; Lee *et al.*, 2002). In addition, bZIP transcription factors have been reported to regulate the expression of at least one type of metallothionein genes in humans (Lee *et al.*, 1987), an observation that has not been made in plants.

Colletotrichum coccodes (strain DAOM 183088) is a host-specific pathogen of velvetleaf (*Abutilon theophrasti* Medik), a notorious broad-leaf weed in soybean and corn cropping systems (Watson *et al.*, 2000). Typically, this strain causes grey-brown foliar lesions on velvetleaf leaves leading to rapid senescence and premature shedding of leaves. Because of its restricted host range, *C. coccodes* DAOM 183088 has been considered a potential mycoherbicide for velvetleaf, and continued research to enhance the weed control strategy has focused on laboratory and field studies targeted at optimization of inoculum production (Yu *et al.*, 1997), mode of application (Hodgson *et al.*, 1988; Wymore and Watson, 1989), efficacy (Ahn *et al.*, 2005a; Amsellem *et al.*, 2002), molecular

detection and quantification methodologies (Dauch *et al.*, 2003; Dauch *et al.*, 2006) and elucidation of the plant's defence mechanism (Ahn *et al.*, 2005b). Despite all of these advances, knowledge on the molecular determinants of virulence and pathogenicity involved in velvetleaf-*C. coccodes* interaction is still lacking.

In an attempt to better understand these determinants that are crucial to the development of a safe mycoherbicide strategy, we initiated a large scale EST study and characterized 139 plant ESTs that were preferentially induced in response to *C. coccodes* infection. Among them, type 3 MT and bZIP transcription factor were identified and their induction in velvetleaf leaves upon *C. coccodes* infection was confirmed (Dauch *et al.* submitted to Molecular Plant Pathology). Based on our findings and the involvement of MT and bZIP transcription factors in plant senescence and stress, the current study was conducted to investigate whether metallothionein encoding genes and bZIP transcription factor respond directly to *C. coccodes* infections. To do so, we monitored the temporal gene expression ratios of type 2 and type 3 metallothionein, and bZIP transcription factor in leaves of velvetleaf infected with *C. coccodes* by quantitative reverse-transcription (QRT)-PCR.

7.3. Materials and methods

7.3.1. Biological material and growth conditions

Colletotrichum coccodes (DAOM 183088) strain, deposited at the Biosystematics Research Institute (Ottawa, ON, Canada), was cultured on potato

dextrose agar (PDA, Becton Dickinson Microbiology Systems, Sparks, MD) for one week at 28°C in the dark. Conidial production was initiated by transferring five agar plugs into 600 ml of modified Richard medium (MRM) (DiTommaso and Watson, 1995) and incubated at 24°C with agitation (200 rpm) for 7 days. The conidia were harvested through filtration and centrifuged at 4°C for 10 min at 6,000 x *g*. The conidial pellet was resuspended and adjusted with ddH_2O to a final concentration of 10^9 conidia/ml, using a hematocytometer.

Velvetleaf (*Abutilon theophrasti* Medik.) seeds, collected from fields located in Ste-Anne de Bellevue (Qc., Canada) were placed in 100 x 15 mm Petri dishes on Whatman papers no.1, moistened with 4 ml of distilled water and pre-germinated in the dark for three days. Two pre-germinated seeds were planted in CONE-TAINERS (Stuewe and Sons, Corvallis, OR) of 164 ml capacity. Prior to use, the CONE-TAINERS were surface sterilized for 45 min in 3% solution of sodium hypochlorite, rinsed with distilled water and filled with moist non-sterile Promix (Premier Tech, Rivière du Loup, QC, Canada). The CONE-TAINERS were placed on racks, randomized and placed in growth chambers with conditions adjusted to 24/18°C for 10/14 h (day/night) with a light intensity of 300□M/m^2. One week after seeding, plants were thinned so as to leave one seedling per Cone-tainer™. Plants were watered and fertilized two weeks after the initial seeding with 20 ml (3g/l) solution of 20:20:20 (N:P:K) until 4 weeks old (5-leaves stage). Spraying of velvetleaf plants was performed in a spray chamber (Research Instrument MFG. Co. Ltd., Guelph, ON, Canada) to ensure uniform coverage of the leaves. The sprayed plants were placed in a dew chamber (90% humidity, 24°C) for 18 h to maximize fungal infection. All plants received a constant rate of

10^9 conidia/ml (50 ml/m^2) of *C. coccodes*, while uninfected plants received ddH_2O (mock treatment). There were 3 replicates per treatment, each replicate consisted of one CONE-TAINER each containing one plant. The 3rd leaf from each plant was excised at the base of the petiole at 5 different time points (1, 2, 5, 7, and 14 days after inoculation (DAI)), immediately flash-frozen in liquid nitrogen (N), and stored at -80°C prior to being processed.

7.3.2. Isolation of genomic DNA and RNA, reverse-transcription and conventional PCR

Genomic DNA (gDNA) from *C. coccodes* was isolated from 20 mg of freeze-dried 12-day-old mycelial mats as previously described (Dauch *et al.*, 2003). Total gDNA from velvetleaf leaves was extracted using the Qiagen DNeasy Plant Mini-Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's protocol except, that all pipetting steps were carried on ice, centrifugation steps at 4°C and an additional washing step was performed. DNA isolated from all biological material were quantitatively and qualitatively estimated by spectrophotometry, and run on 1% agarose gels (1 x TAE) to verify the absence of DNA degradation.

For RNA extraction purposes, leaf samples were ground in liquid N with the addition of 0.5 g NaCl and 0.33 g PVPP/replicate to prevent the mucilaginous material from interfering in the nucleic acid extraction processes. Total fungal and plant RNA were extracted from 100 mg conidial powder and 100 mg leaf tissue, respectively with the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada) with the following modifications: two lilac columns were loaded with 700 □l RLT

buffer re-suspended fungal or plant powder, and their precipitate was pooled in a single pink column for each sample unit. RNA extracts were treated with TURBO DNase (Ambion Inc., Austin, TX) to remove any potential DNA contaminant, and the integrity of the isolated nucleic acids were checked on 1.2% agarose denaturing formaldehyde gels. The absence of DNA contamination in the total RNA extracts was also verified by PCR amplification of total RNA with the VvactF/R primer set (Table 1) which amplifies a segment of the actin gene from velvetleaf. Reverse transcription of 500 ng of DNA-free RNA into cDNA was accomplished with the QuantiTect Rev. Transcription kits (Qiagen, Mississauga, ON, Canada).

Conventional PCR was performed in an Applied Biosystems 9600 (Foster City, CA) as follows: the reaction mix (25 µl) consisted of 2 µl RNA template, 0.2 µM each of the primers, 2.5 µl of 10 x buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.5 U of *Taq* polymerase (Invitrogen, Burlington, ON, Canada) and 200 nM each of dNTP (Invitrogen, Burlington, ON, Canada). Reactions were prepared on ice and cycled for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, and a final extension step at 72°C for 10 min. A positive and a negative control containing 2 µl of velvetleaf gDNA (10 ng/µl) and ddH_2O, respectively, were included in each run. PCR products were resolved on 1% agarose gels (1xTAE) with a Gene Ruler™ 100bp DNA Ladder (Invitrogen, Burlington, ON, Canada), stained with ethidium bromide and pictures were recorded by a gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada).

7.3.3. Primer Design

Primer pair sets for three target genes and one housekeeping gene (HKG) were designed with the software Primer 3 (Rozen and Skaletsky, 2000) and submitted to Nucleotide Blast at NCBI to confirm specificity (Table 1), and were custom synthesized by AlphaDNA (Montréal, QC). The type 2 metallothionein primers (VvMet23F/R) were designed based on the sequences of the type 2 metallothionein gene from *Gossypium hirsutum* (Genbank accession numbers DT051903, DT051624, and AI732023). The primer sets for type 3 metallothionein (AtMt3F/R) and bZIP transcription factor (VvbZIP3F/R) were designed based on ESTs previously identified from a subtractive library constructed from velvetleaf-*C. coccodes* interaction (Genbank accession numbers DV767807 to DV767846, and DV970112 to DV970114 for type 3 metallothionein, and DV767736 for bZIP). The identity of each of the target sequences was inferred by BLAST (Altschul *et al.*, 1997) (blastn and blastx) analysis conducted through the web interface at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) on sequences from Viridiplantae. Type 3 metallothionein and bZIP sequences were identified by sequence similarity to *Gossypium hirsutum* metallothionein (GenBank accession number AAW47577) and *Arabidopsis thaliana* bZIP transcription factor (GenBank accession numbers AAG25727 and AAG25728). Conceptual amino acid sequences were predicted using EXPASy (Expert Protein Analysis System) translate tool (Gasteiger *et al.*, 2003) available at http://ca.expasy.org/tools/dna.html).

The HKG tubulin was amplified with the primer pair Vvtub3F/R that were designed based on a *Gossypium hirsutum* □-tubulin sequence (GenBank accession

number AF487511). Several primer pairs targeting other potential velvetleaf house-keeping genes such as actin, HPPD, and RNA polymerase, were also designed and tested in conventional reverse transcription (RT)-PCR, but found unreliable during quantitative real-time reverse transcription (QRT)-PCR optimization. The actin gene primers, used to check for DNA contamination of RNA extracts, were designed based on *Malva pusilla* actin gene sequence (GenBank accession number AF112538). They amplify a product of 159 bp and 250 bp from velvetleaf cDNA and gDNA, respectively.

All primers were tested on velvetleaf and *C. coccodes* gDNA and cDNA in conventional PCR and (RT)-PCR assays, respectively followed by gel electrophoresis to confirm the plant origin of the amplified products. Melting point analyses were systematically performed at the end of the real-time PCR to confirm the amplification of a unique product for each of the target and housekeeping genes.

7.3.4. Expression analysis by real-time QRT-PCR

Real-time Quantitative Reverse Transcription (QRT)-PCR was performed, for each of the target gene (type 2 MT, type 3 MT, and bZIP) and the HKG (β-tubulin) in a Mx3000p thermocycler (Stratagene, La Jolla, CA). Each real-time PCR reaction mix (final volume of 20 µl) consisted of 10 µl Brilliant SYBR Green master mix (Stratagene), 125 nM of VvMet23F/R (type 2 MT), 200 nM of AtMt3F/R (type 3 MT), 175 nM of VvbZIP3F/R (bZIP transcription factor), or 275 nM of Vvtub3F/R (β-tubulin) primers, 30 nM of ROX reference dye

(Stratagene), and 4 µl of template ($1/20^{th}$ diluted of cDNA from velvetleaf inoculated with *C. coccodes*-or mock-treated plants) or 4 µl ddH_2O (no template control). Thermo cycling was performed with an initial denaturation at 95°C (10 min) followed by 40 to 50 cycles at 95°C (30 s), 58°C (for type 2 MT and tubulin primer sets) or 59°C (for type 3 MT) and bZIP transcription factor primer sets) (15 s), 72°C (30 s) (2.5°C/s). The reading of fluorescence was done at 77°C (for type 3 MT primer set), 78°C (for □-tubulin and bZIP primer sets), or 80°C (for type 2 MT primer set) and lasted 11 s. Finally, a melting curve was generated by programming the thermocycler to reach 95°C (60 s), 55°C (30 s) (2.5°C/s) and 95°C (0 s) (0.1°C/s). Data generated by real-time RT-PCR were estimated using Stratagene analysis software. Each run included a negative control and was repeated two times on two different days. Data were presented as averages of three biological replicates and two technical replicates.

7.3.5. Data analysis

Relative expression of the type 2 MT, type 3 MT, and bZIP genes were normalized against the expression of the housekeeping gene (HKG) tubulin. Relative expression ratios were calculated for the three target genes in velvetleaf inoculated with *C. coccodes* (sample) versus the controls (mock treated) at 1, 2, 5, 7, and 14 DAI using equation [1] (Pfaffl, 2001). E_{target} and E_{ref} represent the PCR amplification efficiencies for target and HKG genes, respectively, and were calculated from equation [2] (Pfaffl 2002, http://www.gene-quantification.info/) in which RnB and RnA are two fluorescence levels taken during the exponential

phase at two crossing points (CP) CPB and CPA. Amplification efficiencies for each gene were first calculated for all individual samples of the run and then averaged for each gene. ΔCP_{target} is the CP deviation of control – sample of each target gene; ΔCP_{ref} is the CP deviation of control – sample of the HKG.

$$Ratio = (E_{target})^{\Delta CP_{target}\,(control\text{-}sample)} / (E_{ref})^{\Delta CP_{ref}\,(control\text{-}sample)} \quad [1]$$

$$E = (RnB/RnA)^{\wedge[\,1\,/\,CPB\,-\,CPA]} \quad [2]$$

The fold change of each target gene were tested for significant difference between treatments using one-way analysis of variance (ANOVA) in the Statistical Analysis System (SAS, 1999). Treatment means comparisons at each time-point were made using the least significant difference (LSD) test at $P = 0.05$ level.

7.4. Results

7.4.1. Metallothionein and bZIP gene sequences from *Abutilon theophrasti*

Metallothionein (MT) type 3 cDNAs contain 180-bp ORFs encoding 60 amino acids with a predicted molecular mass of 6635 Da. Type 3 MT proteins are characterized by four conserved cysteine residues at the N-terminal end, the first three being part of a conserved domain arranged as Cys-Gly-Asn-Cys-Asp-Cys, and six cysteine residues in the C-terminal cysteine-rich domain arranged as Cys-Xaa-Cys (Fig. 1B). By comparison with other plant MT sequences, velvetleaf type 3 MT was found more similar to *Gossypium* (71% identity), another *Malvaceaeous* plant, and to *Actinidia* (59% identity), than to *Oryza*, *Arabidopsis* or poplar type 3 MT sequences.

Despite the fact that the complete ORF of type 2 metallothionein could not be obtained, the predicted amino acid composition shows features that undoubtedly identify the sequence as a type 2 MT. Seven conserved cysteine residues are located in the N-terminal end that begin with the conserved motif MSCCGGNCGCGS, characteristic of this type of MT (Fig. 1A).

The ESTs for bZIP transcription factor (Genbank accession numbers DV767735 and DV767736) are 100% identical to each other and lack the N-terminal coding sequence. Based on the available C-terminal translated sequence, velvetleaf bZIP shares 29 and 46% identity with *Arabidopsis thaliana* BZ02H1 (Genbank accession number AAG25727), and BZ02H2 (Genbank accession number AAG25728) bZIP proteins, respectively (data not shown). In addition, the 118 amino-acid deduced sequence of velvetleaf bZIP transcription factor could contain two casein kinase II phosphorylation sites, which is similar to the bZIP transcription factor proteins of *Arabidopsis*.

7.4.2. Quantification of target and the house-keeping gene transcripts

Using primers specifically developed for each of the three plant target genes and for the house-keeping gene □-tubulin (Table 1), the amplification of velvetleaf cDNA (Fig. 3A, C, E, G) showed single expected amplicons with melting temperatures (Tm) of 81.3, 82.3, 79.6, and 81.7°C, respectively (Fig. 3B, D, F, H) and with an expected putative size of 200, 130, 106, and 120 bp, respectively (Fig. 2). Prior to reverse-transcription of total RNA to cDNA, all RNA samples were treated with RNase-free DNase and tested for the absence of

DNA contamination by PCR using primers targeting the actin gene of velvetleaf (Table 1). Depending on the gene, titers as low as 10 ag of target gene fragment could reproducibly be detected using SYBR Green chemistry (data not shown). Amplification plots were highly reproducible between duplicate samples, and fluorescence data from negative controls containing no templates always remained below the detection threshold (Fig. 3A, C, E, G; data not shown). Primer dimers (Tm = 77 °C) were occasionally detected in the negative control when using type 2 MT primers, although this never occurred in the samples (Fig. 3C, D). Tubulin and bZIP primers also sporadically generated primer dimers (Tm = 74.5°C and 74°C, respectively; Fig. 3B, H) during PCR amplification of samples or control, however measurements of fluorescence was systematically performed at 78°C, a temperature at which potential primers dimers.

7.4.3. Temporal quantification of genes during disease development

Compared to uninfected plants, the expression ratios of the three target genes significantly ($P < 0.05$) changed in response to *C. coccodes* infection (Fig. 4A-C). The expression ratios were significantly ($P < 0.05$) upregulated by 8-fold for type 2 MT, 16-fold for type 3 MT, and 4-fold for bZIP transcription factor one day after inoculation (Fig. 4A-C), a time period at which velvetleaf leaves appeared symptomless (Fig. 4D). Although the leaves still showed no visual symptoms after 2 days of infection (Fig. 4D), the expression ratios of type 3 MT and bZIP transcription factor significantly dropped ($P < 0.05$) by 2- and 6-fold, respectively below the expression ratio of control plants (Fig. 4B-C). Necrotic lesions became visible 5 days after inoculation and increased with time (Fig. 4D),

during which the expression ratios varied depending on the gene. The expression of bZIP-encoding gene consistently remained downregulated 5 days after inoculation and onwards, as compared to uninfected leaves (Fig. 4C). In contrast, the expression ratios of types 2 and 3 MT transcripts showed a similar pattern of induction 5 and 7 days after inoculation followed by a 1.5-fold drop 14 days after inoculation. However, these differences were not significant ($P > 0.05$) compared to the control plants (Fig. 4A, B).

Table 7.1. PCR primer characteristics.

Target gene	Primer	Size (bp)	Sequence (5'-3')	Tm (°C)[a]	Amplicon size (bp)[b]
Type 2 metallothionein	VvMet23F	23	GAGTTCTCAGTTGTCTCGGCAAT	68	130[c]
	VvMet23R	20	GTCTTGCTGTGGTGGAAACT	60	
Type 3 metallothionein	AtMt3F	19	GTGCGTGAAGCAAGGAAAC	58	106
	AtMt3R	20	TCCACACTTGCAATCGTTCT	58	
bZIP transcription factor	VvbZIP3F	20	CTGAGGAGATGCTTGCTGGA	62	120
	VvbZIP3R	20	TGGATGGTAATGGTGGGTGA	60	
β-tubulin	Vvtub3F	20	TCCCAACAACGTGAAATCAA	56	200
	VvTub3R	20	CATCCATTCCTTCCCCTGTA	60	
Actin	VvactF	18	AATGGCCGATGGTGAGGA	56	159 (250[d])
	VvactR	20	TCCTTCTGACCCATCCCAAC	62	

[a] Tm is provided as Tm = 2x(A+T)+4x (C+G).

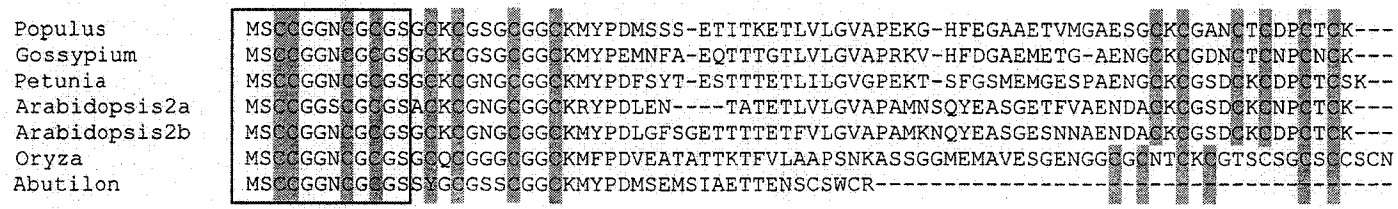
[b] amplicon size on cDNA.

[c] approximate size.

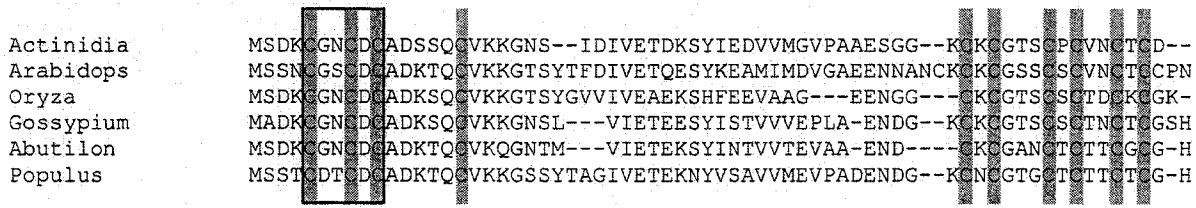
[d] amplicon size on gDNA.

Figure 7.1. Multiple alignment of velvetleaf (*Abutilon theophrasti*) type 2 (**A**) and type 3 (**B**) metallothionein (MT) amino acid sequences with other plant metallothionein amino acid sequences. (**A**) Type 2 MT: *Populus balsamifera* subsp. *trichocarpa* x *Populus deltoides* (GenBank accession number: AAT02524), *Gossypium hirsutum* (DT051624), *Petunia hybrida* (AAG39645), *Arabidopsis thaliana* MT2A (P25860), *Arabidopsis thaliana* MT2B (NP_195858), and *Oryza sativa* (P93433). (**B**) Type 3 MT: *Actinidia deliciosa* (P43389), *Arabidopsis thaliana* (AAB67234), *Oryza sativa* (AAB53811), *Gossypium hirsutum* (AAW47577), *Populus balsamifera* subsp. *trichocarpa* x *Populus deltoids* (AAT02527). Conserved cystein residues are shown in gray boxes. Conserved motifs for each type are boxed with a solid line.

A. Metallothionein type 2



B. Metallothionein type 3



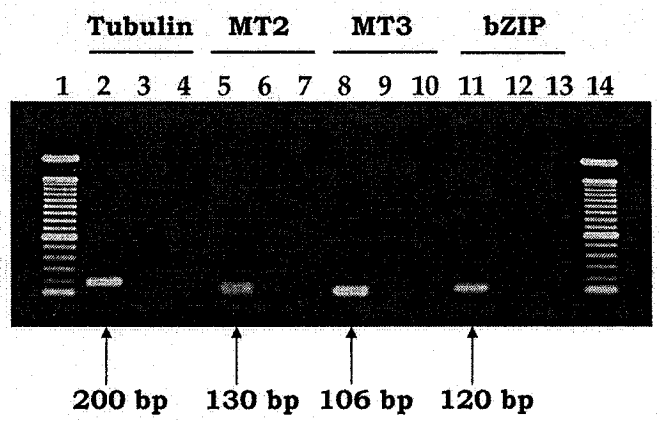
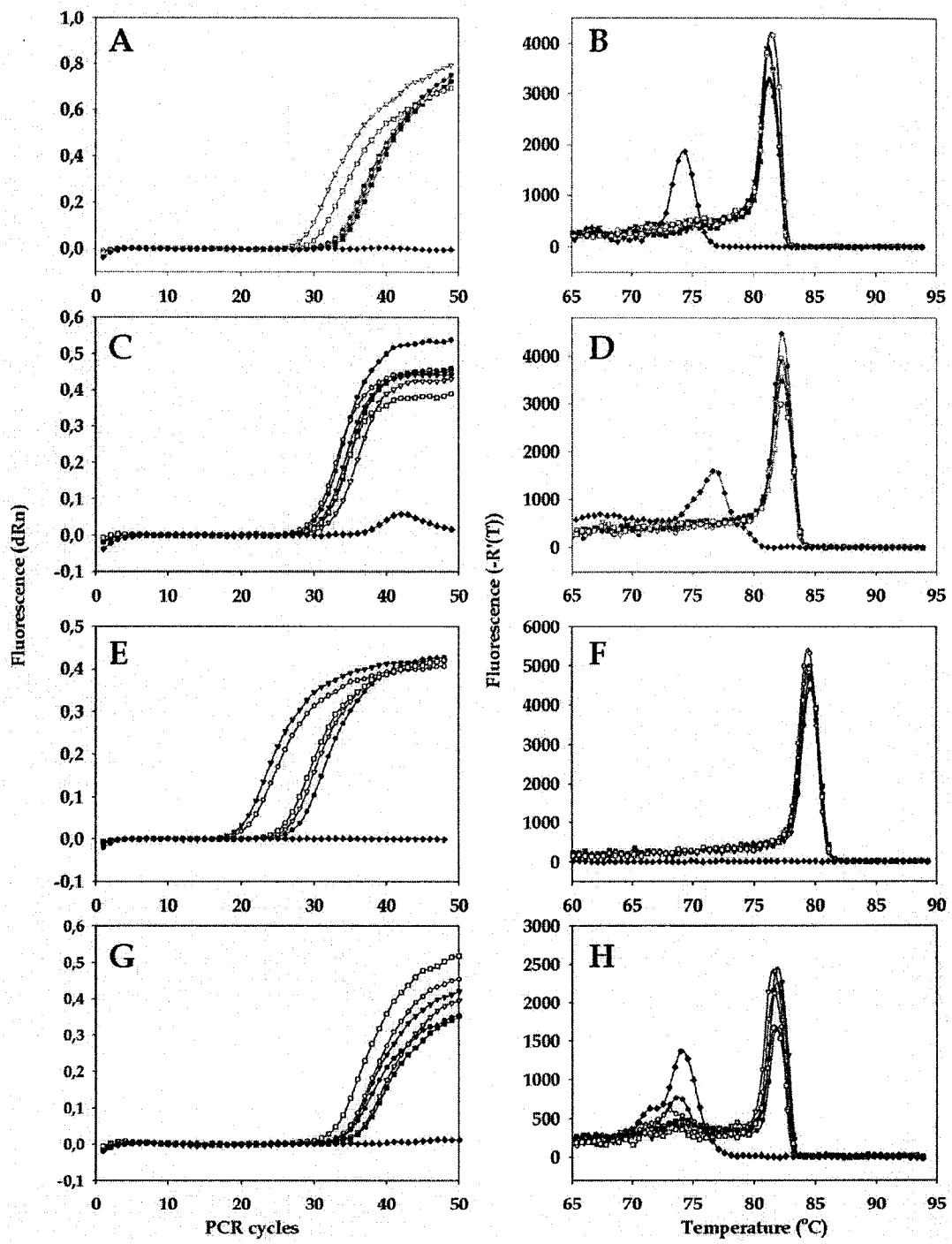


Figure 7.2. Specificity of gene expression analysis by QRT-PCR. Lanes: 2 to 4 = amplification of □-tubulin; lanes 5 to 7 = amplification of type 2 metallothionein; lanes 8 to 10 = amplification of type 3 metallothionein; lanes 11 to13 = amplification of bZIP transcription factor using primers listed in Table 1. Lanes 2, 5, 8, and 11 = velvetleaf cDNA; lanes 3, 6, 9, and 12 = *Colletotrichum coccodes* cDNA; lanes 4, 7, 10, and 13 = negative controls; lanes 1 and 14 = 100-bp ladder.

Figure 7.3. Real-time QRT-PCR quantification example of □-tubulin, metallothionein type 2, type 3, and bZIP transcription factor genes: (**A** and **B**) □-tubulin; (**C** and **D**) type 2 metallothionein; (**E** and **F**) type 3 metallothionein; (**G** and **H**) bZIP transcription factor, using primers listed in Table 1. Amplification (**A**, **C**, **E**, and **G**) and melting peak (**B**, **D**, **F**, and **H**) profiles of selected samples. Symbols: cDNA from velvetleaf leaves harvested two (●), five (○) and fourteen (▼) days after *Colletotrichum coccodes* infection. cDNA from velvetleaf leaves mock-infected and harvested two (▽), five (■) and fourteen (□) days after treatment. Negative control (◆).


A
B
C
D
E
F
G
H
Fluorescence (dRn)
Fluorescence (-R'(T))
PCR cycles
Temperature (°C)

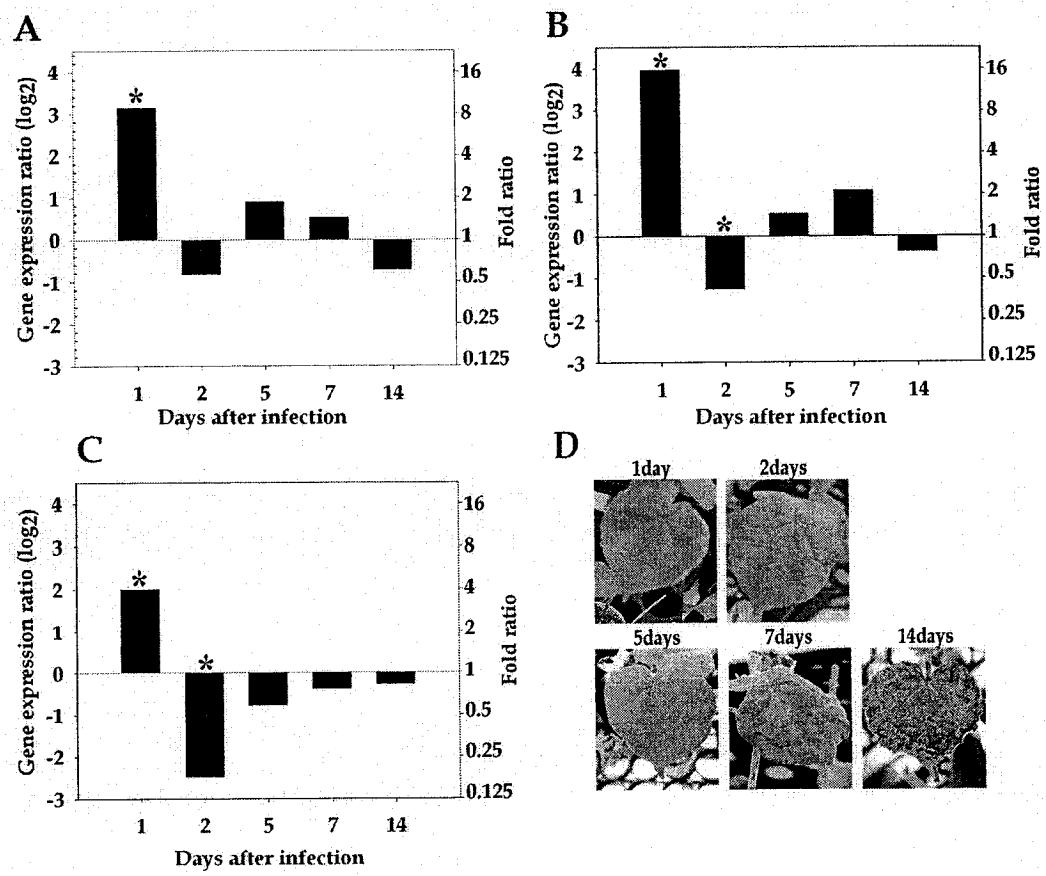


Figure 7.4. Temporal expression of the type 2, type 3 metallothionein, and bZIP transcription factor genes in velvetleaf leaves infected with *Colletotrichum coccodes*. (**A**) type 2 metallothionein; (**B**) type 3 metallothionein; (**C**) bZIP transcription factor. Data obtained by QRT-PCR quantification was normalized to the □-tubulin gene expression. Y-axis represents the $\log_2$ ratio of gene expression from 1 to 14 days after fungal infection. X-axis represents the time points of harvest. * Significant differences of gene expression between *C. coccodes* treated plants and control plants ($P < 0.05$); (**D**) velvetleaf leaves 12 h, 24 h, 2, 5, 7 and 14 days after *C. coccodes* infection.

7.5. Discussion

We studied the temporal gene expression of metallothionein (MT) and bZIP transcription factor during the pathogenic interaction between velvetleaf and the biocontrol agent *C. coccodes*. The choice of these genes was based on a previous expression profiling study using high through-put microarray analyses in which type 3 MT and bZIP transcription factor genes were found preferentially induced in response to *C. coccodes infections* (submitted to Molecular Plant Pathology).

In this study we characterized velvetleaf cDNA encoding two types of MT. This is the first investigation of the occurrence of MTs in velvetleaf in response to pathogen attack. Based on the arrangement of cysteine residues, the deduced polypeptide can be classified as a class II type 2 and type 3 MT-like proteins. Like other plant MT-like proteins, velvetleaf MT proteins are encoded by a small gene family, that are distributed across different chromosomes at least in *Arabidopsis*, *Oryza* and *Lycopersicon* (Cobbett and Goldsbrough, 2002). Although we did not obtain the full-length sequence of velvetleaf bZIP gene, the cDNA sequence was found most similar to *Arabidopsis* bZIP genes that belong to group C of the bZIP superfamily. Protein members of this group have been linked to abiotic stress responses (Weisshaar *et al.*, 1991)and to pathogen attack (Drogelaser *et al.*, 1997).

Several factors that could have affected the accuracy of MT and bZIP gene quantification were carefully controlled when conducting the experiments. (i) The design of gene specific primers was based on velvetleaf and *Gossypium* sequences deposited in the GenBank database. (ii) Statistical validation of the housekeeping

gene □-tubulin whose expression remained constant among treatments ($P < 0.05$). (iii) Individual QRT-PCR efficiencies for target and house-keeping genes were estimated on a sample per sample basis (Pfaffl 2002, http://www.gene-quantification.info/) in order to take into account inherent sample and gene PCR efficiency variations. (iv) The application of a mathematical model combined with statistical analysis that accounts for both differences in amplification efficiencies and threshold cycle numbers (Pfaffl, 2001). .

In this study, substantial upregulation of type 2 MT-encoding genes in response to *C. coccodes* is in agreement with previous studies in which over-expression of plant type 2 MT has been reported in tobacco-virus and potato-*Phytophthora infestans* pathosystems(Choi *et al.*, 1996). Interestingly, our results indicate that significant over expression of velvetleaf type 3 MT transcripts occurred one day after inoculation of velvetleaf leaves by *C. coccodes*. The very high abundance in expression ratio of type 3 MT in this study represents the first report that type 3 MT in plants is inducible in response to pathogen attack. This was highly unexpected because upregulation of plant type 3 MT has so far been observed as part of senescence-related responses in plants (Bhalerao *et al.*, 2003; Breeze *et al.*, 2004).

Disease caused by *C. coccodes* on velvetleaf involves the formation of necrotic senescent-like lesions that begin to appear on the leaves 5 days after inoculation followed by complete senescence after 14 days. The temporal, but differential expression of type 3 and type 2 MT over 14 days of infection might represent a combined response of the plant to pathogen attack and to the process of leaf senescence. In agreement with this observation, Quirino and colleagues

demonstrated that a diverse range of genes, including those linked to the plant's defense response, were expressed during leaf senescence of *Arabidopsis thaliana* (Quirino *et al.*, 1999).

In this study, an important decline in expression ratio of type 3 MT, and to a lesser extent of type 2 MT, was observed two days after inoculation followed by a slight increase at 5 and 7 days after inoculation. Why such decline occurred is hard to explain, but a plausible explanation could be that the plant accumulated large amounts of MT transcripts in response to the pathogen within early stages of infection, and then suppressed and maintained to levels comparable to those expressed in control plants. A similar trend of metallothionein differential gene expression in tobacco plants following wounding or tobacco mosaic virus (TMV) infection over a period of 72 hours was observed (Choi *et al.*, 1996). Relative amounts of metallothionein mRNA were highly induced at 12 h, sharply dropped after 24 hours following wounding and TMV infection, and remained the same thereafter only for the wounding treatment.

Plant response to pathogen attack is known to be well orchestrated by transcription factors including the ethylene-responsive-element-binding factors (EREBF), WRKY and MYB proteins, salicylic acid-inducible proteins, and basic domain leucine zipper (bZIP) proteins (Singh *et al.*, 2002). In this study, the expression levels of velvetleaf bZIP transcription factor followed the same trend as that of type 2 and type 3 MT-like genes during the first 2 days of infection, and remained well below the levels found in uninfected plants. These results clearly suggest that velvetleaf bZIP factors responded specifically and directly to the presence of *C. coccodes*. Preferential induction of bZIP transcription factors by

plant pathogens has been recently reported in pepper plants infected by bacterial and viral pathogens (Lee *et al.*, 2002). Depending on the pathogen, northern blotting experiments showed an upregulation of bZIP transcription factors as early as 3 h in response to pathogen inoculation, then declined 5 days after viral infection (Lee *et al.*, 2002), a trend similar to what we have obtained in this study. Interestingly, in this study, the sensitivity of QRT-PCR technique enabled us to detect low levels of bZIP transcription factor which otherwise would not have appeared if the same data had been analyzed by northern blotting.

In conclusion, this study showed that similar patterns of bZIP transcription factor and metallothionein gene expression in velvetleaf occurred at 24 h (upregulation) and 48 h (downregulation) following fungal infection. After this period and depending on the gene, expression levels were maintained either below or at similar levels observed in the control treatment. Future work should be devoted to identify other genes that are tightly linked to the regulation of metallothionein and bZIP transcription factor in response to *C. coccodes* attack.

CHAPTER VIII

GENERAL CONCLUSION

An important research effort has been devoted to the biological control of the noxious weed velvetleaf (*Abutilon theophrasti*, Medik.) with *Colletotrichum coccodes*. For instance, aspects related to the inoculum production, and modes of application have been well researched in the last twenty years. However, the ability to monitor *C. coccodes* after environmental release and to understand the molecular determinants expressed during velvetleaf-*C. coccodes* interaction have received little attention. Chapters III to VII of this thesis report on these two aspects.

Two sets of strain-specific PCR primers were developed for *C. coccodes* DAOM 183088. The target DNA locus was identified through random amplified polymorphic DNA (RAPD) screening performed on a range of 38 different organisms comprising several *C. coccodes* strains, 15 other species of *Colletotrichum*, a variety of heterogeneous organisms frequently encountered on velvetleaf phylloplane, and various plant species. Analysis of the electrophoretic profiles identified a unique product amplified from *C. coccodes* strain DAOM 183088 only. Cloning and sequencing of this product revealed a non-significant similarity with fungal ribosomal genes suggesting that the fragment was not related to any known DNA sequence. Two new primer sets, N5F/N5R and N5Fi/N5Ri referred to as sequence-characterized amplified region (SCAR) primers, were designed on this sequence for use in PCR detection assays. Strain-specificity of the primers was confirmed by the absence of DNA amplified

products from all other *C. coccodes* strains, *Colletotrichum* species and heterogeneous organisms. The SCAR primer sets N5F/N5R and N5Fi/N5Ri successfully detected *C. coccodes* from infected velvetleaf plants and from soil samples originating from biocontrol field-release experiments, while control plants and natural field soil samples were tested negative for the presence of *C. coccodes*.

Given the sensitivity of real-time PCR technique to soil inhibitory compounds which may lead to biases in the estimation of target organism quantities, chapter IV describes an assay in which biases in PCR efficiency are monitored on a sample per sample basis. The method was based on the construction of an external control (EC) which consists of a DNA molecule introduced at a specific concentration to soil DNA extracts from experimental samples suspected to contain PCR inhibitors. The EC is run in parallel with the target DNA quantification assay and has its own standard curve that corrects for differences in amplification efficiencies between EC and target DNA. All soil samples from deliberate release field trials were found positive for the presence of PCR inhibitors, and with substantial variability in the magnitude of PCR efficiency (from 12 to 82%). The differences in PCR efficiency observed with the EC were then used to estimate target *C. coccodes* DNA quantities in the same samples through normalization for the presence of PCR inhibitory compounds.

Chapter V of this thesis reports on the molecular monitoring of two strains of *Colletotrichum coccodes*, the wild-type (DAOM 183088) and the T-20a, engineered with *NEP1* (necrosis and ethylene inducing peptide) gene to increase the virulence of the strain on velvetleaf. In this chapter, real-time quantitative

PCR (QPCR) was used to assess the extent of colonization of both strains on velvetleaf during the first two weeks of infection. Both *C. coccodes* strains could be detected as soon as the conidia were sprayed on the leaves and up to 14 days after inoculation (DAI). Increased amounts of fungal DNA paralleled the appearance of necrotic lesions on velvetleaf leaves infected with the wild-type strain, but unexpectedly, the wild-type strain was more efficient at infecting velvetleaf than the transgenic T-20a strain. In contrast to what has been published on T-20a, necrotic lesions were scarce on velvetleaf leaves infected with the T-20a strain, an observation that was accompanied by a steady but insignificant increase in T-20a DNA amounts during the first two weeks of infection. Concurrently, quantification of host DNA revealed that the amounts of plant DNA decreases in responses to the wild-type strain infection only. The expression of the T-20a *NEP1* transgene could neither be detected in agar growing mycelium nor in T-20a infected plants, suggesting that the introduced gene may not be transcriptionally active in the transformed strain.

The molecular dialogue occurring during the interaction of the wild-type *C. coccodes* (183088) with velvetleaf was thoroughly studied in Chapter VI. A suppression subtractive hybridization (SSH) cDNA library, in which velvetleaf and *C. coccodes* up-regulated genes expressed 12 and 24 h after infection, was cloned and differentially screened by microarrays. A total of 139 expressed sequence tags (ESTs), representing truly enriched *in planta* up-regulated genes were sequenced of which 91 and 3 were assigned to putative plant and fungal functions. Overall, the transcripts with identified functions were categorized into 5 main functional categories: transcription, energy, signaling, cell growth and

maintenance, and oxidative stress and defense. Eight percent of the ESTs had high similarity scores in the databases but no associated known function, while 24% of the ESTs showed either no significant homology with sequences deposited in the public databases or no hit, and were thus considered as novel ESTs. The largest category of genes associated with known functions belonged to oxidative stress and defense, and included 79% of metallothioneins (MT) type 3 transcripts, which, according to our knowledge, is the first report of a type 3 plant MT induced in response to fungal infection. The expression of seven genes (type 3 metallothionein, EREB, WRKY, and bZIP transcription factors, reticuline oxidase, ascorbate peroxidase, and ACC oxidase) selected from different functional categories, was further validated by real-time quantitative QRT-PCR assays. Relative to uninfected velvetleaf leaves, all seven genes were significantly induced by the presence of *C. coccodes* on velvetleaf leaves.

Chapter VII reports on the temporal induction of velvetleaf genes encoding type 2, type 3 MT and bZIP transcription factor genes in response to pathogen attack. Compared to uninfected plants, substantial accumulation of the three gene transcripts was observed just one day after inoculation (DAI), a time at which the plant appeared symptomless. After 2 DAI, a significant decrease in transcript level was observed for type 3 MT and bZIP transcription factor, while type 2 MT abundance was decreased, although insignificantly. Necrotic symptoms appeared 5 DAI and increased with time, during which gene expression levels were maintained either below or at levels similar to those observed in the control. These findings indicate that *C. coccodes* altered the expression of type 2

and 3 MT and bZIP genes. In addition, the study demonstrates for the first time that type 3 MT is substantially induced due to a biotic stress.

CONTRIBUTIONS TO SCIENCE

The work presented in this thesis advanced scientific awareness in several ways:

1) Chapter III: The PCR primers developed in Chapter III are strain specific. Under greenhouse and field conditions, they successfully detected the mycoherbicide strain *C. coccodes* (DAOM 183088) in plant and soil samples. Because of their specificity, the primer sets have the potential to be used not only in experimental field release trials of the mycoherbicide but also in large-scale epidemiological and risk assessment studies.

2) Chapter IV: A method was developed for the quantification of *C. coccodes* in soil which takes into account the deleterious effects that PCR inhibitors exert on the efficiency of the real-time PCR amplification. The external control enabled the quantitative estimation of PCR inhibitory effects occurring in DNA extracts from 18 different field soil samples and highlighted for the first time the important heterogeneity of soil in that respect. The method can be used as such for the quantification of other microorganisms in soil or can be implemented in a multiplex PCR assay to assess both target DNA quantities and PCR inhibitory effects. It was successfully applied to quantify the mycoherbicide in experimental field plots treated for several years with *C. coccodes*. The method is unique in this field and represents a novel approach that greatly improves precision and accuracy for the quantification of microorganisms by real-time PCR.

3) Chapter V: Molecular monitoring of *C. coccodes* strains on velvetleaf leaves combined with the construction of kinetics growth curves allowed to

establish that *C. coccodes* T-20a strain, genetically transformed with the *NEP1* gene, was not an efficient colonizer of velvetleaf leaves as compared to the wild-type strain. These findings suggest that the *NEP1* transgene had been silenced and raises questions concerning the feasibility and safety of genetically created hypervirulent mycoherbicides. The developed assay not only allowed the detection of *C. coccodes* in symptomless plants, but also represents a novel method for the quantification of *C. coccodes in planta.*

4) Chapter VI: An inventory of genes specifically up-regulated during the pathogenic interaction between velvetleaf and *C. coccodes* was established. Several of these genes had functions similar to genes already reported in other compatible pathosystems, while others were identified as novel genes whose role in disease had so far remained unsuspected. These are certainly novel genes that have not been reported in other pathosystems and therefore potentially offer new challenges for the field of plant microbe interaction. Among the genes whose functions had been identified were the type 3 metallothioneins which so far have never been implicated in plant's defence against pathogens. To the best of our knowledge, this is the first report on the use of microarrays to study weed-mycoherbicide interactions and the library constitutes a first step in the analysis of the molecular determinants of virulence and pathogenicity in velvetleaf-*C. coccodes* interaction that had not been examined so far.

5) Chapter VII: Differential gene expression profiles of velvetleaf metallothionein, and bZIP transcription factor in response to *C. coccodes* infection were established over a period of 14 days after inoculation. The results of this work will increase our knowledge on how velvetleaf counteracts *C.*

coccodes attack at the transcriptional level. Such an approach had not been exploited so far to unravel the ballet of genes during the weed-mycoherbicide interaction.

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APPENDIX 1

Genbank accession numbers generated in the thesis: AF 448480; DQ287984; AY211498; DQ287985; DQ287988; DQ287986; DQ287987; DQ287989; DQ287990; DV767725; DV767726; DV767727; DV767729; DV767728; DV767732; DV767731; DV767733; DV767734; DV767735; DV767736; DV767737; DV970106; DV767738; DV767739; DV767793; DV767740; DV767741; DV767742; DV970108; DV767743; DV767744; DV970115; DV767745; DQ296478; DQ296475; DQ296479; DQ296480; DQ296477; DQ296476; DV767807 to DV767846; DV970112 to DV970114; DV767754; DV767753; DV767770; DV767746; DV767747; DV767748; DV767749; DV767750; DV767751; DV767752; DV970109; DV767755; DV767756; DV767757; DV767758; DV767759; DV970107; DV767760; DV767761; DV767801; DV767762; DV970110; DV767763; DV767764; DV767765; DV767766; DV767767; DV767768; DV767769; DV767771; DV767772; DV767773; DV767774; DV767775; DV767776; DV767777; DV767778; DV970111; DV767779; DV767780; DV767781; DV767782; DV767783; DV767784; DV767785; DV767786; DV767787; DV767788; DV767789; DV767790; DV767791; DV767792; DV767794; DV767795; DV767796; DV767797; DV767798; DV767799; DV767800; DV767802; DV767803; DV767804; DV767805; DV767806.

APPENDIX 2

Email received from Dr Zamir Punja (punja@sfu.ca) on January 18th 2006:

Dear Amelie,

You have the permission of the Canadian Phytopathological Society, to co-publish the following paper in your thesis: "Real-time PCR quantification of Colletotrichum coccodes DNA in bioherbicide release field soils with normalization for PCR inhibition" and the authors are Dauch, A. L., Watson, A. K., Seguin, P., and Jabaji-Hare, S. H. You may acknowledge the page numbers in the thesis once they become available in March.

Zamir K. Punja, PhD
Professor, Plant Pathology/Biotechnology,
Dept. of Biological Sciences,
Simon Fraser University,
Burnaby, B.C. V5A 1S6, CANADA
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On Jan 18, 2006, at 2:49 AM, Amélie wrote:
> Dear Zamir,
> Suha and I recently got a paper accepted for publication in the CJJP (on Nov 28
> 2005, manuscript number 172-05) that should come out in the March issue. I
> would like to request your permission to co-publish this manuscript in my
> PhDthesis. The final version of my thesis will not be publicly available before
> the month of April 2006.
>
> The manuscript is entitled "Real-time PCR quantification of Colletotrichum
> coccodes DNA in bioherbicide release field soils with normalization for PCR
> inhibition" and the authors are Dauch, A. L., Watson, A. K., Seguin, P., Jabaji-
> Hare, S. H. I previously asked this permission to Roxanne Landriault from NRC
> research Press thinking that NRC would be the authority giving this kind of
> authorization but I was apparently wrong ...
> Thank you in adavnce, and best wishes for this new year,
> Amélie,
>
> Amélie Dauch
> Department of Plant Science
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