TITLE

Identification and characterization of genes involved in pathogenicity of *Colletotrichum* spp. on strawberry by isolation of impaired pathogenicity mutants

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by

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TABLE OF CONTENTS

List of abbreviations

aa	Amino acid
ANOVA	Analysis of variance
cDNA	Complimentary deoxy nucleic acid
CD	Conserved domains
2-DE	Two-dimensional gel electrophoresis
DIC	Differential interference contrast microscopy
dNTP	Deoxy nucleic three phosphate
DSR	Disease severity rating
H_2DCFDA	2', 7'-dichlorofluorescin diacetate
inPCR	Inverse polymerase chain reaction
EtOH-	Ethanol
h	Hour
IEF	Isoelectric focusing
LC	Liquid chromatography
LSD	Least significance difference test
LSCM	Laser-scanning confocal microscope
min	Minute
M3S	Modified Mathur's medium (M3S)
MM	Minimal medium
MS	Mass spectrometry
PKA	cAMP-dependent protein kinase A
Reg	Regeneration medium
REMI	Restriction-enzyme mediated integration
rpm	Round Per minute
ROS	Reactive Oxygen Species
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SOD	Cu Zn superoxide dismutase
S/N	Supernatant
TE	Tris (pH 8) and EDTA (pH 8)
QRT-PCR	Quantitative real time polymerase chain reaction
wk	week
kDa	Kilo Dalton

ABSTRACT

Colletotrichum acutatum, the cause of strawberry anthracnose, is one of the most important fungal pathogens of this crop worldwide. However, the molecular basis of the infection process and pathogenicity components involved in the establishment of host plant colonization is poorly understood. Disruption of genes that result in the reduction or complete loss of disease symptoms by generation and screening for nonpathogenic mutants is a powerful tool for identifying pathogenicity-related genes. Successful application of this technique for plant fungal pathosystems requires reliable and rapid screening procedures.

Development of a rapid *in vitro* bioassay in this study, enabled large scale screening and isolation of nonpathogenic mutants of *Colletotrichum acutatum* and *C. gloeosporioides* on strawberry seedlings. Inoculation was carried out on strawberry seedlings at two different developmental stages: 12-week-old (young) and 15-week-old (older) seedlings. A comparison was made between two inoculation techniques; (i) foliar dip and (ii) root soak, at two incubation temperatures (19ºC and 25ºC). Mortality of young seedlings was observed 4 days after inoculation with both species, reaching 50% within 10 days, using both techniques, at 25ºC. However, mortality of older seedlings was delayed by 4 days compared to that in the young seedlings when using the root soak method. Disease development decreased in young and older seedlings at the lower temperature. This method, utilized for screening for non-pathogenic mutants, was also reliable in determining pathogenicity of the cucurbit-specific *C. magna* that did not cause disease symptoms on strawberry by either inoculation method.

A random mutagenesis technique termed restriction-enzyme mediated integration (REMI) was used in order to identify genes that are important for colonization of strawberry. Over 1000 randomly mutated REMI strains were produced and screened

for pathogenicity by the proposed method, resulting in a selection of five reduced-virulence isolates. Initial characterization of the reduced mutants included morphological characterization, molecular characterization of the insertion event and isolation of the flanking regions disrupted by the insertion vector.

One of the five reduced pathogenicity mutants, designated Ca5, was the focus of this research. This mutant exhibited epiphytic hyphal growth and did not cause lesions on strawberry plants but grew necrotrophically when inoculated directly onto wounded stolons. In the absence of an external nitrogen source, the mutant exhibited extended germ-tube growth prior to appressorium formation. The deduced product of the impaired gene (*nir1*) is a C6 zinc-finger protein which is similar to NirA, an *Aspergillus nidulans* transcriptional regulator of nitrogen metabolism. Based on the molecular characterization of the Ca5 strain, the perception of nitrogen deprivation as a signal for induction of pathogenicity related morphogenesis development was studied. Inoculation of leaves with wild type (w.t) or non-pathogenic Ca5 conidia in the presence of a preferred nitrogen source resulted in massive epiphytic hyphal production, appressorium formation and rapid symptom development. In an attempt to determine nitrogen availability during the infection process, the expression of nitrate reductase (*nit1*) and glutamine synthetase (*gln1*) was investigated. Expression of *C. acutatum* w.t *nit1* and *gln1* was induced by nitrate but only *nit1* expression was repressed while growing in complete nutrient supply. Furthermore, *nit1* transcription increased during the appressorium-production stage, indicating that nitrogen starvation constitutes a cue for the regulation of appressorium development. Presence of the *nit1* transcript during various phases of infection is indicative of partial nitrogen starvation *in planta*. cAMP-dependent protein kinase A (PKA) was determined to be a negative regulator of immediate post-germination appressoria formation in the w.t.

Since inhibition of PKA activity in the *nir1* mutant did not affect appressoria formation, it is suggested that NIR1 acts either in parallel or downstream of the PKA pathway. The current results show that *nir1* is a pathogenicity determinant and a regulator of preinfection development under nitrogen-starvation conditions and that nitrogen availability is a significant factor in the pre-penetration phase.

Given that the *nir1* gene plays a role in a nitrogen-regulated signaling pathway and that *C. acutatum* pathogenicity can be attributed to its ability to sense and respond to nitrogen limitation, the relevance of nutrient regulation of morphogenesis for the pathogenicity of *C. acutatum* was further studied by performing a proteomic-based approach. To determine whether nitrogen starvation constitutes a cue for regulating developmental processes and pathogenicity, the cellular outcome of changes to nitrogen deprivation, in context to development and early stages of pathogenicity, was investigated. Quantitative analysis of proteins synthesized during appressoria formation, under nitrogen limiting conditions or complete nutrient supply, compared to the Ca5 non-pathogenic mutant, revealed significant changes in the abundance of different proteins. The function of the proteins that were up-regulated or down-regulated can be grouped into the following main categories: energy metabolism, nitrogen and amino acid metabolism, protein synthesis and degradation, response to stress and reactive oxygen scavengers. Differential display analysis of proteins involved in growth under limiting conditions and during appressoria formation could be useful to further understand changes leading to establishment of the early infection stage in additional host pathogen interactions.

Proteins patterns of different developmental stages and growth conditions revealed variation in abundance of proteins belonging to the ROS (Reactive oxygen species) scavenger machinery. Members belonging to the ROS scavenger machinery,

CuZn superoxide dismutase (SOD) and glutathione peroxidase (GPX) were up-regulated at the appressoria formation stage and nitrogen limiting conditions, compared to growth in complete nutrient supply, whereas the bifunctional catalase (CAT2) was predominantly detected at the appressoria stage. To follow changes of ROS levels during fungal development, qualitative and quantitative ROS production was measured in response to different nutritional conditions. *In vitro* fungal ROS generation was markedly increased under nitrogen limiting conditions, a precondition for appressorium formation, compared to growth in complete nutrient supply. Plant ROS accumulation *in vivo* was detected at the stolon appoplastic space, exclusively at sites of direct physical contact between appressoria structures and the host. Fungal ROS production within germinating conidia during the pre-penetration phase and over the course of penetration and colonization was prominent. The role of ROS as a regulator of appressoria development was investigated using a pharmacological based approach. Application of an exogenous antioxidant *in vitro*, such as N-acetyl-L-cysteine or mannitol, reduced ROS production as well as appressorium formation. These results suggest that ROS production is an early fungal response to nitrogen starvation, modulating initial stages of pathogen development.

A general up-regulation in metabolic activity during appressoria formation and nutrient deficiency conditions was observed via the proteomic-based analysis. Abundance of proteins belonging to the glyoxylate cycle and lipid metabolism, such as malate dehydrogenase (MDH), formate dehydrogenase (FDH) and acetyl CoA acetyltransferase was observed during the course of appressoria production. In contrast, down-regulation of isocitrate dehydrogenase (IDH), detected during appressoria formation may indicate that the organism is preparing for lipid-based energy supply. The present study demonstrates that the developmental processes

occurring under nutritional deprivation are facilitated by metabolic shifts which may be mediated by ROS production.

Regarding the *nir1* mutation, findings pertaining to the Ca5 non-pathogenic mutant are intriguing and represent the initial steps in defining the factors controlling virulence in *C. acutatum* infecting strawberry at the molecular level. This study utilizing the Ca5 mutant demonstrates that many diverse physiological and morphogenetic processes may be classified as being mediated by pathogenicity genes and emphasize the perception of nitrogen starvation regulation on development and pathogenicity in *C. acutatum*.

INTRODUCTION AND LITERATURE REVIEW

1.1. Epidemiology of *Colletotrichum acutatum* J.H. Simmonds

Colletotrichum acutatum is an ascomycete filamentous fungus with a broad geographic and host range. This pathogen is the primary causal agent of strawberry anthracnose, causing petiole, stolon, crown and root infections and has become a major constraint in worldwide strawberry (*Fragaria* × *ananassa* Duchesne) production (Freeman and Katan; 1997; Kenneth *et al*., 2002). The dominant species causing disease symptoms on strawberry are *Colletotrichum acutatum*, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz and Sacc. in Penz. [Teleomorph *Glomerella cingulata* (Stonem) Spauld. & Schrenk] (Howard and Albregts, 1983; Smith and Black, 1990; Legard, 2000). This pathogen's ability to attack different plant parts and the fact that several pathogen species could be involved, adds to the complex nature of strawberry anthracnose (Freeman and Katan, 1997). In Israel, anthracnose is one of the most important diseases affecting strawberry and is caused mainly by *C. acutatum,* but occasionally also by *C. gloeosporioides* (Freeman and Katan, 1997; Freeman *et al*., 2002). The pathogen is apparently introduced into production fields on infected or infested propagation material. During warm and moist periods, lesions formed on transplants produce abundant conidia that are splash-dispersed onto flowers and fruits, resulting in new infections (see disease cycle; Fig. 1). Primary inoculum for dispersal within the field is mostly dependent on the formation of spores in acervuli on petioles and fruit tissues (Peres *et al*., 2005). Secondary conidation can occur on the surface of vegetative tissues, which may serve to augment inoculum levels infecting flowers and fruits (Leandro *et al*., 2001). As far as is known, the sexual stage (*G. acutata* not detected on strawberry) may only serve a minor role in the life cycle of *C. acutatum* (Peres *et al*., 2005). *C. acutatum* is primarily a necrotroph on strawberry tissues

although the specific interaction at the cellular level may include combination of strategies as indicated below (Horowitz *et al*., 2002; Peres *et al*., 2005).

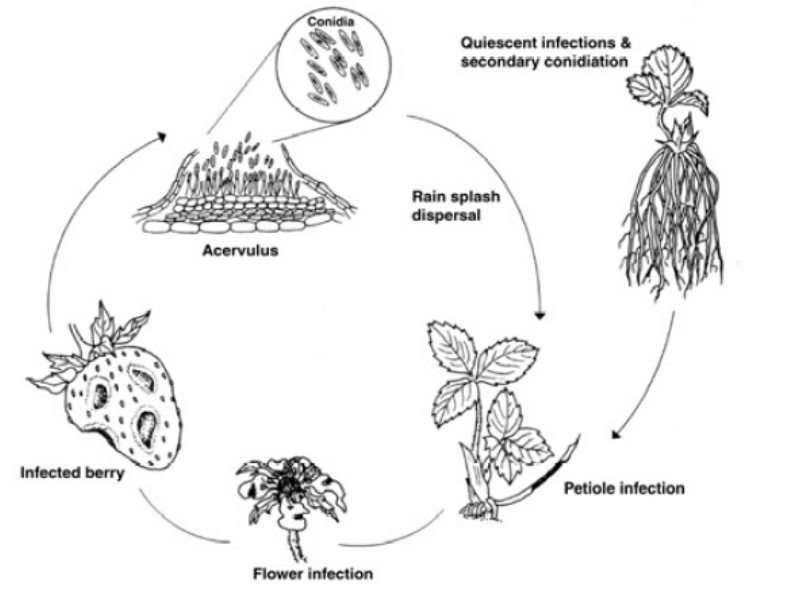

Figure 1. Disease cycle of anthracnose fruit rot of strawberries caused by *Colletotrichum acutatum*. Prolonged necrotrophic phase on flowers, fruits, and petioles. (Illustrated by Lynda E. Chandler; Peres *et al*., 2005).

1.1.1 Infection process of strawberry by *Colletotrichum* spp.

A consideration of the detailed nature of biotrophy, necrotrophy and hemibiotrophy is necessary in understanding the invasion mechanism of *Colletotrichum* spp. Biotrophy is the condition in which the fungus maintains its host cells alive while removing nutrients from them. Necrotrophy is the condition in which the fungus approaches living host cells and kills them before entering their lumina. Hemibiotrophy is the condition in which the fungus initially forms an association with living cells of the host, much like a biotroph, and then in the later stages of infection becomes necrotrophic, actively killing host cells. Bailey *et al*., (1992), described two major invasion strategies of *Colletotrichum* spp., intracellular hemibiotrophic invasion and subcuticular intramural invasion, both avoiding triggering of the resistance responses during the symptomless phase. In the absence of external nutrients, early stages of invasion for both strategies include conidial adhesion to the host surface, germination

of conidia, production of germ tubes, and penetration of the host cuticule via appressoria. Species with an intracellular hemibiotrophic strategy require an intimate cytoplasmic interaction between the plant and the pathogen, invade living cells for a short time maintaining them apparently for nutritional advantage, and then convert to a necrotrophic phase. However, species with a subcuticular intramural strategy do not require such intimacy and grow within the cuticule and the cell walls of epidermal, subepidermal and subtending cells, killing host cells prior to invasion. Ontogeny of the invasion process by *Colletotrichum acutatum* has been studied thoroughly on several hosts (Peres *et al*., 2005) (illustrated in Fig. 2). Although *C. acutatum* is a generalist invader, the fungus possesses a very brief symptomless biotrophic phase (less than 12 hr) before commencing the extended necrotrophic phase which involves major nutritional changes (Horowitz *et al*., 2002; Kenneth *et al*., 2002). During the latter phase, the fungus differentiates secondary hyphae, which are thinner than the primary hyphae and grow extensively, leading to disorganization and mortality of the infected host cell (Kenneth *et al*., 2002; Horowitz *et al*., 2002). Since the brevity of the biotrophic phase in *C. acutatum* leaves some uncertainty about terming these fungi hemibiotrophs, the specific interaction at the cellular level may be a combination of strategies. Despite the well documented infection strategy of *Colletotrichum* species, the molecular bases of pathogenicity are still very much unknown and factors important for virulence and aggressiveness of *Colletotrichum* on strawberry have not been studied in detail.

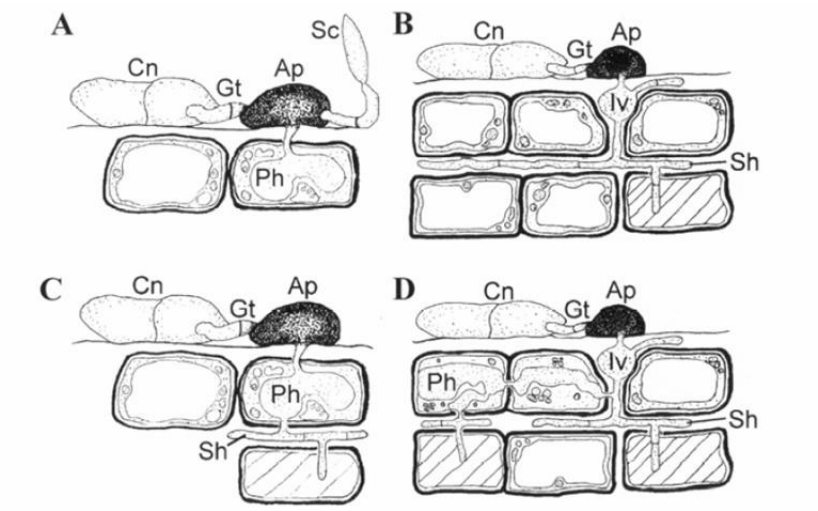

Figure 2. Schematic drawings of different host-pathogen interaction strategies of *Colletotrichum acutatum*. A. Biotrophic growth with secondary conidation. Conidia germinate to form appressoria and quiescent infections. Secondary conidia are formed after germination of the appressorium, predominantly biotrophic disease cycle on citrus leaves. **B.** Subcuticular, intramural necrotrophy with hyphal development within periclinial and anticlinial walls of epidermal host cells. Predominantly necrotrophic disease cycle on strawberry. **C.** Hemibiotrophic interaction with infection vesicle and broad primary hyphae within host cell. Inter-and intracellular secondary hyphal growth is shown illustrating the subsequent necrotrophic phase. A combination of bio-and necrotrophy but mostly biotrophic disease cycle on blueberry fruit. **D.** Hemibiotrophic and subcuticular, intra and intercellular development of *C. acutatum* on almond. A combination of bio-and necrotrophy but mostly necrotrophic disease cycle on almond. **Cn**=conidium; **Gt**=germ tube; **Ap**=appressorium; **Iv**=infection vesicle; **Ph**=primary hyphae; **Sh**=secondary hyphae; **Sc**=secondary conidium; Dead host cells are indicated with diagonal lines. (Illustrated by J.E. Adasksveg; Peres *et al*., 2005).

1.1.2. Appressorium development by *Colletotrichum* spp.

In most, though by no means all, *Colletotrichum* species, differentiation of an appressorium is essential for host penetration. The appressorium, which arises as an apical swelling of the germ tube, promotes adhesion to the host surface and provides the mechanical force and enzymes required for initial penetration (O'Connell *et al*., 2000; Kubo *et al*., 2000). Once formed, appressoria become separated from the germ tube and conidium by a thick septum, and accumulate melanin pigmentation. Maturation of the appressorium involves formation of a penetration pore in the base of the cell, deposition of new wall layers darkly pigmented with melanin, and secretion of extracellular matrix materials. Subsequent penetration of the plant cuticule and cell

wall probably involves a combination of mechanical force, in the form of high turgor pressure, and enzymatic degradation (Hwang *et al*., 1995). The enormous turgor in appressoria is a consequence of the accumulation of very large quantities of glycerol in the cell. Glycerol acts as a highly soluble osmolyte, causing rapid influx of water into the appressorium to generate hydrostatic turgor. The biosynthesis of glycerol occurs rapidly during appressorium morphogenesis and involves mobilization of storage reserves from the conidium. A rapid degradation of lipid bodies occurs during appressorium formation and subsequent penetration of the host plant, implying that lipolysis provides the compounds required for appressorium formation and function (Wang *et al*., 2003; Asakura *et al*., 2006).

According to cellular studies, predominantly performed with *Magnaporthe grisea* and *Colletotrichum* species, appressorium development can be subdivided into three distinct developmental stages: differentiation, maturation and function. Differentiation of the appressorium seems to depend on perception of several putative inductive extracellular signals that are physical and or biological such as surface rigidity, nutrient availability and specific chemical compounds. It has been demonstrated that cyclic AMP, mitogen-activated protein kinases, and Ca^{2+}/calmodulin-mediated signaling pathways are all directly involved in triggering the morphogenesis procedure that leads to appressorium formation (Veneault-Fourrey *et al*., 2005). One of the better-studied components of environmental perception is the cAMP-dependent protein kinase A (PKA) signalling pathway. PKA has been shown to play an important role in nutrient sensing in the saprophytic yeast *Saccharomyces cerevisiae*. Likewise, PKA appears to play an integral role in surface sensing, vegetative growth and appressoria formation in species of the filamentous phytopathogen *Colletotrichum* (Lee *et al*., 2003) and *M.*

grisea (Adachi and Hamer, 1998). Once appressorium differentiation has occurred, maturation and subsequent function take place.

Morphological and physiological transition during the life cycle of *C. accutatum* appears to be induced by the environment encountered during each stage of pathogenesis. The cellular environment represents a challenge to an invading fungus, which must encounter nutrition limitation. Signals that affect initial establishment of the fungus on the host have received limited attention in the area of *Colletotrichum*.

1.1.3. Interaction of *Colletotrichum* on strawberry

During the interaction between a plant and a fungus, a complex molecular dialogue is initiated as soon as the conidium comes into contact with its potential host. Perception of a fungal pathogen leads to rapid induction of defense responses, including generation of reactive oxygen species, cell wall reinforcement, synthesis of phytoalexins, accumulation of pathogenesis-related protein (PR proteins), and a change in protein phosphorylation status (Veneault-Fourrey *et al*., 2005). A broad spectrum of defense responses includes early and late responsive genes perhaps geared towards immediate defense and long-lasting defense strategies. The time course of induction of strawberry defense and resistance responses toward the first steps of infection by *Colletotrichum* is not yet fully understood. Recent studies on expression analysis of two class II chitinase genes from strawberry plants suggested different regulation in response to *C. acutatum* compared to *C. fragariae*. Apparently, activation or induction of different secondary messengers or signaling pathways is responsible for the observed induction kinetics (Khan and Shih, 2004). Findings concerning PR protein genes subsequent to fungal infection could advance our understanding, of the timing of each developmental stage which allows evading or triggering of strawberry defense

responses. In this study, different PR proteins coding genes were used to follow induction of plant defense response at early fungal developmental stages.

1.2. Pathogenicity-related genes in *Colletotrichum* spp.

This study has specifically been aimed to clone and characterize fungal genes that are involved in the ability to sense and respond to the host environment, and/or required during pathogenic development. Recently, some genes that may have important roles in pathogenicity have been cloned from various pathogenic species within the genus *Colletotrichum*. In some instances, definitive roles for these genes in pathogenicity have been shown using gene disruption or mutant complementation. In general, two approaches have been used to identify pathogenicity genes in phytopathogenic fungi. The first approach is first to identify mutants impaired in their ability to infect normally susceptible host plants, while the second direct approach is to identify a candidate gene, then test the role of this gene by targeted gene disruption. Candidate genes that have been isolated from *Colletotrichum* include (a) genes expressed specifically in appressoria development, (b) genes expressed in intracellular hyphae (c) genes encoding a range of extracellular hydrolytic enzymes, (d) genes induced during growth *in planta* which may have roles in fungal nutrition, while others may directly effect pathogenesis (Manners *et al*., 2000).

To advance our understanding of *Colletotrichum* disease of strawberry, pathogenicity genes can be identified by utilizing a large scale forward genetic approach for screening for reduced or impaired virulence mutants. In general, the following procedures should be available for this purpose: (i) a random mutagenesis system, (ii) an efficient transformation system and (iii) a rapid, reliable infection bioassay (Sweigard *et al*., 1998). The restriction enzyme mediated integration (REMI)

technique has been applied successfully to isolate pathogenicity-related genes in several plant pathogenic fungi (Kuspa *et al*., 1992; Bolker *et al*., 1995; Thon *et al*., 2000; Yakoby *et al*., 2001) and may serve as an insertional mutagenesis method. An efficient transformation system was developed for *C. gloeosporioides* (Robinson and Sharon, 1999) and for *C. acutatum* (Horowitz *et al*., 2002) by electroporation of germinating conidia which provided an easy and efficient way to obtain stable transformants of this fungus. However, in the past, pathogenicity assays of *Colletotrichum* spp. were designed especially for breeding programs for anthracnose resistance and were not suitable for large scale screening for reduced pathogenicity mutants. These techniques involve foliar application of conidia by spraying, pipetting, or injection of seedlings or plants, which were ultimately selected for foliage, stolon and crown resistance (Smith and Spiers, 1982; Denoyes-Rothan and Guerin, 1996). Such methods require considerable greenhouse space, are time and labor consuming and carry a high chance of cross contamination during inoculation, thus were not appropriate for a large scale screening procedure for nonpathogenic mutants. Likewise, strawberry fruit inoculation methods have been developed to study the virulence (Maas and Howard, 1985) and resistance to anthracnose fruit rot (Denoyes-Rothan *et al*., 1999). Similarly, an inoculation technique of detached petioles was developed for assessing resistance of strawberry lines to *C. acutatum* (Koch-Dean *et al*., 2002). However, these two latter methods, in which detached organs were used, are inappropriate for assessing pathogenicity, since resistance mechanisms in detached tissues may not be that accurate. Therefore, initially, the main objective of this study was to establish a rapid and reliable bioassay for large-scale screening of putative non-pathogenic *Colletotrichum* mutants on strawberry. The effects of incubation temperature, seedling age, inoculation technique and isolate were evaluated to

determine the conditions best suited for this purpose (Horowitz *et al*., 2004). The second derived objective was to prove the efficacy of the proposed screening method by isolating reduced pathogenicity mutants for future studies. One of the reduced pathogenicity mutants of *C. acutatum*, designated Ca5, was shown to be affected at a key step in the infection process, prompting the research to focus on early stages of the infection process. Molecular analysis of the Ca5 mutant permitted identification of the first pathogenicity gene, *nir1*, isolated from strawberry-infecting *C. acutatum*, which encodes a protein involved in nitrogen metabolism. The results demonstrated that a common regulator of nitrogen metabolism is required for this fungus' growth and development. These findings led to examine the effect of nitrogen availability on fungal morphogenesis and pathogenicity.

1.3. Facing nutritional changes during plant colonization

Molecular communication of *Colletotrichum* species begins as soon as a fungal conidium lands on a plant surface in the absence of external nutrients, and progresses via morphogenetic changes resulting in the development of specific infection structures in this environment. To be a successful pathogen, a fungus must be able to adjust its metabolism to utilize nutrients available within the host tissue. There is much evidence to suggest that fungal metabolism can be divided into at least two phases. The first is based on lipolysis and occurs during germination and penetration of the host. The second phase uses glycolysis and predominates during the invasion of host tissue (Solomon *et al*., 2003). Little is known about metabolic control circuits in phytopathogenic fungi and their roles in regulation of metabolism and/or nutritional signals in disease development (Snoeijers *et al*., 2000). Hemibiotrophic plant pathogens are characterized by an initial biotrophic phase that is followed by a rapid switch to

necrotrophic phase, resulting in typical symptoms such as in the *Colletotrichum* strawberry interaction. Major nutritional changes are believed to occur when maintaining the hemibiotrophic lifestyle, while relatively few pathogenicity genes have been identified that are related to the switch from biotrophy to necrotrophy in hemibiotrophs. Several studies have suggested that lack of nutrients is one of the signals controlling the expression of genes involved in pathogenicity of various microbial plant pathogens (Pellier *et al*., 2003). Genes that are either induced or highly expressed in the pathogen during nutrient limitation may play a direct role in the pathogenesis process and control essential adaptions to the nutritional environment encountered by the pathogen. Moreover, it has been proposed that a subset of signaling pathways that regulate fungal pathogenicity have been co-opted from those involved in nutrient sensing and subsequent fungal response (Alspaugh *et al*., 1997; Pellier *et al*., 2003), as illustrated in the model of nitrogen sensing in (Fig. 3).

Major nutritional changes are believed to occur during: (i) the early pre-penetration phase, during which the fungus is dependent mainly on pre-acquired nutrient supplies, (ii) the onset of biotrophy, during which the fungus presumably acquires nutrients adjacent to living host cells, (iii) the cessation of biotrophy and the initiation of the necrotrophic phase. The infection process requires the spatially and temporally regulated development of appropriate infection structures, suggesting the occurrence of tightly regulated fungal development *in planta*. Carbon and/or nitrogen starvation have been reported to induce or increase the expression of a number of *in planta*-induced genes of several fungal plant pathogens (Snoeijers *et al*., 2000). For example, the *mpg1* gene of the hemibiotrophic rice blast fungus *Magnaporthe grisea* encodes a hydrophobin-like protein involved in appressorium formation that is necessary for pathogenicity and is expressed *in vitro* under conditions of carbon and

nitrogen starvation (Talbot *et al*., 1993). Similarly, the expression of the avirulence gene *avr9* of the biotrophic fungal tomato pathogen *Cladosporium fulvum* is induced under nitrogen, but not carbon starvation (Van den Ackerveken *et al*., 1994; Perez-Garcia, *et al*., 2001), yet nitrogen starvation does not control some other effector genes that are required for full virulence of *C. fulvum* (Thomma *et al*., 2006). The CgGS gene and the CgDN3 gene, which encode a glutamine synthetase (GS) and a product of unknown function, respectively, in *Colletotrichum gloeosporioides*, the hemibiotrophic fungal pathogen of tropical legumes, were obtained through a screen for genes that are specifically induced *in vitro* under nitrogen starvation (Stephenson *et al*., 1997; 2000). These genes have been shown to be expressed *in planta* during the early stages of the *C. gloeosporioides* infection process. On the other hand, examination of the transcriptional adaptation of *Fusarium oxysporum* f. sp. *lycopersici* (the causal agent of vascular wilt in tomato) during nutritional stress and plant colonization indicated that nitrogen starvation partially mimics *in planta* growth conditions (Divon *et al*., 2005). Thus, it is frequently assumed that biotrophic and hemibiotrophic fungal pathogens encounter nitrogen-limiting conditions at the onset of the infection process and that nitrogen starvation constitutes one of the signals involved in the regulation of genes that are induced *in planta* (Snoeijers *et al*., 2000). The host environment and starvation stress, particularly for nitrogen, were found to be important for *Colletotrichum* morphogenesis.

1.3.1. Nitrogen utilization during early infection stages

Fungi are able to utilize a wide array of nitrogen-containing compounds but some nitrogen compounds are more preferentially used than others. The nitrogen sources available for a pathogen in the host plant are dependent on the tissue that is

being colonized. Nitrogen sources used by a foliar pathogen might be different from those used by a soilborne or root-infecting. Similarly, it is likely that a necrotrophic pathogen, which kills tissues, is probably able to use a broader spectrum of nitrogen sources than a biotrophic pathogen which feeds on living host tissue and only has access to nitrogen sources available in the apoplast and/or the haustorial matrix (Snoeijers *et al*., 2000).

In the saprophytic filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* AREA and NIT2 which interact with the pathway-specific regulators NIRA and NIT4, respectively, act to facilitate the expression of genes involved in metabolizing alternative nitrogen sources (Marzluf, 1997). In the latter fungi the major, positively-acting regulatory genes *area* (*A. nidulans*) and *nit-2* (*N. crassa*) mediate global nitrogen repression and derepression. Both AREA and NIT2 are members of the GATA family of transcription factors that bind to promoter domains containing a GATA sequence (Marzluf, 1997). AREA and NIT2 activate the expression of many genes whose products are required for the utilization of nitrogen from various secondary sources or when nitrogen is limited. In pathogenic fungi, the loss of such a major, wide domain nitrogen regulator might repress the expression of genes that are necessary for pathogenicity and could affect the ability of the pathogen to grow and proliferate within the host.

A model for the fungal nitrogen catabolic pathway and the proposed role of the AREA-like protein for induction of nitrogen-dependent pathogenicity genes in fungal pathogens is illustrated (Fig. 3).

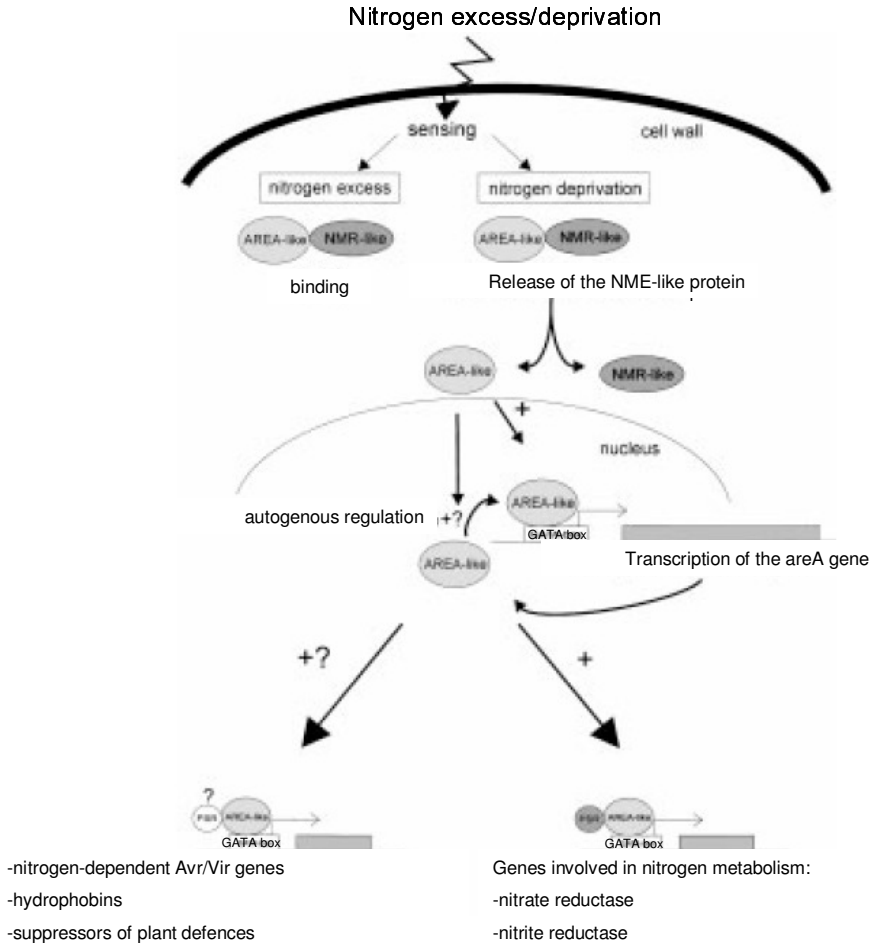

Figure 3. Model for nitrogen-sensing and induction of nitrogen-dependent pathogenicity genes of fungal pathogens. When primary nitrogen sources are available, the negative regulatory (NMR-like) protein binds to the major positive-acting (AREA-like) protein preventing activation of genes involved in nitrogen utilization. The released AREA-like protein induces the expression of a broad range of nitrogen metabolism genes. It is assumed that the AREA-like protein interacts with multiple positive-acting, pathway-specific regulatory proteins in turning on specific sets of nitrogen-catabolic genes, depending upon the availability of substrates and requirement for nitrogen (Snoeijers *at al*., 2000).

1.4. A comparative proteomic investigation as a tool for studing nutritional stress response

Findings indicate that nitrogen-limiting conditions are predominant during the early infection stage, suggesting the potential use of proteomics for analyzing up and down-regulated proteins related to fungal morphogenesis and pathogenicity. Protein analysis has been successfully used for the study of various filamentous fungi of economic interest, such as *Aspergillus oryzae* (Nandakumar and Marten; 2002), *Trichoderma reesi* (Lim *et al*., 2001), *Erysiphe pisi* (Curto *et al*., 2006) and *Botrytis cinerea* (Fernandez Acero *et al*., 2006). Similar studies in *Magnaporthe grisea* (Kim *et al*., 2004) provided important clues regarding developmental changes such as

appressorium generation. In *Phytophthora infestans*, proteomics was utilized to identify proteins involved in the establishment of infection of the host plant (Ebstrup *et al*., 2005). In order to identify novel components that may be linked with the perception of nitrogen limitation and pathogenicity-related development, the protein abundance patterns of w.t and Ca5 non-pathogenic mutant, grown under nitrogen-limiting conditions with those of the w.t grown in a complete nutrient supply and at the appressorium formation stage was compared. The technique used for the separation of sample proteins is 2-dimensional electrophoresis (2-DE). This technique uses the power of both isoelectric focusing (IEF) and SDS-PAGE electrophoresis to separate proteins firstly by their pI and then by their molecular weight. The functional implications of the identified proteins, with special emphasis on the essential role of ROS as an early response to nitrogen deficiency, were studied.

1.4.1. Reactive oxygen species (ROS) and development in microbial eukaryotes

Reactive oxygen species (ROS), in particular the superoxide anion (O_2^-), its conjugated acid, the perhydroxyl radical ($HO_2^{\cdot}$), and their dismutation product hydrogen peroxide (H_2O_2) have been regarded as inevitable harmful by-products of aerobic metabolism. The detrimental affect of ROS on DNA, proteins, lipids and other cell components and their role in pathogenesis are well established (Smirnoff, N., 2005). However, there is increasing evidence supporting an alternative view, in which specific ROS-producing enzymes, produce ROS to regulate different cellular functions, including cell proliferation, cell differentiation, signal transduction and ion transport (Aguirre *et al*., 2005). Most ROS are highly reactive and short-lived and therefore difficult to detect in complex biological matrices. At present, real-time detection of ROS is limited and suffers from low specificity and therefore does not

measure a particular ROS (Shulaev and Oliver; 2006). Various ROS-scavenging systems, including ascorbate peroxidases, glutathione, superoxide dismutases and catalases, maintain ROS homeostasis in different compartments of the cell. These enzymes could restrict the ROS-dependent damage or finely tune ROS-dependent signal transduction (Mittler *et al*., 2004). In tobacco, reduction of catalase and ascorbate peroxidase activities resulted in hyper-sensitive response to pathogens, whereas the overexpression of catalase leads to more disease-sensitive plants. Collectively, these results suggest that ROS-scavenging systems can serve an important role in managing ROS generated in response to pathogens (Torres *et al*., 2006). Several reports in different eukaryotic microorganisms have shown a correlation between developmental processes and the up-regulation of specific antioxidant enzymes. A manganese SOD gene is induced during spore development in *Colletotrichum graminicola* (Fang *et al*., 2002) and one of the developmental pathways in the slime mold *Physarum polycephalum*, is accompanied by a high induction of SOD activity (Aguirre *et al*., 2005). During the stationary phase, many microorganisms develop stress-resistant cells that can be compared to the dormant phase of spores. SOD is essential for stationary-phase survival in *Saccharomyces cerevisiae* (Longo *et al*., 1996), and in *Candida albicans* a cytoplasmic manganese SOD is expressed upon entrance to the stationary phase (Lamarre *et al*., 2001). Several catalase genes, present in *Aspergillus nidulans* and *N. crassa,* are differentially expressed, both temporally and spatially (Kawasaki and Aguirre, 2001). Induction of antioxidant enzymes in structures that are undergoing development and the association of specific antioxidant enzymes with fully differentiated structures suggest that ROS are produced at the start and during cell differentiation in several microbial eukaryotes (Aguirre *et al*., 2005). On the other hand a successful pathogen

must be able to overcome or suppress the complex array of ROS-mediated host defenses. Microbial suppression of ROS-mediated defenses by secretion of ROS-scavenging enzymes such as superoxide dismutase and catalase, which converts ROS into less reactive species, has been documented in plant and animal pathogens (Jennings *et al*., 1998). Despite the evidence indicating that ROS are related and probably required for cell differentiation in eukaryotic cells, the sources of these ROS and their contribution to development remain to be determined. It is well established that mitochondria generate low but continuous levels of O_2^- which might be involved in cell signaling and development, while the primary source of ROS is an O_2^- generating membrane-bound NADPH oxidase (NOX). Fungal NOX enzymes appear to participate in ROS production in several developmental processes such as sexual reproduction, germination and regulation of specific fungal plant interactions (Aguirre *et al*., 2005). Recent research has demonsrated a novel role of the nox_A NADPH oxidase coding gene in regulating the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloë festucae*, and its grass host, *Lolium perenne* (Tanaka *et al*., 2006). These results suggest that fungal ROS production is critical in maintaining this mutualistic fungus–plant interaction.

In microbial eukaryotes, ROS share many similarities with other cell types but possess specific features which may differ from plants and animals. Results presented here indicate that ROS and ROS scavenger machinery may play important roles in mechanisms related to regulation in morphology and host pathogen interaction of *C. acutatum* under nitrogen deprivation. These findings prompted further investigation into the role of ROS in the *C. acutatum* pathosystem.

2. RESEARCH OBJECTIVES

1. Establishing a rapid and reliable bioassay for large-scale screening of putative non-pathogenic *Colletotrichum* mutants on strawberry.
2. Performing forward genetic approach by utilizing REMI mutagenesis to generate reduced pathogeniciy mutants.
3. Molecular cloning and sequence analysis of the disrupted genes flanking the tagged plasmid in the reduced pathogenicity mutants.
4. Molecular characterization of the Ca5 non-pathogenic mutant disrupted in the *nir*1, a *nirA*-like transcription factor, affected at an early stage of infection.
5. Assessing the effect of nitrogen availability or limitation on fungal morphogenesis and pathogenicity.
6. Performing a comparative proteomic investigation of *C. acutatum* under different nutritional conditions and morphologies in order to identify pathogenicity-related developmental components.

2.1. RESEARCH SIGNIFICANCE

Pathogenicity genes are of interest not only to increase our overall knowledge of disease processes, but also since certain genes may become targets for disease control (Indurum and Howlett; 2001). Pathogenicity genes can be identified by utilizing a large scale forward genetic approach for screening for reduced or impaired virulence mutants generated by a random insertional mutagenesis technique. Development of a rapid, reliable and large-scale screening technique for pathogenicity assessment of *Colletotrichum* spp. enabled the selection of five reduced-virulence mutants and could be used potentially for screening additional pathogens of strawberry.

Molecular analysis of the mutant isolate Ca5 impaired in pathogenicity permitted identification of the first *Colletotrichum acutatum* pathogenicity gene, *nir1*, involved in nitrogen metabolism and development. Since nitrogen is crucial as a potentially limiting nutrient, it is important to understand its metabolism in *C. acutatum*. Further investigations were conducted to determine whether *C. acutatum* may be subjected to nitrogen limitation during the early stages of infection and whether the production and maintenance of appressoria is under environmental and genetic control. Results have demonstrated that the ability of *Colletotrichum* spp. to sense and transport nitrogen is crutial, not only for survival, but also as a prelude to a variety of developmental processes. Implications of these findings could facilitate the understanding of *C. acutatum* interaction on strawberry and nitrogenous fertilizers, on disease development.

Given the effect of nitrogen limitation on developmental process in *Colletotrichum*, one more goal was to identify additional proteins involved in nitrogen deprivation and development. Such proteins may play additional roles in the developmental process which is a precondition for pathogenicity. Results obtained in this study shed light on initial metabolic control circuits in *C. acutatum* affecting pathogenicity on strawberry, and may indicate whether or not regulation of metabolism or nutritional signaling is important in disease development. It is anticipated that these results will further the understanding of *Colletotrichum* biology in relation to nutritional adaptation during the early phase of infection and provide new potential targets for disease intervention.

MATERIALS AND METHODS

3.1. Strains, media and growth condition

Colletotrichum acutatum isolate C.a 149 (IMI391664) (Freeman *et al*., 2001), *C. gloeosporioides* isolates C.g 318 (IMI391663), C.g 342 pathogenic on strawberry (Freeman *et al*., 2002), *C. magna* isolate L2.5 (IMI391662) pathogenic and n-path, a u.v. induced non-pathogenic isolate on cucurbits (Freeman and Rodriguez, 1992), were used throughout this study. Approximately 1000 transgenic isolates of *C. acutatum* and *C. gloeosporioides* were generated (see below) and evaluated for pathogenicity. One mutant from *C. acutatum*, was designated Ca5 and analyzed further (Horowitz *et al*., 2004). All of the *Colletotrichum* strains, w.t and transformants, were cultured at 25°C on modified Mathur's medium (M3S) as previously described (Freeman *et al*., 1993) or on regeneration medium (Horowitz *et al*., 2004). Mutants were maintained on M3S or Reg supplemented with 60 μg/ml hygromycin B (Calbiochem, San Diego, CA, USA). Prior to seedling inoculation or fungal culturing, conidia were isolated by flooding 5- to 6-day-old cultures with distilled water and adjusted to a final concentration of 10^5 with a hemacytometer (Brand, Wertheim, Germany). For induction of nitrogen limiting conditions, w.t or mutant strain cultures were incubated at 25°C in M3S medium flasks with horizontal shaking at 150 rpm for 2 days. Cultures were then filtered through Whatman no. 54 filter paper and subcultured to flasks with minimal medium (MM), 20 g/l D-glucose, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.5 g/l KCl, 0.15 g/l $CaCl_2(2H_2O)$, 3 mg/l $FeSO_4(7H_2O)$, 3 mg/l $ZnSO_4(7H_2O)$, 1.25 mg/l $CuSO_4(5H_20)$, 350 μg/l $MnSO_4(H_20)$, 250 μg/l $Na_2MoO_4(2H_2O)$, 6.25 $\times 10^{-6}$ μg/l biotin and 1.25 mg/l thiamine, supplemented with various nitrogen sources at 5mM concentration. For growth in complete growth medium, mycelia were subcultured on Regeneration medium (Reg) [145.7 g/l mannitol (Sigma Aldrich, Steinheim, Germany), 4 g/l yeast extract (Difco

Laboratories, Detroit, MI, USA), 1 g/l soluble starch, 50ml/l pea juice (Robinson *et al*.,. 1999)]. Mycelia in Reg and MM medium were harvested by passing through filter paper at various time points, lyophilized, frozen in liquid nitrogen and stored at -80°C, prior to RNA or protein extractions.

Bacteria *E.coli* strain: DH5α, was grown on either liquid or solid LB media (Sambrook *et al*., 1989) and supplemented with the antibiotic Ampicillin at appropriate concentrations.

3.1.1. Production of nitrate-non-utilizing mutants

Chlorate medium, based on minimal medium amended with 15 g/l $KClO_3$, was used to generate *nit* mutants (Correll *et al*., 1987). Puhalla's minimal nitrate agar (MM) (Puhalla, 1985), a sucrose-salt medium containing nitrate as the nitrogen source, was used to detect *nit* mutants and for complementation (heterokaryon) tests. Mycelial plugs were placed in the middle of plates containing chlorate medium and incubated at 25°C. Fast-growing sectors emerging from the restricted colonies were transferred to MM plates and examined after a 4-day incubation period. Colonies with a thin expansive mycelium, which were unable to utilize nitrate as a sole nitrogen source and did not produce aerial hyphae (consequently grew as thin expansive colonies) on MM, were considered to be *nit* mutants. Nitrate, nitrite and hypoxanthine media were used for partial phenotyping of *nit1*, or *nitM,* respectively (Correll *et al*., 1987). Complementation between *nit* mutants was tested on MM and heterokaryons were usually evident within 10 days. Mutants capable of forming prototrophic heterokaryons were classified as harboring mutations at different loci.

3.1.2. Appressoria induction assay

For appressorium-RNA and protein preparation, conidia of *C. acutatum* (10^6 conidia/dish) were spread onto Petri dishes (13 cm diameter) containing 10 ml of water and were incubated for 12 h. The conidia were then harvested by scraping them off the Petri dishes and centrifuging at 10,000 rpm for 15 min. The pellet containing conidia and appressoria was harvested by filtering through filter paper, lyophilized and subjected to RNA or protein extraction. Germination and appressorial formation by *Colletotrichum* mutants impaired in pathogenicity and comparative w.t isolates were observed on glasstic slides (HYCOR, Biomedical Inc., Garden Grove, CA, USA). Briefly, 20 µl of a conidial suspension of 10^5 conidia/ml of each isolate was placed into five cells of the glasstic slide and kept on moistened filter paper in a moist chamber. After 12 to 14 h of incubation, conidial germination and appressorial development were observed in three microscopic fields in each of five cells per slide. Three germination and appressorial formation stages were documented: conidial germination and immediate appressorial formation (appressoria), conidial germination and appressorial formation after more than double the conidia length (germination + appressoria) and conidia germination without appressorial formation (conidia + germination). Frequency of germination and appressorial formation were pooled for each of three replications per isolate after *t* tests were conducted to determine homogeneity of variance among the three independent experiments and no significant statistical difference among experiments was found ($P > 0.1$). Mean comparisons of germination and appressorial formation were calculated with the combined data using LSD, according to the Tukey-Kramer multiple comparison test at $P < 0.05$. To confirm isolate phenotype, strawberry seedlings were inoculated with conidial droplets then germination and appressorial formation was monitored microscopically after 12 to 14 h on leaves.

3.2. Strawberry plant material and inoculation techniques

3.2.1. Propagation of strawberry seedlings

Seeds of strawberry cv. Malach, an anthracnose susceptible strawberry cultivar, were produced from fruits harvested from December to March from a commercially cultivated field (Tzofit, Central Sharon region, Israel). The seeds were collected after peeling and drying the skins for 72 h at 25 ±1ºC. The seeds were then surface-disinfested and soaked overnight in sterile deionized water (dH_2O). Seeds were germinated on moist, finely ground peat-vermiculite medium (vol/vol; 1:1) in containers and overhead-irrigated with KNO_3 (2 g/liter) soluble fertilizer solution. Containers were covered with plastic bags to maintain 90-100% relative humidity and kept at 25±1°C under continuous fluorescent light for 2 weeks. Thereafter, the plastic bags were removed and the germinated strawberry seedlings were transferred to a greenhouse with supplemental lighting (16-h photoperiod) and temperature of 25±1°C. Plants in the greenhouse were overhead irrigated weekly with a soluble fertilizer (N-P-K 4: 2.5: 6 + 6% microelements, Haifa Chemical Corp., Haifa, Israel). After 12 weeks, most of the seedlings used for inoculation experiments consisted of 4 to 5 true leaves.

The effect of seedling age on disease response was evaluated at two developmental stages: (i) young, 12-wk-old seedlings and (ii) older, 15-wk-old seedlings. Both sets of seedlings were grown for the first 12 weeks as described above. Older seedlings were transplanted at 12 weeks to polyethylene trays containing peat-vermiculite medium (vol/vol; 1:1) and were grown for an additional 3 weeks in a greenhouse with a 16-h photoperiod. Conidial suspensions of isolates C.a 149, C.g 318 and n-path were used separately to inoculate young or older seedlings at 19±1ºC and 25±1ºC and mortality was recorded over a 3-wk period. Experiments examining the

effect of seedling age were repeated twice for each control isolate at each incubation temperature (50 replicate seedlings were tested for each treatment).

3.2.2. Inoculation techniques

Two inoculation methods were evaluated for their effect on subsequent disease development. The methods included a root soak and foliar dip of strawberry seedlings in conidial suspensions at varied concentration. For the root soak method, conidial suspensions from isolates C.a 149, C.g 318 and n-path were used separately by inserting seedling roots into 2 ml Eppendorf tubes containing 1.5 ml of conidial suspensions at different concentrations 10^4, 10^5, 10^6 conidia/ml. Seedlings inoculated by foliar dip were inverted and inserted into a conidial suspension of the three control isolates and inoculum was applied to all the seedling parts, above the roots. Seedlings were then inserted into Eppendorf tubes filled with 1.5 ml sterile dH_2O. Sterile water was used as a control for noninoculated seedlings in both methods. To maintain high relative humidity, inoculated and control seedlings were transferred immediately into boxes covered with plastic bags, and incubated for a period of 20 days in environmental chambers at 19°C or 25°C. In each experiment, 50 seedlings were tested for each control isolate at each temperature. Twenty seedlings treated with sterile dH_2O served as noninoculated controls. Experiments determining the effect of inoculation method on seedling mortality were repeated twice for each control isolate (50 replicate seedlings were tested for each treatment). To assess the contribution of external nitrogen sources to disease development, either glutamine, ammonium chloride, nitrate (10mM, each), or water, were added to the conidial suspensions used in the foliar-dip inoculation method.

3.2.2.1. Pathogenicity assays for isolation of putative nonpathogenic mutants

REMI mutant isolates were tested for their ability to induce anthracnose symptoms on strawberry seedlings. Young seedlings with 4 to 5 true leaves were inoculated by the foliar dip method with 10^5 conidia/ml of each REMI isolate and inserted into Eppendorf tubes. Seedling mortality was assessed after 7 to 10 days. Transformants that did not cause disease on any of the five seedlings were subjected to an additional pathogenicity screening, using 15 seedlings. REMI transformants exhibiting reduced pathogenicity were screened a third time in the same manner as the second test. All experiments included a positive control that consisted of 15 seedlings inoculated with w.t isolates of *C. gloeosporioides*, C.g 318, C.g 342 and of *C. acutatum*, C.a 149. Negative controls consisted of 15 replicate seedlings inoculated with n-path of *C. magna* specific to cucurbits and a water control. Pathogenicity of the putative mutants was further verified on mature daughter plants (cv. Malach). Mature plants were inoculated by foliar dip or sprayed with conidial suspensions of 10^6 conidia/ml and were maintained under 90-100% relative humidity by covering with plastic bags at 25±1°C for 2 weeks. Disease severity of daughter plants was assessed after 2 weeks according to the following disease severity rating (DSR) scale: 0=plants with no visible lesions, 1=one small (<0.5 cm) lesion on leaves or petioles, 2=two small lesions on leaves or petioles, 3=one or more large, actively developing lesions on leaves or petioles, 4=developing necrotic and wilt symptoms, 5=dead plant with necrotic lesions. Pathogenicity of potential nonpathogenic mutant and w.t isolates was expressed as the mean DSR of 10 mature strawberry plants per isolate.

3.2.3. Statistical analysis

Data were analyzed using the JMP software package (version 3.2.6; SAS Institute, Inc. Cary, NC, USA). Each *in vitro* seedling inoculation experiment was

repeated once and data from each experimental repeat were subjected to ANOVA and error variances were evaluated for homogeneity by pairwise *t* tests. ANOVA was performed separately for each seedling age × incubation temperature × inoculation method × incubation temperature of each isolate combination. Because there were no significant statistical differences among experiments and the error variances were homogeneous ($P > 0.1$), data from individual experiments were combined. The combined data were separated using the Tukey-Kramer multiple comparison test, $P < 0.05$ to detect differences among treatments. Additionally, the time period required to reach 50% seedling mortality was calculated from the respective regression equation of disease progress for each isolate combination. Significant differences of time to reach 50% mortality were calculated by contrast *t* tests ($P < 0.05$), in an analysis of variance for the linear regression.

3.3. Fungal transformation procedure

Electroporation of germinating conidia was performed essentially as previously described (Horowitz *et al*., 2002). Briefly, isolates C.a 149, C.g 342 and C.g 318 were cultured on solid M3S medium for 5 days. Conidia were collected in pea juice (Robinson and Sharon, 1999), adjusted to 10^6 conidia/ml, and incubated at 28ºC for 4.5 to 5 h to initiate germination. The germinated conidia were collected, washed with cold electroporation buffer [1mM n-2-hydroxyethlpiperazine-N-2-ethanesulfonic acid (HEPES), 50 mM mannitol, pH 7.5] and concentrated to 10^8 conidia/ml, and 100-µl aliquots were distributed in cold electroporation cuvettes (Bio-Rad, Hercules, CA, USA). The 5.13-Kb pGH-1 plasmid (Yakoby *et al*., 2001) was used as a transformation vector. Restriction enzyme mediated integration (REMI) was performed with 5 µg of linearized *Xba*I or *Hind*III pGH-1 plasmid for each transformation event (performed in

separate cuvettes) in the presence of 24 units of the same enzyme used to linearize the plasmid. Electroporation was performed with a gene pulser (Bio-Rad, Hercules, CA, USA) operating at 1.4 kV, 800Ω, and 25 µF. After application of the electric pulse, the conidia were transferred to regeneration (Reg) medium [145.7 g mannitol, 4 g yeast extract (Difco Laboratories, Detroit, MI, USA), 1 g soluble starch, 16 g agar (Difco), and 50 ml pea juice per liter]. After 10 h, a top overlay (2 mm) of water agar with 125 µg/ml of hygromycin B (Calbiochem, San Diego, CA, USA) was added. Transgenic colonies appeared 4 to 5 days after transformation. Stability of transformants was assessed by serial transfer of mycelial plugs of representative REMI transformants onto fresh Reg medium containing or devoid of 60 µg/ml of hygromycin B, every 2 weeks for a 2-month period.

3.4. DNA modification and cloning procedures

PCR from genomic DNA was performed in a reaction volume of 50 µl with 1 ng of DNA, 50 pmol of oligonucleotide primers, 25 µM dNTPs, and 2.5 U of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) in 1×PCR buffer (Boehringer Mannheim, Mannheim, Germany). For cloning of the PCR products, the reaction mix was separated on an agarose gel, and the fragment was purified with DNA extraction kit (Biological Industries, Kibbutz Beit Haemek, Israel) and cloned into the T-cloning vector or pGEM-Teasy (Promega, Madison, WI, USA). Bluescript KS (Strategene, La Jolla, CA, USA) or pGEM-Teasy were used for cloning and preparation of various constructs. All DNA modification and cloning procedures were carried out as described (Sambrook *et al*, 1989).

3.4.1. Plasmids used and construction of gene replacement vector

The fungal transformation vector for generating REMI mutants was pgH-1 (Yakoby *et al*., 2001). The plasmid pHA-1.3 (Redman and Rodriguez, 1994) was used as a source for the *E. coli* hygromycin phosphotransferase (*hph*) gene regulated by promoter and terminator elements from the *trpC* gene of *A. nidulans*. The *hph* gene was excised from pHA-1.3 by digestion with *Sal*I, and subcloned into corresponding sites of pBluescript II KS to increase the number of available restriction sites. The resulting vector was named ks:hyg and used as a PCR template to introduce new *Bgl*II and *Kpn*I sites (for primers used, see Table 1). The product was cloned into a pGEM-T vector to form pGEM:hyg. The *hph* gene was then excised with *Bgl*II and *Kpn*I and subcloned into the corresponding sites in the *nir1* plasmid to produce *nir1*:hyg3. In the replacement vector *nir1*:hyg3, the *hph* cassette is flanked on the 5` and 3` ends by 2.7 kb and 0.9 kb, respectively, of sequences homologous to the *nir1* locus. The *nir1*:hyg3 vector was digested with *Bal*I and *SnaB*I at positions 375 bp and 5666 bp respectively, and the resulting 5.3 kb linear fragment was used to transform germinated conidia of *C. acutatum.* The *nir1*:hyg3 vector is shown diagrammatically in Figure 13.

3.5. Isolation and analysis of nucleic acids from *Colletotrichum acutatum* and strawberry plants

3.5.1. Isolation of DNA from *C. acutatum*

Total DNA was extracted based on published protocols (Rodriguez, 1993). Freeze-dried mycelia were ground by mortar and pestle in liquid nitrogen to obtain powdered mycelia. Powdered mycelia were transferred to Falcon tubes and 5 ml of lysis buffer to prepare liquid slurry was added (150mM EDTA, 50mM Tris pH 8, 2% Sarkosyl). Samples were mixed and incubated at 65°C for 30 min, and centrifuged 5 min at 10000

rpm. The supernatant (S/N) was transferred to a new tube containing 0.7 volumes of 20% polyethylene glycol solution (PEG, Mr 8000). The precipitated DNA was centrifuged for 5 min at 7500 rpm, and resuspended in 500 µl of TE buffer (10mM Tris, 1mM EDTA, pH 8). For the removal of RNA, polyphosphates and protein from samples, 0.5 volumes of 7.5 M ammonium acetate were added, samples were mixed, placed on ice for 5 min, and centrifuged for 5 min at 10,000 rpm. The S/N was transferred to a new tube containing 0.6 volumes of isopropanol, mixed gently, and centrifuged for 5 min at 7500 rpm. The DNA pellet was resuspended in 500 µl TE buffer, NaCl to 0.1M and 400 µl of 95% ethanol was added, samples mixed and centrifuged for 5 min at 7500 rpm. The purified DNA pellet was resuspended in 500 µl of TE buffer and maintained at 4°C.

3.5.2. Isolation of RNA from *C. acutatum* and strawberry plants

Total RNA was extracted from fungal mycelia, conidia and appressoria using Tri-reagent (Sigma-Aldrich, Steinheim, Germany). Extraction from strawberry plants at various time points after inoculation and from control non-inoculated plants was performed using the following method described by Folta *et al.*, 2005. Briefly, 1 g of tissue was ground in liquid nitrogen using a mortar and pestle, then incubated in extraction buffer [2% CTAB, 2% polyvinylpyrrolidone, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/ml spermidine, and 2.0% β-mercaptoethanol] at 65°C for 10 min. The samples were cooled to room temperature, an equal volume of chloroform: octonol (24:1) was added and the mixture was homogenized. The organic and aqueous phases were separated by centrifugation at 8000 rpm and the supernatant was vortexed with an equal volume of chloroform: octonol. The phases were again separated by centrifugation and the supernatant was transferred to a clean tube. LiCl

was added to a final concentration of 2.5 M and RNA was precipitated on ice overnight. RNA was then collected by centrifugation at 8000 rpm. The pellet was resuspended in 500 µl SSTE [1 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA] and extracted with an equal volume of chloroform: octonol. The S/N was precipitated with two volumes of ethanol containing 0.3 M sodium acetate, the pellet was washed with 76% ethanol, dried in a Speed Vac, and resuspended in 50 µl 10 mM Tris-HCl (pH 8.0) 2.5 mM EDTA before quantification by spectrophotometry. PolyA$^+$ RNA was isolated from total RNA using the PolyA Kit (Qiagen, Hilden, Germany).

3.5.3. Southern hybridization

For Southern hybridization, 10 µg of digested genomic DNA were subjected to electrophoresis on 1% agarose gels, and transferred to nylon membranes (Hybond N$^+$; Amersham, Little Chalfont, UK). ECL-based Southern hybridizations were performed o.n according to the manufacture's instructions (Amersham Biosciences, Amersham, UK).

3.5.4. Plasmid rescue procedure

To recover pGH-1 (Yakoby *et al*., 2001) and flanking fungal genomic DNA of the integration site, genomic DNA of REMI mutants were digested with *Xba*I or *Hind*III. The digested products were ethanol-precipitated (0.1 M NaCl, two volumes of 95% v/v ethanol) and subsequently subjected to ligation (T4 DNA ligase) in a 100-µl reaction volume. The ligated product was used as a template for inverse PCR (inPCR) with a pair of primers inHPHfor and inHPHrev (Table 1) designed according to the flanking regions of the *hph* cassette. The inPCR was performed in 50-µl mixtures, with the Expand long template PCR system (Roche, Mannheim, Germany). Amplification conditions were as follows: template denaturation for 2 min at 94°C, 10 cycles of 10

sec denaturation at 94°C, 30 sec annealing at 65°C and 30 sec elongation at 68°C, 20 cycles of 10 sec denaturation at 94°C, 30 sec annealing at 65°C and 30 sec elongation at 68°C. The elongation phase was extended by 20 sec in each cycle and the final elongation phase was 7 min at 68°C. The ~2.5-kb PCR product was cloned into a pGEM-T vector and sequenced. Based on the sequences of the flanking region, a genome walking procedure was performed using the Universal genome walker kit (Clontech, Palo Alto, CA, USA). Additional sequence information allowed the design of two sets of terminal primers to isolate full-length clones of the disrupted gene.

3.5.5. Molecular analysis of targeted gene-disruption mutants

Inactivation of *nir1* was performed by one-step gene replacement. To screen for the replacement of *nir1*, genomic DNA of 120 independent *C. acutatum* transformants was analyzed by PCR. Two pairs of primers were used to evaluate gene-disruption events or ectopic integration. The first set consisted of the hphf, based on the *hph* sequence (within the hyg cassette), and Catf2 based on the endogenous *nir1* sequence which is not present in the gene-replacement construct. An event of homologous recombination occurred when a band of 2.8 kb appeared. A second pair of primers was designed, flanking the *hph* cassette (149WT4 at position 2481 and 149HpaI2B at position 4959; Table 1 and Fig.13). In the case of ectopic integration, two bands were expected, at ~2500 bp and 565 bp, whereas homologous recombination occurred when the small band was no longer evident. Subsequently, the *nir1*-replaced strains were tested for pathogenicity and growth properties on minimal medium supplemented with nitrate as the sole nitrogen source.

3.6. Gene expression analysis

3.6.1. Gene expression analysis by relative RT-PCR

Reverse transcription was carried out on 1 µg total RNA treated with RQ1 DNASE (Promega, Madison, WI, USA) using Reverse-iT^{tm} RTase Blend (ABgene, Epsom, UK) with an anchored oligo-dT primer. Reverse-transcribed RNA (cDNA) was stored at -20°C**.** For relative RT-PCR experiments amplification was performed in 50-µl reactions with the following components: 4 µl cDNA, 5 u/µl Taq DNA polymerase, 1x Taq polymerase buffer, 0.2 mM dNTP and upstream and downstream primers to final concentrations of 0.2 µM. RNA samples were tested for the presence of genomic DNA contamination by using extracted treated RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. RT-PCR products were resolved on 1.4% agarose gels, recorded, and saved in tagged information format files for quantification using the NIH Image program software (Macintosh, North Mathews, Urbana, IL, USA).

The genes encoding *C. acutatum* NR (*nit1*) and GS (*gln1*) were cloned by PCR, using primers (MgNR1/MgNR3 and Moco/Nadr for *nit1* and GSfor/GSrev for *gln1*, respectively; Table 1) designed on the basis of conserved sequences. For RT-PCR-based analysis of *nit1* and *gln1* expression levels, we used primers CanRfor/CanRrev and GSfor/GSrev, respectively (Table 1). A fragment of the *C. acutatum* ß-tubulin-encoding gene (*tub2*) was amplified and cloned using primers tub2b and genec (Yarden and Katan, 1993; Table 1). Nucleotide sequence data reported here are available in the GenBank database under the accession numbers GenBank accession numbers DQ192665 (*nir1*), DQ192666 (*nit1*) and DQ192667 (*gln1*).

Table 1. Primers used throughout this study. Suffix of dg referrs to degenerate primers while qrt suffix refers to primers used in quantitative real time (QRT)-PCR experiments.

Primer name	Sequence (5' to 3')	Primer name	Sequence (5' to 3')
tub2B	gacrtccttcatrgcga	Catalase.dg.F	gchtggttcaagctc
genec	gaggaattcccagaccgtatgatg	Catalase.dg.R	acraagaagtcgttsgtca
β-tubulin. qrt.F	taccgacaaaggtggaggac	GPX.dg.F	gccgactacaagggcaaggtc
β-tubulin.qrt.R	aggatgtcgaggaccagatg	GPX.dg.R	ctcrggcttggtsgtgctsgc
T7BglII	taatacagatctcactataggg	GPX.dg.F2	ggncargargtnccnctngcn
SP6KpnI	ccaagctatttaggtaccacta	GPX.dg.R2	yttnagccanacccanagnggrtt
149WT4	tcgatcagaaacttctcgacaga	SOD.qrt.F	ttgtggggattgaagtgagg
149HpaI2B	ggaaacgtccactccaagaca	SOD.qrt.R	ccaccattacctgggacatc
Inhph/for	tcgatcagaaacttctcgacaga	HSP.qrt.F	aagtcctcaccacccaagtg
Inhph/rev	gtactcgccgatagtggaaac	HSP.qrt.R	agaaggaggaggaccgtgtt
CaRTfor	gtaactggtgtcagctccctgac	Proteasome.qrt.F	caagttgartaygcyttcaagg
CaRTcDNA	cgtaggtacctcagtggtgggc	Proteasome.qrt.R	gtgtaracytggctgatgttkgc
CaRTrev	cgacggcggcttcacagcacgcg	Catalase.qrt.F	gtcgcctgctctcaagaagg
C6RT1.for.qrt	gttccaagaatcgacctcca	Catalase. qrt.R	cccaggcagtagagatgagctt
C6RT2.rev.qrt	taataggcggttagccatgc	GPX.qrt.F	gggcaaggtcttctaccaca
MgNR1	aagggyttctchtggggwgcy	GPX.qrt.R	gacatcggtagcgctcttgt
MgNR3	accawgggttrttcatcattc	GPX.qrt.F2	gaggagtcttaccgcgacag
Moco	gaatgatgaayaacccwtggt	GPX.qrt.R2	gattctcaggcttggtggtg
Nadr	gtdatnccngtaccaccrcadatcat	PR3F515	agggcggcaagaaagtttac
CaNRfor	ggaggaaacctggccaacgga	PR3 R-712	gtgcaggaatggctgaagtc
CaNRrev	cgatttaccgcagatcatgac	PR2 R-702	ctggtgaagtgaacaaggcat
GSfor	cccaacattctcgtcctctcc	PR2F575	tcacctttrgggcaacaatg
GSrev	ctcgaagtaaccgtagccct	PR1-R112	cccacattcatcaccaac
Nit1 qrt-for	tcctctagacagcgaaa	PR1-F-28	ggctacggagagatgacag
Nit1 qrt-rev	aggatcttttgggctgctga	ACTIN R-212	agaaggtgtgatgccaaatct
Gln1 qrt-for	atgagtgcgccaaattgatg	ACTIN F-104	gccagaaagatgcttatgtcg
Gln1 qrt-rev	actcctgctcgagaccgaac	CHT1 F-449	gaccgattcaactatcttgg
Catf2	acaaacggtctacttacattgta	CHT1-R569	ctccgtcatccagaaccata
Ca5(1A)	tcatgtgctcttgtaccgct	CHT2 F-520	tggttctggatgactcctcag
hphf	ctgtcgagaagtttctgatcg	CHT2-R683	ttatcttgtccacgcccac
Sod.dg.F	ctttgagcaggagtccgagtc	PR4-R207	tagcaggcatcttccacagg
Sod.dg.R	acccttgccragrtcgtcrgtrcc	PR4-F57	gccacgtaccacctctacaa
Hsp.dg.F	aagatgaaggagaccgcygag		
Hsp.dg.R	acagcctcatcggggttgacggac		
Proteasome.dgF	caagttgartaygcyttcaagg		
Proteasome.dgR	gtgtaracytggctgatgttkgc		

3.6.2. Gene expression analysis by quantitative real-time PCR (QRT-PCR).

For QRT-PCR, reverse transcription was carried out as described for the relative RT-PCR procedure. cDNA samples were diluted 1:10 to the final template concentration for real time PCR. Real time detection was performed with ABsolute SYBR green ROX mix (ABgene, Epsom, UK) in a Rotor-Gene 3000 machine (Corbett Research, Sydney, Australia) and results analyzed with the rotor-gene 6 software. Regions of the genes encoding *C. acutatum* superoxide dismutase (*sod1*),

glutathione peroxidase (*gpx*), bifunctional catalase (*cat2*), heat shock protein (*hsp70*) and proteasome alpha subunit were cloned by primers based on peptide sequences (Table 1). The endogenous control was the β-tubulin gene. The calibrator used was either the appressoria-extracted sample in experiments where nitrogen availability was monitored or extracts from strawberry seedlings at 2-days post–inoculation phase in experiments involving *in planta nit*1, *gln*1 and *tub* gene expression. Primers (10 µM) used for real time experiments are listed in Table 1. Primer efficacy was examined by performing reactions with different concentrations of template and verifying an appropriate slope for a curve of cycle threshold (C_T) values versus the log values of template concentrations. A mixture of all cDNAs of all the treatments was used as template for calibration curves. Relative quantification was calculated on the basis of $\Delta\Delta C_T$. The ΔC_T value was determined by substracting the C_T results for the target gene from the the endogenous control gene C_T and then normalizing as suggested by Rotor Gene ($\Delta\Delta C_T$). The final relative quantification value is $2^{-\Delta\Delta CT}$, which represents the level of expression of the gene in relation to the control (appressoria stage or inoculated seedlings 2-days post-inoculation) treatment. Each experiment was conducted 4 times, with similar results, and results from one such experiment are presented. Mean values of $2^{-\Delta\Delta CT}$ ± standard deviation of the differences of the same cDNA measurements of each treatment were used to compare the means of treatments by LSD, according to the Tukey-Kramer multiple comparison test at $P < 0.05$.

3.7. Protein-related methodologies

3.7.1. Protein extraction

Protein extraction of *C. acutatum* mycelium and appressoria was carried out according to Hurkman and Tanaka (1986) with minor modifications. Dry mycelia,

conidia or appressoria (0.25 g) were ground and homogenized to a very fine powder in a chilled mortar and pestle with liquid nitrogen in 5 mL of the extraction buffer (0.1 M Tris, pH 8.0, 5% sucrose, 2% SDS, 50 mM DTT, 2 mM PMSF) and 100 μL of Complete Inhibitor [1 tablet of complete protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in 1 ml ddH_2O]. The homogenates were kept on ice for 10 min and centrifuged at 10,000 rpm for 30 min at 4ºC. The S/N fraction was collected and transferred into a new tube, and 5 mL of water-saturated phenol solution was added and the mixture was kept on ice, with shaking, for 10 min. The phenol fraction was collected and transferred to a new tube, to which 5 mL of extraction buffer was added. The tube was then kept on ice, shaken for 10 min, re-centrifuged at 10,000 rpm for 30 min at 4°C, and the phenol fraction was again transferred into a new tube. The proteins were precipitated overnight with 25 mL of 0.1 M ammonium acetate in cold methanol at –20 ºC, followed by centrifugation at 10,000 rpm for 30 min at 4°C. The pellet was washed three times with 15 mL of 0.1 M ammonium acetate in cold methanol, then with 15 mL of cold acetone. The air-dried pellet was dissolved in 600 μL of rehydration solution containing 9 M urea, 3% CHAPS, 0.5% Triton X-100, 2% (v/v) IPG buffer, 0.3% (w/v) DTT, and 0.002% bromophenol blue. The protein content was measured with the Bicinchoninic acid protein assay kit (Sigma, St Louis, MO, USA), according to the manufacturer's instructions.

3.7.2. 2-DE Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed with immobilized pH gradients according to the manufacturer's recommendations (Amersham Biosciences, Amersham, UK) with minor modifications. For analytical and preparative gels, the 13-cm IPG strips (pH 4-7) (Amersham Pharmacia Biotech,

Amersham, UK) were rehydrated overnight with 250 µL of rehydration solution containing 50 µg protein at room temperature. The isoelectric focusing step was conducted at 18°C with a Multiphor II apparatus (Amersham Biosciences, Amersham, UK), The running conditions were as follows: 300 V for 15 min, 500 V for 15 min, 1,000 V for 15 min, 1,500 V for 15 min, 2,000 V for 15 min, 2,500V for 15 min, 3,000V for 15 min and 3,500V for 4 h. The focused strips were equilibrated twice for 15 min in 10 mL of equilibration solution; the first equilibration solution contained 50 mM Tris-HCl, pH 8.8, 6 M urea, glycerol 30% (v/v), SDS 2% (w/v), bromophenol blue 0.002%, and 2mM tributylphosphine (TBP); the second equilibration solution was the same except that TBP was replaced with 2.5% (w/v) iodoacetamide. The second dimension was performed by SDS-PAGE in a vertical slab of 12.5% acrylamide with a SE 600 Series Vertical Slab Gel Unit (Hoeffer Scientific Instruments, San Francisco, CA,USA). The protein spots were visualized by staining with Colloidal Coomassie blue G-250. With each protein sample, analysis was performed 3 times, with accurate and reproducible protein pattern produced among technical replicates from independent extractions.

3.7.2.1. Image acquisition and analysis

Gel images were analyzed with Gel matching and data analysis, were performed with Z3 software (Compugen Inc., Nes Ziona, Israel). To ascertain quantitative changes in proteomic maps of each treatment, comparison of absolute quantity of each spot among the treatments were generated by the Delta2D software (version 3.2) (Decodon, Greifswald, Germany). The relative quantity of the spot was computed by setting total spot quantity on a gel to 100%, excluding background. Three images for each treatment were grouped to calculate the averaged quantity of

all the individual protein spots. The numerical expression ratio is the mean of the relative quantity of the 'sample spot' divided by the mean relative quantity of 'master spot', calculated for each gel and spot. These values were used to designate the significant differentially expressed spots. Proteins were regarded as significantly regulated if (i) the corresponding ratios referring to the relative quantity of the spots were changed more than two-fold and if (ii) this regulation pattern was found in all biological and technical replicates. All other proteins were classified as "not regulated". The analysis was re-evaluated by visual inspection, focusing on those spots most dramatically altered between treatments, and consistent in all replicates. Standard deviation of the mean of relative quantity of each spot was determined. Spots showing consistent differential expression patterns between treatments were excised from gels, digested and subjected to LC-MS/MS analysis to determine protein identity.

3.7.2.2. In gel proteolysis

The proteins in the gel were reduced with 10 mM DTT (60ºC for 30 min) and modified with 100 mM iodoacetamide in 10mM ammonium bicarbonate (room temperature for 30 min). The gel pieces were dehydrated with acetonitrile and rehydrated with 10% acetonitrile in 10 mM ammonium bicabonate containing trypsin [modified trypsin (Promega, Madison, WI, USA) at a 1:100 enzyme-to-substrate ratio. The gel pieces were incubated overnight at 37^{o}C and the resulting peptides were recovered and analyzed.

3.7.2.3. Mass-spectrometry analysis of proteins

Electrospray-ion-trap analysis of proteins was performed at the Smoler Proteomics Center (Department of Biology, Technion – Israel Institute of

Technology, Haifa, Israel). The tryptic peptides were resolved by reverse-phase chromatography on 0.1 X 200-mm fused silica capillaries (J&W, 100 micrometer ID) packed with Everest reversed phase material (Grace Vydac, CA, USA). The peptides were eluted with linear 50 minute gradients of 5 to 95% of acetonitrile with 0.1% formic acid in water at flow rates of 0.4 µl/min. Mass spectrometry (MS) was performed by an ion-trap mass spectrometer (LCQ-DecaXP, Finnigan, San Jose, CA, USA) in a positive mode, using repetitively full MS scan followed by collision induced dissociation (CID) of the 3 most dominant ions selected from the first MS scan. The MS data was clustered and analyzed using the Sequest software (J. Eng and J.Yates, University of Washington and Finnigan, San Jose, CA, USA) or Pep-Miner (Beer *et al*., 2004) searching the NR-NCBI or specific databases. In addition, accuracy of identification of individual peptides was assessed visually by a trained operator. A peptide was considered as high quality if its Pep-Miner identification score was greater than 80, corresponding to the sequest Xcore of >1.5 for single charged peptides, >2.5 for double charged peptides and >3 for triple charged peptides. To determine the possible functions and classification of hypothetical proteins, the sequence information was used to search for conserved domains (CDs) on-line CD-search in the NCBI database. The CD with the highest score was listed as the CD for the respective hypothetical protein.

3.7.3. Staining to detect SOD activity

The activities of all three types of superoxide dismutase [$CuZn^-$, Mn^- and Fe^- SOD] were detected on a non-denaturing polyacrylamide gel by negative staining (Hwang *et al*., 2002). The gel was incubated with gentle shaking in 50mM phosphate buffer (pH 7.8) for 10 min, in nitro-blue-tetrazolium solution (1 mg/ ml) for 10 min

and then in 50 mM phosphate buffer (pH 7.8) containing 0.01 mg/ ml riboflavin (Sigma Aldrich, Steinheim, Germany) and 3.25 mg/ml N,N,N',N'-tetramethylethylenediamine (TEMED) for 10 min at room temperature. Areas of SOD activity remained clear when the gel was exposed to the light. Two bands of SOD activity were identified according to Dolashka–Angelova *et al*., (1999). Gel was scanned immediately after the photochemical reaction and pictures were saved in tagged information format files for quantification using the NIH Image program.

3.8. Intracellular ROS and lipid body detection

Intracellular levels of ROS in *C. acutatum* were monitored with the oxidant-sensitive probe 2', 7'-dichlorofluorescin diacetate (H_2DCFDA) (Molecular Probes, Leiden, The Netherlands) an established compound used to detect and quantify intracellular produced H_2O_2. Ten µM H_2DCFDA in 50mM MES buffer pH 6.2 (Duchefa Biochemie, Haarlem, The Netherlands) was used to detect ROS generation. For measurement of ROS generation upon nutritional induction, 80 µl of conidia were resuspended in Reg medium or in water containing 5mM of the various nitrogen-containing compounds (glutamine, urea, nitrate, nitrite or ammonium sulfate), allowed to germinate in Microtest plates (Sarstedt, Inc. Newton, NC, USA) at various intervals during a 16 hr period, before staining for 1 hr with the H_2DCFDA probe. Then, fluorescence levels were measured by a Fluorimeter (Microplate Fluorescence Reader FL600; BIO-TEK, Winooski, Vt., USA). Fluorescence levels were corrected by substracting the fluorescence background of each medium and were divided by ROS levels measured for water treatment, to get relative fluorescence values. Following ROS production *in planta***,** slices were prepared from inoculated strawberry stolons and leaves 12-72 hr post inoculation (p.i) and subjected to the fluorescent dye for 1 hr

before confocal microscopic observation. Lipid droplets in germinating conidia and appressoria were visualized by staining with a Nile Red solution (Sigma Aldrich, Steinheim, Germany) as described by Thines *et al*. (2000). Cytological analysis was performed with freshly harvested conidia, incubated under different nutritional conditions. Suitable material was mounted directly in 5 µg mL^{-1} Nile Red in glycerol solution. This solution was prepared from an initial solution of 1 mg/mL Nile Red in acetone. Within a few seconds lipid droplets began to fluoresce.

3.9. Pharmacological applications

3.9.1. Antioxidant treatments

For all pharmacological treatments, conidia were resuspended with antioxidant solutions at various concentrations: N-acetyl-L-cysteine (0.005 -1mM), L-proline (0.5-4mM) and mannitol (1-1000mM) (Sigma Aldrich, Steinheim, Germany) and allowed to germinate for 4-12 hr in Microtest plates (Sarstedt, Inc. Newton, NC., USA) for fluorescent measurements or in glasstic slides for determining percent appressoria formation. Assays for induction of appressorium formation and germination upon antioxidant treatments, were performed as indicated for the appressoria induction assay.

3.9.2. cAMP, caffeine, and PKA inhibitor KT5720 pharmacological treatments

cAMP was purchased from Calbiochem, caffeine and KT5720 were purchased from Sigma. Stock solutions of cAMP and caffeine were prepared in dH_2O whereas stocks of KT5720 were prepared in dimethyl sulfoxide. Assays for induction of appressorium formation and germination by Ca5 mutant and w.t strains in the

presence of PKA agonists and the inhibitor were performed on glasstic slides, as previously described.

3.10. Light, fluorescence and confocal imaging

For infection assays, stolons and leaves of 2-wk-old strawberry daughter plants cv. Malach, an anthracnose-susceptible strawberry cultivar, were inoculated with a suspension of *C. acutatum* conidia at a concentration of 10^5 conidia mL^{-1}. Individual 10 µl drops of inoculum suspension were placed on strawberry stolons and leaves and incubated for 12-72 hr at 25°C in a moist chamber, and samples were removed at 12, 24, 48 and 72 h intervals for observation. Slices were sampled from beneath the site at which inoculum droplets were applied and directly mounted, in H_2DCFDA probe for microscopic examination of ROS. Alternatively, *in-vitro* germinating conidia upon different nutritional treatments were examined for ROS production or lipid bodies detection by microscopic observation. All microscopic observations and image acquisitions were performed using the Olympus IX-81 FluoView FV500 confocal microscope (Olympus Optical Co., Tokyo, Japan) equipped with a 488nm argon-ion laser. Transmitted light images were obtained using Nomarski differential interference contrast (DIC). For ROS detection samples were excited by 488nm light and the emission was collected through a BA 505-525 filter. For autofluorescence, a BA 660 IF emission filter was used. For lipid bodies detection samples were excited by 543 HeNe laser and image acquisition was accomplished using an E600LP (long pass) emission filter.

RESULTS

4.1. DEVELOPMENT OF A ROBUST SCREENING METHOD FOR PATHOGENICITY OF *COLLETOTRICHUM* SPP. ON STRAWBERRY SEEDLINGS ENABLING FORWARD GENETIC STUDIES

In order to establish a rapid and reliable bioassay for large-scale screening of putative non-pathogenic *Colletotrichum* mutants on strawberry the effect of incubation temperature, seedling age, inoculation technique and isolate species were evaluated to determine the conditions best suited for this purpose. The efficacy of the proposed method was verified, culminating in the isolation of reduced-pathogenicity mutants.

4.1.1. Disease incidence in strawberry seedlings

Disease incidence and symptom development in the inoculated seedlings were monitored over 20-day periods. After this period, lower leaves closest to the water began to senesce and rot in water and n-path *C. magna* control treatments. Therefore, the experiments were terminated at this stage. Seedling mortality was determined as a binary test (dead or live seedling) and not by a disease severity rating (DSR) scale, which was used later on mature plants inoculated with putative non-pathogenic mutants. Local anthracnose lesions on leaves and petioles were monitored but seedling mortality was recorded only when all seedling petioles collapsed, which led to seedling death. Disease progress in the seedlings inoculated with the n-path isolate was similar to water controls and was significantly lower than in seedlings inoculated with the pathogenic w.t isolates (Fig. 4A).

4.1.1.1. Effect of seedling age on disease response

The effect of seedling age on disease response was evaluated using 12–wk-old (young) and 15-wk-old (older) seedlings. A rapid disease response, similar for young

and older inoculated seedlings was observed within 4 days, by the foliar dip method, with isolates C.g 318 of *C. gloeosporioides* and C.a 149 of *C. acutatum* (Fig. 5A). No significant differences were observed in time required to reach 50% seedling mortality of young and older seedlings, inoculated by the foliar dip method at 25ºC (Table 2).

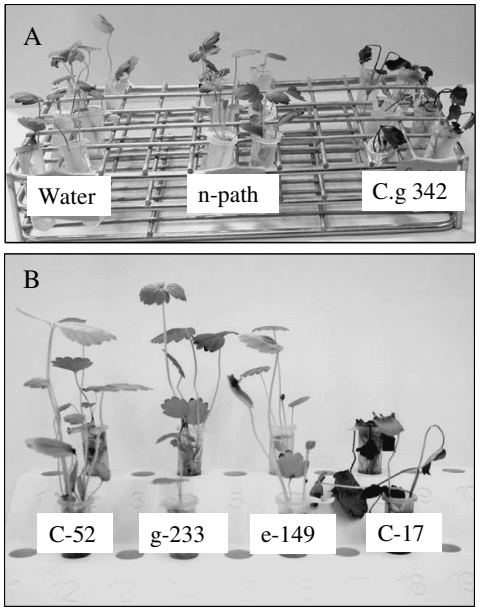

Figure 4. Disease incidence 16 days after inoculation with different *Colletotrichum* spp. and strains. (A) Young seedlings inoculated with nonpathogenic isolate (n-path) of *Colletotrichum magna*, pathogenic isolate C.g 342 of *C. gloeosporioides*, and water control, and (B) reduced pathogenicity mutants (c-52, g-233, e-149) and pathogenic mutant (C-17) of C.g 342 of *C. gloeosporioides*. Seedlings were inoculated by the foliar dip method in a conidial suspension (10^5 conidia/ml) of each isolate and incubated at 25ºC.

However, seedling mortality decreased significantly in older seedlings compared to young seedlings inoculated by the root soak method, with both pathogenic isolates (Fig. 5B). Mortality of young seedlings was observed from 4 days after root inoculation, whereas that of older seedlings was delayed, occurring 8 days after inoculation (Fig. 5B).

4.1.1.2. Effect of inoculation method on disease response

Foliar dip and root soak inoculation methods were evaluated for their effect on subsequent disease development. Young and older seedlings inoculated by the foliar dip method developed typical anthracnose lesions on foliage and petioles within 2 to 3 days. However, seedlings inoculated by the root soak method did not develop lesions on foliage but exhibited active necrotic lesions on lower petioles that resulted in rapid

seedling collapse at 25ºC. The root soak technique resulted in rapid seedling collapse but minimal anthracnose symptoms were observed on foliage. No significant differences were observed in time required to reach 50% disease incidence of young seedlings inoculated by the two methods at 25°C (Table 2). In older seedlings, foliar dip produced more rapid disease progress than root soak (Figs. 5A and B) and time required for 50% mortality was significantly less for seedlings inoculated with most isolates by foliar dip than by root soak at 25ºC (Table 2).

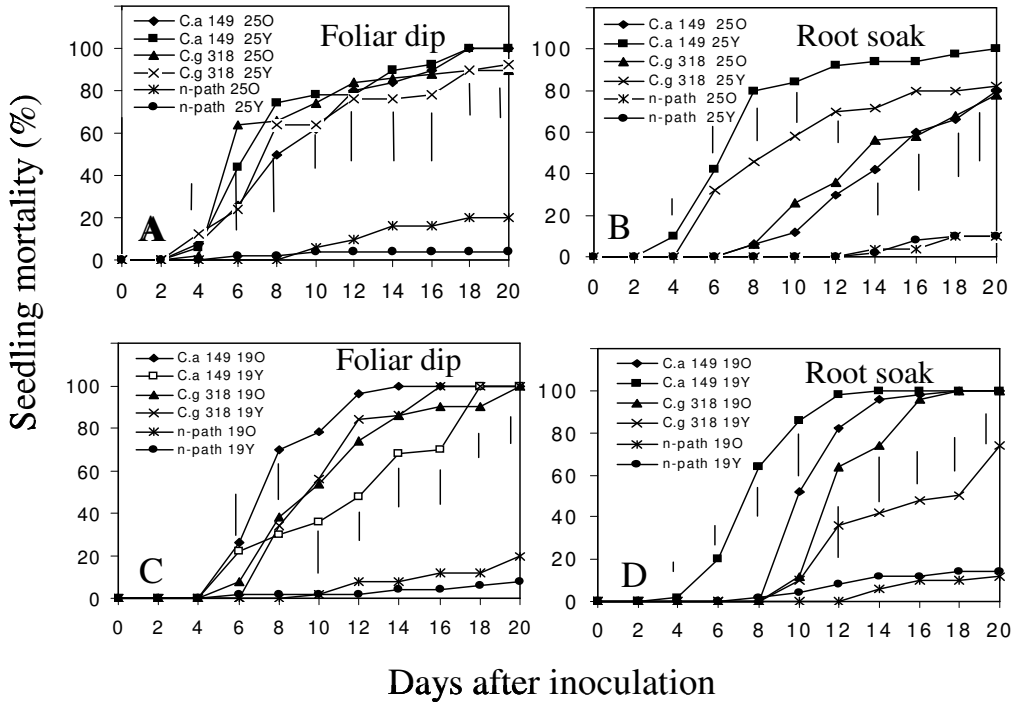

Figure 5. Disease incidence in strawberry seedlings inoculated with wild type isolates C.g 318 of *Colletotrichum gloeosporioides*, C.a 149 of *C. acutatum* and non-pathogenic u.v. mutant, n-path of *C. magna* at both inoculation method, seedling ages and incubation temperatures. A. Foliar dip of young (Y) and older (O) seedlings at 25ºC. **B.** Root soak inoculation of young (Y) and older (O) seedlings at 25ºC. **C.** Foliar dip of young (Y) and older (O) seedlings at 19ºC. **D.** Root soak inoculation of young (Y) and older (O) seedlings at 19ºC. Each datum point is the mean of two individual experiments. Distances, larger than the vertical bars between symbols at each point are significant $P<0.05$, according to Tukey Kramer multiple comparison test.

Table 2. Time required to reach 50% mortality in young (12 wk) and older (15 wk) strawberry seedlings. Seedlings were inoculated by the root soak and foliar dip at different incubation temperatures with isolate C.a 149 of *Colletotrichum acutatum* or isolate C.g 318 of *C. gloeosporioides*.

Isolate	Inoculation method	Incubation temperature (°C)	Days to reach 50% mortality[x] young	older
C.a 149	Foliar dip	25	8.2 a[y]	9.2 ab
C.a 149	Root soak	25	7.7 a	15.3 c
C.a 149	Foliar dip	19	11.2 b	8.2 a
C.a 149	Root soak	19	8.2 a	10.2 b
C.g 318	Foliar dip	25	9.5 ab	8.3 ab
C.g 318	Root soak	25	10.5 b	14.5 c
C.g 318	Foliar dip	19	9.8 b	10.1 b
C.g 318	Root soak	19	16.9 c	11.4 b

[x]Values for 50% seedling mortality were calculated from the respective regression equation of disease progress for each seedling age × inoculation method × isolate × incubation temperature combination.
[y]Values with the same letters do not differ significantly ($P < 0.05$) according to contrast *t*-test.

4.1.1.3. Effect of incubation temperature on disease response

Temperatures of 19ºC and 25ºC were evaluated for their effect on disease development. The onset of seedling mortality occurred 2 days later at 19ºC compared to 25ºC for both young and older seedlings inoculated by foliar dip (Fig. 5A, C). Significant differences in time required to reach 50% mortality of seedlings were observed only for young seedlings inoculated with C.a 149 by the foliage method at both incubation temperatures (Table 2). Disease progress was slower for young and older seedlings inoculated with *C. gloeosporioides* at 19ºC by the root soak method, than that in young seedlings inoculated with isolate of *C. acutatum* (Fig. 5D).

4.1.1.4. Effect of inoculum concentration on disease response

Inoculum concentrations of 10^4, 10^5 and 10^6 conidia/ml of three isolates C.a 149, C.g 318 and n-path of *C. magna* were assessed for disease response (Table 3). Significant differences in disease progress were observed for isolate C.g 318 of *C.*

gloeosporioides at all three inoculum concentrations. Significant differences were also observed for C.g 318 in the time required to reach 50% seedling mortality of 13.8, 9.5 and 6.4 days for 10^4, 10^5 and 10^6 conidia/ml, respectively. Similar trends were observed for isolate C.a 149 of *C. acutatum* at 10^4 and 10^5, 10^6 conidia/ml, corresponding to 10.4, 8.2 and 8.0 days, respectively (Table 3).

Table 3. Time required reaching 50% mortality in young strawberry seedlings inoculated by the foliar dip method with three different inoculum concentrations. *Colletotrichum* isolates used were C.a 149 of *Colletotrichum acutatum* and C.g 318 of *C. gloeosporioides*

Isolate	Species	Inoculum concentration (conidia/ml)	Time (days)[x]
C.g 318	*C. gloeosporioides*	10^4	13.8 a[y]
C.g 318	*C. gloeosporioides*	10^5	9.5 b
C.g 318	*C. gloeosporioides*	10^6	6.4 c
C.a 149	*C. acutatum*	10^4	10.4 d
C.a 149	*C. acutatum*	10^5	8.2 b
C.a 149	*C. acutatum*	10^6	8.0 b

[x]Values for 50% seedling mortality were calculated from the regression equation of disease progress for each inoculum concentration and isolate combination.
[y]Values with different letters are significantly different based on results of contrast *t* tests ($P < 0.05$).

4.1.2. Screening for reduced-pathogenicity mutants

The foliar dip method was evaluated for detecting reduced pathogenicity of REMI mutants in comparison to the n-path mutant of *C. magna*. Significant differences in mortality were observed in seedlings inoculated with the w.t C.g 342 of *C. gloeosporioides* compared to REMI mutant isolates c-52, c-63, e-149 and g-233 (Fig. 4B, Fig. 6A). Furthermore, w.t isolate L2.5 of *C. magna,* pathogenic on cucurbits, did not cause disease in strawberry seedlings (Fig. 6A). REMI mutants that exhibited reduced pathogenicity after three seedling screening tests were used to inoculate mature daughter plants. Disease severity values were significantly lower for plants inoculated with c-52, c-63, e149, and g233 compared to w.t isolate C.g 342 (Table 4).

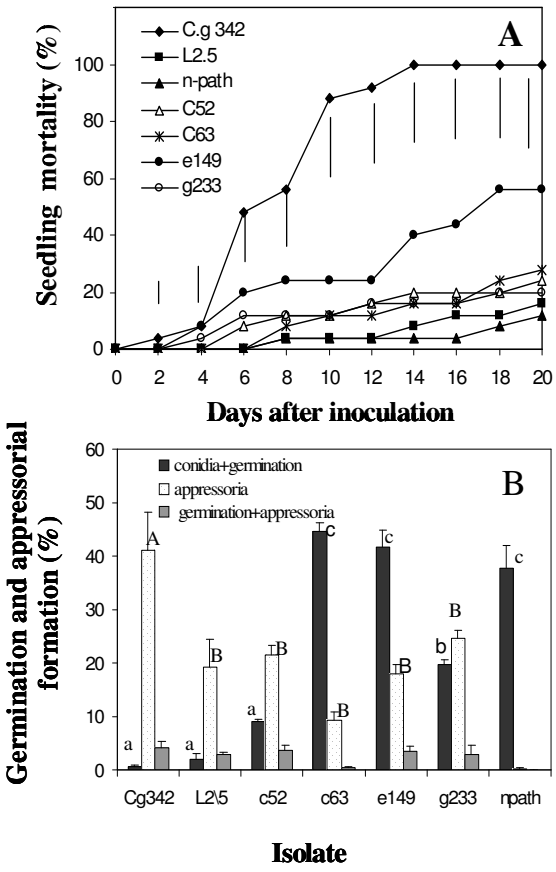

Figure 6. Disease response and germination phenotype of reduced pathognicity mutant and control isolates. **A**. Disease response of strawberry seedlings inoculated with mutant isolates of *Colletotrichum gloeosporioides* and control isolates L2.5, a nonpathogenic mutant (n-path) of *C. magna* and C.g 342 of *C. gloeosporioides*. Distances between symbols at each point that are larger than the vertical bars are significant, $P \leq 0.05$. **B.** Assessment of conidial germination and appressorial formation compared to w.t isolates. Each column in B is the mean of three individual experiments. Bars represent standard error of the mean and common letters are not significantly different ($P \leq 0.05$), according to the Tukey-Kramer multiple comparison test, for each of the parameters (conidia + germination, appressoria) between isolates.

4.1.2.1. Assays for germination and appressorium formation

Significant differences in appressorium formation were observed between the n-path mutant compared to that of w.t L2.5 isolate of *C. magna.* Significant differences were also observed between the REMI reduced-pathogenicity mutants and the corresponding w.t C.g 342 isolate of *C. gloeosporioides*. In n-path and REMI mutants c-63, e-149 and g-233, conidia tended to form long germ tubes without appressoria at significantly higher rates than their corresponding w.t isolates (Fig. 6B).

4.1.2.2. Molecular characterization of reduced-pathogenicity mutants

Five significantly reduced virulence REMI mutants were identified by screening above 1000 transformants. As the efficient recovery of disrupted genes relies on the insertion of a single plasmid into an appropriate restriction site, putative REMI

mutants were screened by Southern blot analysis. Southern blot analysis permitted the estimation of the vector copy number present in the genomes of each mutant. Except for mutant c-63, all the rest of the transformants comprised a single insertion vector. Isolation of the tagged genes was performed by inPCR reactions and subsequent elongation of the gene by a genome walker technique.

Table 4. Response of mature strawberry daughter plants to inoculation with various REMI mutants of *Colletotrichum gloeosporioides* and *C. acutatum* reduced in pathogenicity, relative to wild-type isolates.

Species	Isolates	DSR[x]
C. gloeosporioides	C.g 342	4.25 a[y]
C. magna	N-path	0.00 b
C. gloeosporioides	c-52	0.50 b
C. gloeosporioides	c-63	1.25 b
C. gloeosporioides	e-149	0.75 b
C. gloeosporioides	g-233	0.25 b

[x]Disease severity rating (DSR) of daughter plants was scored 2 weeks after inoculation for disease symptoms according to a 0-5 scale (0=no visible lesions, 5=dead plants), values represent the means for 10 plants per isolate.
[y]Values with different letters are significantly different at $p \leq 0.05$ according to the Tukey-Kramer multiple comparison test.

The Disrupted genes were identified in these reduced pathogenicity mutants. In mutant g233, the transforming vector was inserted in a gene encoding a protein (274 aa) which is similar to a conserved hypothetical protein of *Gibberella zeae* (XP-384540). Mutant e149 was disrupted in a gene encoding a protein similar to hypothetical protein of *Gibberella zeae* (XP-384894) with unknown function. The tagged gene of mutant c-44 had no known homologs in filamentous fungi and in the database. Mutant Ca5 was disrupted in a gene encoding a transcription factor involved in nitrogen metabolism. The Ca5 mutant became the focus of this research. Further characterization of these genes may advance our knowledge in factors affecting pathogenicity and perhaps reveal novel candidates of fungal virulence factors.

4.2. A DEFECT IN *nir1,* A *nirA*-LIKE TRANSCRIPTION FACTOR, CONFERS MORPHOLOGICAL ABNORMALITIES AND LOSS OF PATHOGENICITY IN *COLLETOTRICHUM ACUTATUM*

Molecular analysis of the Ca5 mutant permitted identification of the first pathogenicity gene *nir1*, isolated from strawberry-infecting *C. acutatum*, which encodes a protein involved in nitrogen metabolism. These findings and the fact that the Ca5 mutant is blocked at a very early phase of the infection cycle, led to examination of nitrogen availability effect on fungal morphogenesis and pathogenicity.

4.2.1. Characterization of the Ca5 mutant phenotype

The Ca5 REMI mutant, impaired in its pathogenicity on strawberry, did not exhibit any significant differences in radial growth or conidiation compared to the w.t, when cultured on M3S, considered to be a rich growth medium. Unlike the w.t, which produced symptoms within 4-5 days on strawberry seedlings, the mutant strain was unable to produce symptoms 2 wk following spray inoculation (Fig. 7A, B). Inoculation of stolons with the Ca5 strain produced epiphytic hyphae, in contrast to the necrotic lesions formed by the w.t, one wk after inoculation (Fig. 7C, D). The Ca5 strain was able to grow necrotrophically and conidiate on leaves and stolons when conidia were inoculated directly, by drop inoculation, onto wounded sites. Furthermore, inoculation with M3S medium plugs of w.t and the Ca5 strain, supported massive epiphytic growth which culminated with entry to the plant tissue and development of necrotrophic symptoms (Fig. 7E, F). When placed on a plastic surface or strawberry leaves, most of the germinating w.t conidia produced appressoria in close proximity to the conidia, within 12 h (Fig. 8A, C). In contrast, the Ca5 mutant formed elongated germ tubes and underwent branching prior to

appressorium formation on plastic and strawberry leaf surfaces (Fig. 8B, D). At all tested times between 1-14 day post-inoculation, the Ca5 mutant was recovered from strawberry tissues on selective media containing hygromycin B, indicating that it can survive on the host without causing plant necrotrophy and wilting. Microscopic observation of strawberry inoculated with the Ca5 mutant revealed that fungal hyphae were restricted to the host cuticle, the epidermis and subepidermal layers. The Ca5 mutant was unaffected in its germination rates on rich media (95.3% ±3.4) compared to the w.t (92.5%±2.5), or on MM supplemented with urea (92.2%±5.4 for the w.t and 90.5%±4.3 for the Ca5 mutant). In both strains, presence of preferred nitrogen sources increased conidial germination (characterized by the formation of long germ tubes and branching) compared to that observed in water (Fig. 8E, F).

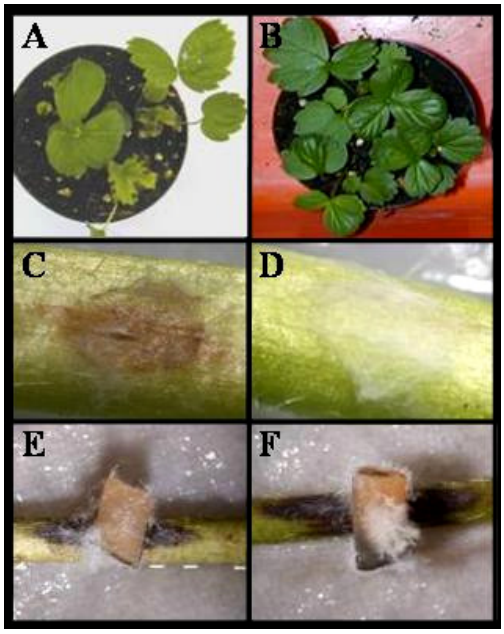

Figure 7. Pathogenicity phenotypes of wild type and Ca5 mutant. **(A)** w.t and non-pathogenic mutant Ca5 **(B)** strains of *Colletotrichum acutatum* on a susceptible strawberry cultivar, 2 wk after spray inoculation of non-wounded plants with 10^5 conidia/ml. Drop inoculation of non-wounded strawberry stolons with conidia of w.t **(C)** and Ca5 mutant **(D)**, and M3S-medium plugs of w.t **(E)** and Ca5 mutant **(F)** on non-wounded stolons, one wk after inoculation.

The ability of Ca5 and the w.t to form appressoria was further examined on a plastic surface (in the presence of water). Conidia of Ca5 germinated normally in water but formed appressoria only at a low frequency (Fig. 8B, D): 88% (±6.6) of the germinating Ca5 conidia germinated without appressorium formation, while 6.5% (±4.1) germinated and formed elongated germ tubes with subsequent appressorium

formation. This is in contrast to the w.t strain, where 94.9 % (±4.1) of the germinating conidia produced adjacent appressoria and only 2.6% (±0.9) germinated with subsequent appressorium formation. Similar results were also observed when conidial suspensions of the w.t and Ca5 strains were placed on strawberry plants (Fig. 8C, D).

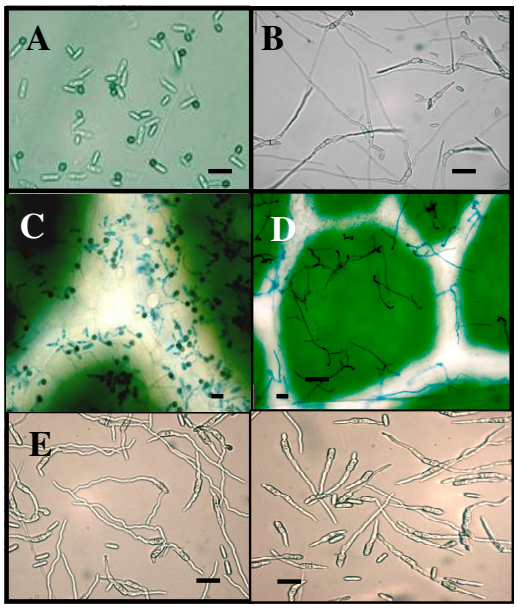

Figure 8. Germination phenotype of the wild type and Ca5 mutant. W.t and non-pathogenic mutant Ca5 strains of *Colletotrichum acutatum* conidia were resuspended in minimal medium lacking a nitrogen source. **A** and **B** – w.t and Ca5, respectively, on a plastic surface. **C** and **D** – w.t and Ca5, respectively, on strawberry leaves, 12 h after drop inoculation. **E** and **F** – w.t and Ca5, respectively, on a plastic surface, in the presence of urea. Bar = 20 μm.

4.2.1.1. Ca5 is affected in a putative $Zn(II)_2Cys_6$ transcriptional activator encoding gene

To determine the genetic nature of the mutant strain, Ca5 genomic DNA was digested with *Xba*I (the enzyme used for linearization of the REMI vector was *Hind*III) and subjected to Southern analysis. The presence of one hybridizing band (~7.2 kb) indicated the occurrence of a single-copy insertion event (data not shown). Isolation of flanking genomic DNA was conducted by inverse PCR with inverted primers originating from the *hph* gene cassette. The 2.5-kb PCR product was cloned, sequenced and used to design primers for walking downstream along the genomic DNA. Hybridization patterns of genomic DNA digested with several restriction enzymes consistently indicated the presence of a single copy of this sequence in the *C. acutatum* genome. The entire gene (~3.8 kb in length) was cloned and fully sequenced. The genomic DNA sequence indicated that the gene contains seven

putative introns (based on conserved splice signals) and encodes a protein of 793 aa (Fig. 9). The deduced protein displays the characteristics of a transcriptional activator belonging to the zinc cluster family. The first domain is a typical zinc binuclear cluster ($Zn(II)_2Cys_6$) comprising six cysteine residues located at the N terminus of the protein followed by a basic and acidic region. Downstream of the zinc cluster, a fungal transcriptional activator motif is present, starting at position 299. The zinc cluster domain is highly similar to the *Aspergillus fumigatus* putative C6 transcriptional activator (60% identity within the zinc cluster domain), indicating that the *C. acutatum* protein is a putative transcriptional activator belonging to the fungal zinc cluster family. Moreover, the predicted protein had high sequence similarity to specific fungal transcription factors analyzed in *N. crassa* (NIT4; Fu *et al*., 1989) and *A. nidulans* (NIRA; Burger, *et al*., 1991) with 48% and 51% identity, respectively, and may be involved in nitrogen metabolism (Fig. 9). The *C. acutatum* gene was thus designated *nir1*. Sequencing results indicated that the REMI vector was inserted in the Ca5 mutant at nucleotide 1156 of the predicted *nir1* gene. To assess whether the mutation in *C. acutatum nir1* affects nitrogen metabolism in this fungus, the mutant was cultured on minimal media containing nitrate, nitrite or hypoxanthine as the sole nitrogen source. The Ca5 mutant grew as well as the w.t on hypoxanthine but failed to grow on nitrite or nitrate, in contrast to the w.t, which developed normally under the latter conditions (Fig. 10).

4.2.1.2. Expression analysis of *nir1* in the wild type and mutant strains

To assess *nir1*'s involvement in nitrogen metabolism, we followed its expression in cultures grown in minimal medium supplemented with different nitrogen sources. Quantitative RT-PCR (QRT-PCR) experiments with primers

C6RT1qrt listed in Table 1 demonstrated that *nir1* is expressed at low levels (as indicated from later cycle threshold values (C_{TS}). *nir1* expression increased in the presence of available glutamine, or nitrate, as sole nitrogen sources. *nir1* expression levels measured after prolonged growth in the presence of nitrate as the sole nitrogen source were significantly ($p<0.05$) higher than those detected in cultures grown in M3S or Reg rich medium or in dormant conidia (Table 5).

```
gal4  9  QACDICRLKKLKCSKEKPKCAKCL-KNNWECRYSPK 43
quta  45 RACDSCRSKKDKCDGAQPICSTCA-SLSRPCTYRAN 80
nir1  25 RACQECRRRRAKCDGQTPSCARCQ-RRDKTCVFTNE 60
c6    16 KACEECRRRRAKCDGKKPSCSRCL-QWSISCQYSST 49
nit4  52 TACIACRRRKSKCDGALPSCAACASVYGTECIYDPN 86
nirA  40 TACIACRRRKSKCDGNLPSCAACSSVYHTTCVYDPN 75
         **  **  ::  **.   * *:. *       * .:
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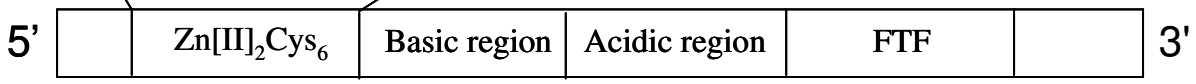

Figure 9. Schematic illustration of *nir*1 gene along with sequence alignment of the fungal zinc finger cluster. The predicted NIR1 protein of *Colletotrichum acutatum* harbors a zinc finger cluster domain followed by a basic and acidic region. At position 299 a fungal transcription factor (FTF) motif is present. Sequence alignment of Zn(II)2Cys6 domains from NIR1 and comparable subdomains (35 residues in total) from other fungal zinc cluster transcriptional activators were analyzed by a CLUSTALW program. Sequences shown are from: NIRA, *Aspergillus nidulans*; NIT4, *Neurospora crassa*; QUTA, *Aspergillus nidulans*; Gal4, *Saccharomyces cerevisiae*; C6, *Aspergillus fumigatus*; and NIR1, *Colletotrichum acutatum.* Identical amino acids are marked by asterisks and similar residues indicated by dots.

Expression of *nir1* at the appressorium-formation stage increased by 12-fold, relative to expression in dormant conidia (Table 5). Under similar experimental conditions, *in planta nir1* transcript levels were undetectable. To determine whether *nir1* was at all expressed in the Ca5 mutant, we performed RT-PCR analysis (utilizing primers CaRTfor and CaRTrev; Table 5, Fig. 11).

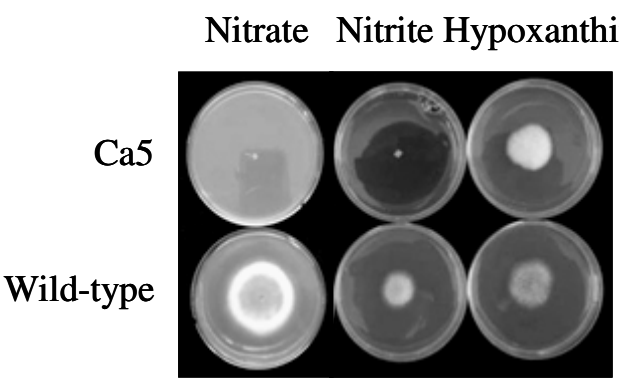

Figure 10. Growth phenotype of w.t of *Colletotrichum acutatum* and non-pathogenic mutant Ca5 strains on minimal media containing nitrate, nitrite or hypoxanthine as sole nitrogen sources.

Table 5. Changes in gene expression levels of fungal *nit1*, *gln1* and *nir1*, relative to the β-tubulin reference gene *in planta* and in culture (under different nutritional conditions) as determined by QRT-PCR.

	Fold Change ($2^{-\Delta\Delta C_T}$)[a]		
Gene Treatment	*nit1*	*gln1*	*nir1*
in planta			
Non-inoculated plants	nd[b]	nd	nd
2 days p.i.[c]	1	1	nd
3 days p.i.	1.27±1	0.54±0.07	nd
4 days p.i.	2.08±0.54	2.51±0.72	nd
In culture[d]			
Reg	0.36±0.03	1.13 ±0.04	0.09±0.04
M3S	0.29±0.1	5.9±0.8	0.12 ±0.02
Nitrate	2.7±0.22	4.32±0.09	13±4.02
Glutamine	2.6±0.4	0.47±0.16	2.49±0.1
Nitrate 24h	7.4±2.3	13.7±0.52	1.11±0.2
Appressoria	1	1	1
Conidia	0.12 ±0.03	6.3±0.7	0.08±0.2

[a] cDNA was synthesized from 1 μg total RNA. All samples were analyzed in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template). C_T, the cycle at which the amplification curve reaches threshold fluorescence, was used to determine the relative amount of transcript. Averaged Ct values were then normalized (to adjust for different amounts of cDNA within each reaction) to the endogenous control gene, β-tubulin. Fold change in target genes was determined by the $2^{-\Delta\Delta Ct}$ method, where $-\Delta\Delta Ct = (C_{tTarget} - C_{t\beta\text{-tubulin}})_{treatment} - (C_{tTarget} - C_{t\beta\text{-tubulin}})_{control}$. The appressoria-extracted sample was used as the control in experiments where nitrogen availability was monitored or extracts from seedlings at the 2-days post–inoculation phase in experiments involving *in planta* expression analysis. Mean values of $2^{-\Delta\Delta Ct}$ ± standard deviation of the differences of the same cDNA measurement of each treatment are presented.

[b] nd=not detected

[c] p.i.=post inoculation

[d] Unless stated otherwise, RNA was extracted from 5 day-old cultures. Appressoria were collected from 16 h-old conidial germlings forming appressoria on glass plates.

Results (Fig. 11) indicated that the gene is expressed but the transcript size (500 bp) is smaller than that of the w.t (720 bp). Thus, the insertion event of vector pGH-1 in Ca5 altered expression of the *nir1* gene, but did not totally abolish it. In addition, *nit1* transcript was not detected in the Ca5 strain (unlike in the w.t cultured under the same

conditions), suggesting insufficient activity of the NIR1 transcriptional activator in the mutant. In all cases, the expression of *gln1* was evident (Fig 11).

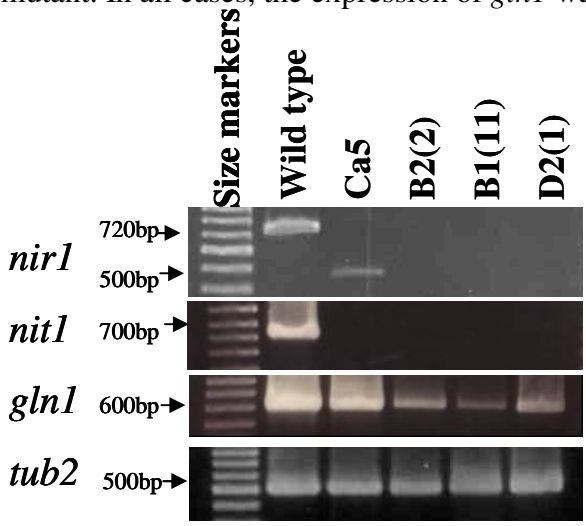

Figure 11. Expression profiles of *nit*1, *nir*1 and *gln*1 in wild type and gene disruption mutants. Expression of *nit1* and *gln1* in the Ca5 mutant and gene disrupted mutants compared to the w.t strain. Cultures were initially grown in M3S rich medium and subsequently transferred to minimal medium supplemented with 5 mM glutamine for 2 days, prior to RNA extraction. Primers used for the specific amplification are listed in Table 1. RT-PCR products were analyzed on 1% agarose gel after 30 cycles.

4.2.2. Effect of nitrogen source on morphology and pathogenicity of *C. acutatum*

W.t conidia resuspended in water or minimal medium lacking a nitrogen source tended to form appressoria adjacent to the conidia on strawberry leaves within 12 h, followed by the appearance of typical anthracnose symptoms (Fig. 12A). However, inoculation with the Ca5 mutant under the same conditions did not result in lesion development (Fig 12B).

Figure 12. Germination and appressorium formation of *Colletotrichum acutatum* wild type and Ca5 mutant strains on strawberry leaves, 4 days post inoculation. A. Drop inoculation with conidia of w.t resulted in massive appressoria formation adjacent to strawberry leaf cells, leading to necrotic lesions (further magnified on upper left). **B.** Drop inoculation with the Ca5 strain was not accompanied by appressorial formation. Epiphytic germination of w.t (**C**) and Ca5 strain (**D**) conidia, resuspended in minimal medium supplemented with urea.

Addition of organic or inorganic nitrogen supplements to minimal medium inhibited the characteristic immediate appressorium formation and promoted extended germ-tube growth. Furthermore, addition of external nitrogen sources such as glutamine or urea to the w.t strain and the Ca5 mutant, induced massive epiphytic

hyphal growth and subsequent formation of appressoria, followed by rapid symptom development (Fig. 12C, D). Thus, exogenous supplements of appropriate nitrogen sources complemented the non-pathogenic phenotype of the *nir1* mutant.

In order to determine the effect of nitrogenous fertilizers application, the influence of different nitrogen sources on efficacy at inciting disease on strawberry was determined. Significant differences were observed in the time required to reach 50% mortality of seedlings inoculated with w.t conidia resuspended in ammonium sulfate (5.2 ±1.2 days) and glutamine (4.1 ±1.5 days), compared to w.t conidia resuspended in nitrate (10.5 ±2 days) or water (11.2 ±1.2 days).

4.2.2.1. Expression profile of nitrogen-metabolism-related genes

To assess the availability of nitrogen source during strawberry colonization, we tested the expression of nitrate reductase and glutamine synthetase (designated *nit1* and *gln1*, respectively) by QRT-PCR. Regions of the genes encoding *C. acutatum nit1* and *gln1* were cloned from *C. acutatum* cDNA by PCR amplification with degenerate primers (Table 1). On the basis of the sequenced cDNA clones, primers for relative RT-PCR and QRT-PCR with *C. acutatum nit1* and *gln1* were designed (Table 1). Transcriptional regulation of *C. acutatum nit1* and *gln1* in response to different nitrogen sources and in the M3S and Reg rich growth medium was examined. *nit1* expression was down-regulated in M3S and Reg rich medium and up-regulated in minimal medium containing nitrate (Table 5). Expression of *gln1* was highest within 24 hours after addition of nitrate and decreased after 5 days of growth in the presence of nitrate. Expression of *gln1* was not significantly altered after prolonged growth in minimal or rich medium. In addition, the presence of glutamine in the medium repressed *gln1* expression. To further investigate the availability of nitrogen sources

in conidia during appressorium formation and host colonization, expression levels of fungal *nit1* and *gln1* were monitored. Transcript levels of *nit1* increased eight-fold during the appressorium-formation stage in comparison to dormant conidia, as opposed to *gln1* expression which was six-fold higher in dormant conidia when compared to the appressorium formation stage. Expression of fungal *nit1* and *gln1* relative to fungal β-tubulin *in planta* was determined from 2 days after colonization until the onset of the symptomatic phase. The relative transcript abundance of both *nit1* and *gln1* was similar during all phases of plant colonization (Table 5).

4.2.2.2. Nitrate-non-utilizing mutants of *C. acutatum* and pathogenicity

To further examine whether the loss of pathogenicity and the developmental defect (impaired appressorium formation) were linked to impaired nitrogen utilization, *C. acutatum* nitrate-non-utilizing mutants (*nit*) were produced. Mutant phenotyping was assessed on the basis of growth on nitrate-, nitrite- and hypoxanthine-supplemented media. The largest group consisted of *nit1* mutants (in which mutations occurred within the Nitrate reductase structural locus), and one group of *nitM* mutants (affected at the molybdenum cofactor loci). Pathogenicity of all *nit* mutants on strawberry seedlings and stolons was significantly reduced when compared to w.t. However, in contrast to the Ca5 mutant, all the tested *nit* mutants produced appressoria in a manner similar to that of the w.t.

4.2.2.3. Targeted disruption of *nir1* by homologous recombination

To inactivate *nir1* by homologous gene replacement, a linear *nir1*-disruption construct, designated *nir1*:hyg3, was obtained by introducing the hygromycin B resistance expression cassette (*hph*), including the promoter and terminator sequences of the *trpC* gene of *A. nidulans*. This cassette was flanked by *nir1* genomic DNA

(positions 1-2751 and 2968-3753, for the 5' and 3' flanks, respectively; Fig. 13A). A double-recombination event between the homologous regions of the *nir1*:hyg3 vector and the *nir1* gene would be expected to replace ~300 nucleotides of the endogenous *nir1* gene with the *hph* gene cassette. Several putative transformants were selected after plating on a medium containing hygromycin. Analysis of gene-disruption events was first performed with primer set 149wt4 and 149HpaI2B, designed on the basis of regions flanking the *hph* cassette. In the case of an ectopic integration, two amplicons (~2500 bp and 565 bp) were produced, whereas in the case of homologous recombination the shorter amplicon was no longer evident (Fig 13). These results were confirmed with an additional set of primers - hphf, and Catf2. Among the 120 transformants generated with *nir1*:hyg3, 11% harbored gene-replacement events.

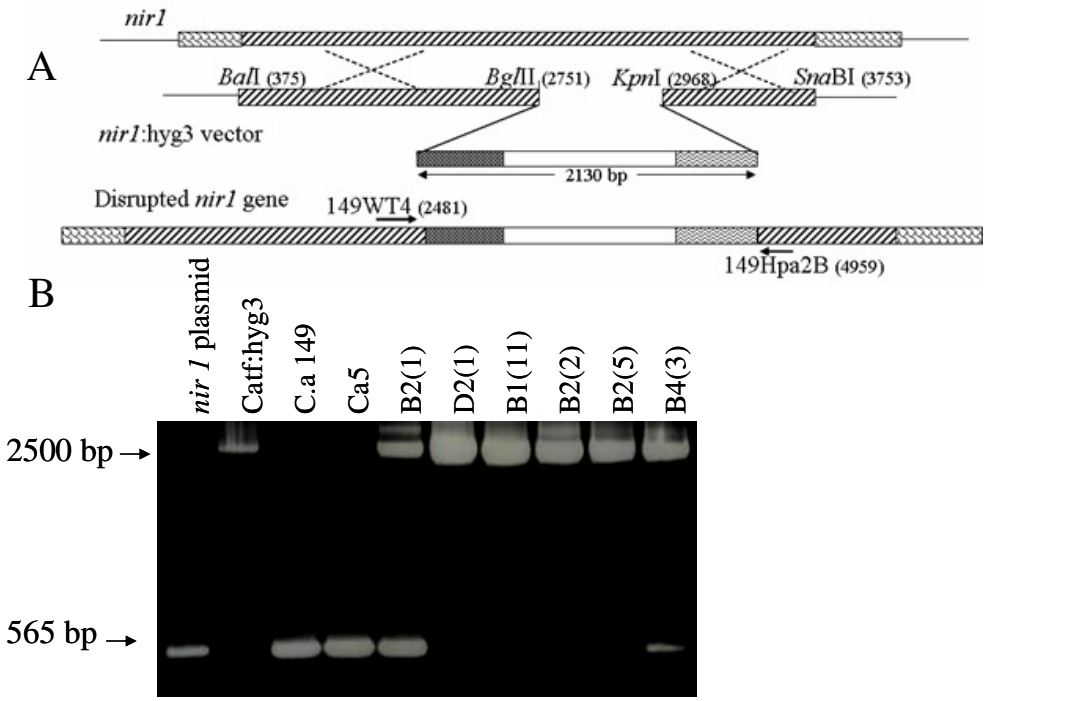

Figure 13. Analysis of gene disrupted mutants. A. Diagrammatic representation of homologous recombination events associated with replacement of part of the *nir1* gene of *Colletotrichum acutatum* with the hygromycin resistance (HygR) gene cassette by the disruption vector *nir1*:hyg3. *nir1*:hyg3 contains a heterologous *hph* gene cassette flanked by 5' and 3' *nir1* genomic fragments. The *hph* gene cassette comprised the promoter and terminator of the *trpC* gene of *Aspergillus nidulans* with the coding region of the *hph* gene of *Escherichia coli* (HygR). Positions of primers 149WT4 and 149HpaI2B used for gene-disruption evaluation are shown.
B. PCR analysis for potential gene-disruption mutants of wild-type *Colletotrichum acutatum* with primers 149WT4 and 149HpaI2B, flanking the *hph* gene cassette. The 565-bp fragment indicates ectopic integration of the *nir1*:hyg3 construct. Absence of the 565-bp fragment and appearance of only a 2500-bp fragment is indication of a homologous recombination event.

4.2.2.3.1. Disruption of *nir1* affects appressorium-formation rates and abolishes pathogenicity

Radial growth rate on solid M3S agar plates indicated that the hyphal extension rates of the *nir1* disruption mutants were equal to those of the w.t culture. The cultures of the *nir1*-disrupted mutants [B1(11), D2(1) and B2(2)] conidiated normally. When conidial suspensions were applied to glass slides they adhered, germinated and then produced elongated germ tubes, a morphological feature distinct from the w.t, which immediately produced melanized appressoria. In additional experiments performed on leaf and plastic surfaces, significant differences existed in the extent of appressorium formation by the gene-replacement mutants compared to that of the w.t. Thus, 7.2% (±1.2) of the germinating conidia of B1(11) produced appressoria adjacent to conidia and 75% (±3.5) germinated without forming an appressorium. Conidia of the gene replacement mutant D2(1) produced 9.5% (±2.4) appressoria adjacent to conidia and 66.8% (±5.4) germinated without forming an appressorium, compared to approximately 95% production of appressoria by the germinating w.t conidia (as described earlier, in comparison to the Ca5 strain). All gene-replacement mutants were tested for their ability to cause disease on strawberry compared to the w.t and Ca5 mutant strains. Within 2 weeks of inoculating host leaves with conidial suspension droplets, typical disease symptoms were produced by the w.t isolate and non-disrupted transformants [B2(1) and B4(3)], with large, clearly visible, necrotic lesions at the sites of inoculation (Fig. 14). In contrast, leaves inoculated with the *nir1*-disrupted mutants [D2(1), B1(11) and B2(2)] remained healthy and were symptomless. When analyzed *in vitro*, growth of the gene-disrupted mutants on minimal medium containing nitrate as the sole nitrogen source was restricted relative to the profuse growth of the w.t strain (Fig. 12A), as expected of

strains lacking NIR1 function. Expression of *nir1* was no longer evident in the gene replacement mutants, unlike in the Ca5 strain, in which an insertion event of vector pGH-1 altered expression of the *nir1* gene. Expression of the structural gene *nit1* was no longer evident in all gene knockout mutants (Fig 12).

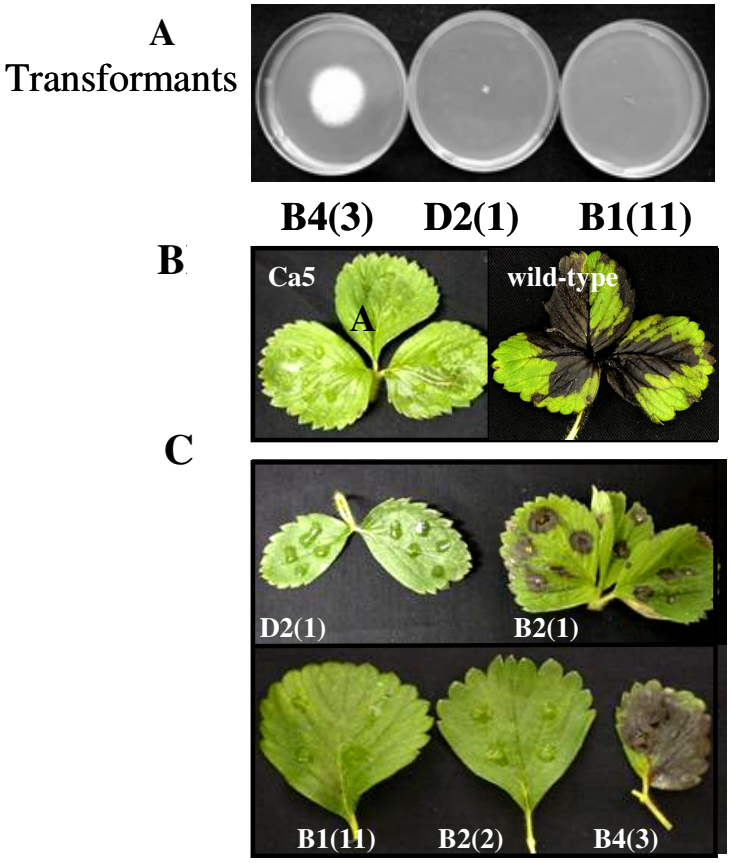

Figure 14. Gene disruption mutants phenotypes. A. Growth of *nir1* gene-disruption mutants D2(1) and B1(11) compared to a non-disrupted strain B4(3), on minimal medium containing nitrate. **B.** Pathogenicity of different *Colletotrichum acutatum* strains on strawberry leaves, as determined by the drop inoculation method. The Ca5 mutant and w.t strains. **C.** Gene-disruption mutants D2(1), B1(11), B2(2) compared with non-disrupted mutants B2(1) and B4(3).

4.2.2.3.2. Factors affecting cAMP protein kinase A (PKA) activity alter appressorium formation patterns

The impaired appressorium formation pattern in the Ca5 strain along with the fact that PKA has been shown to be involved in appressorium formation in various *Colletotrichum* species such as *C. lagenarium and C. trifolli*, led to the examination whether *nir1* and PKA signaling are linked. For this purpose, a pharmacological-based approach, taking advantage of documented effects of cAMP, caffeine (Rollins and Dickman, 1998) and the PKA specific inhibitor, KT5720 on PKA-regulated

fungal development, was utilized. Amending the conidial suspension with the KT5720 (5-25μM) antagonist did not significantly affect the immediate appressoria formation phenotype characteristic of the w.t strain. In contrast, application of the PKA agonists cAMP (5-25mM) or caffeine (2.5-50mM) reduced normal appressorium formation by approximately 75%. These results suggest that PKA is a negative regulator of immediate appressoria formation. When applied to the Ca5 strain, which does not produce appressoria after germination, neither the agonists nor the inhibitor had a significant effect on development (Fig 15). Lack of a phenotypic response of both the Ca5 strain as well as the D2(1) *nir1* gene replacement mutant (data not shown) to the various PKA effectors suggests that both *nir1* and PKA are involved in the normal appressorium formation process. However, whether they function in parallel or *nir1* is downstream of PKA, has yet to be determined.

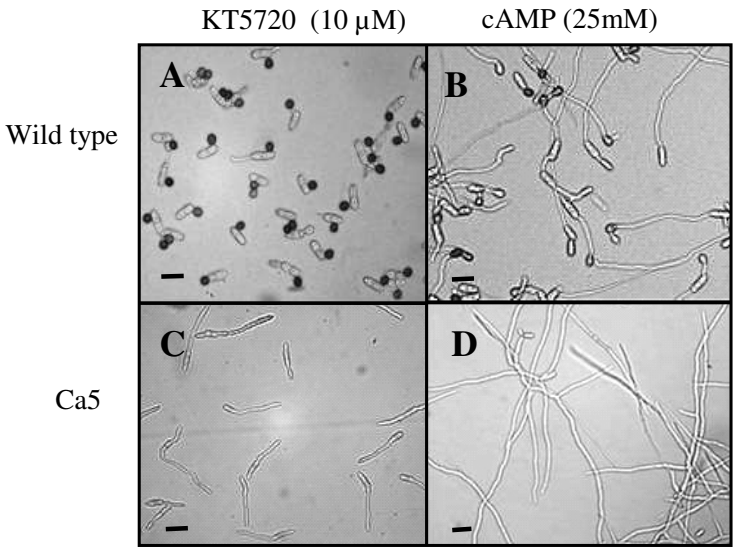

Figure 15. Effect of cyclic AMP (cAMP), Caffeine and the PKA inhibitor KT5720 on the conidial germination phenotype of *C. acutatum*. **A** and **B** – w.t conidia in the presence of 10 μM KT5720 or 25 mM cAMP 25mM , respectively. **C** and **D** - Germination of the Ca5 conidia in the presence of 10 μM KT5720 10μM or 25 mM cAMP, respectively. Bar = 20 μm.

4.3. DIFFERENTIAL PROTEIN EXPRESSION IN *COLLETOTRICHUM ACUTATUM*: CHANGES ASSOCIATED WITH NITROGEN STARVATION AND DEVELOPMENT IMPLICATED IN PATHOGENICITY

The predominance of nitrogen limitation during appressorium formation and lack of pathogenicity in the Ca5 mutant, associated with the nitrogen utilization transcription factor *nir1*disruption, promoted a proteomic based analysis. In order to identify additional novel components that may be linked with the perception of nitrogen limitation and pathogenicity-related development, differential protein expression was studied. Protein abundance patterns were compared among four relevant conditions: w.t and the Ca5 non-pathogenic mutant, grown under nitrogen-limiting conditions, w.t grown in a complete nutrient supply and w.t during appressoium formation stage.

4.3.1. 2-DE, spot analysis

A total of 12 2-DE gels were run, three gels per treatment covering the pH range of 4-7. Representative gels are shown in Fig. 16. Analysis of the 2-DE gels using the Delta2D software (version 3.2) followed by a visual confirmation, revealed 42 spots showing qualitative or quantitative differences between treatments (Fig. 16). The relative quantity of each spot and the fold change presented as expression ratio are presented in Table 6. We focused our analysis of spots on those whose relative quantity ratio differed between the treatments at least 2 fold. Six relevant protein subsets were defined: (1) proteins induced only during the appressoria formation stage (2) proteins induced only by nitrogen limiting conditions (3) common proteins induced during the appressoria formation stage and under nitrogen limiting conditions (4) proteins induced during nitrogen limiting conditions as well as by complete

nutrient supply though not induced during the appressoria formation stage (5) proteins induced primarily under growth in complete nutrient supply (6) proteins induced during nitrogen limiting conditions though not induced in the Ca5 mutant impaired in pathogenicity, while growing under nitrogen limiting conditions.

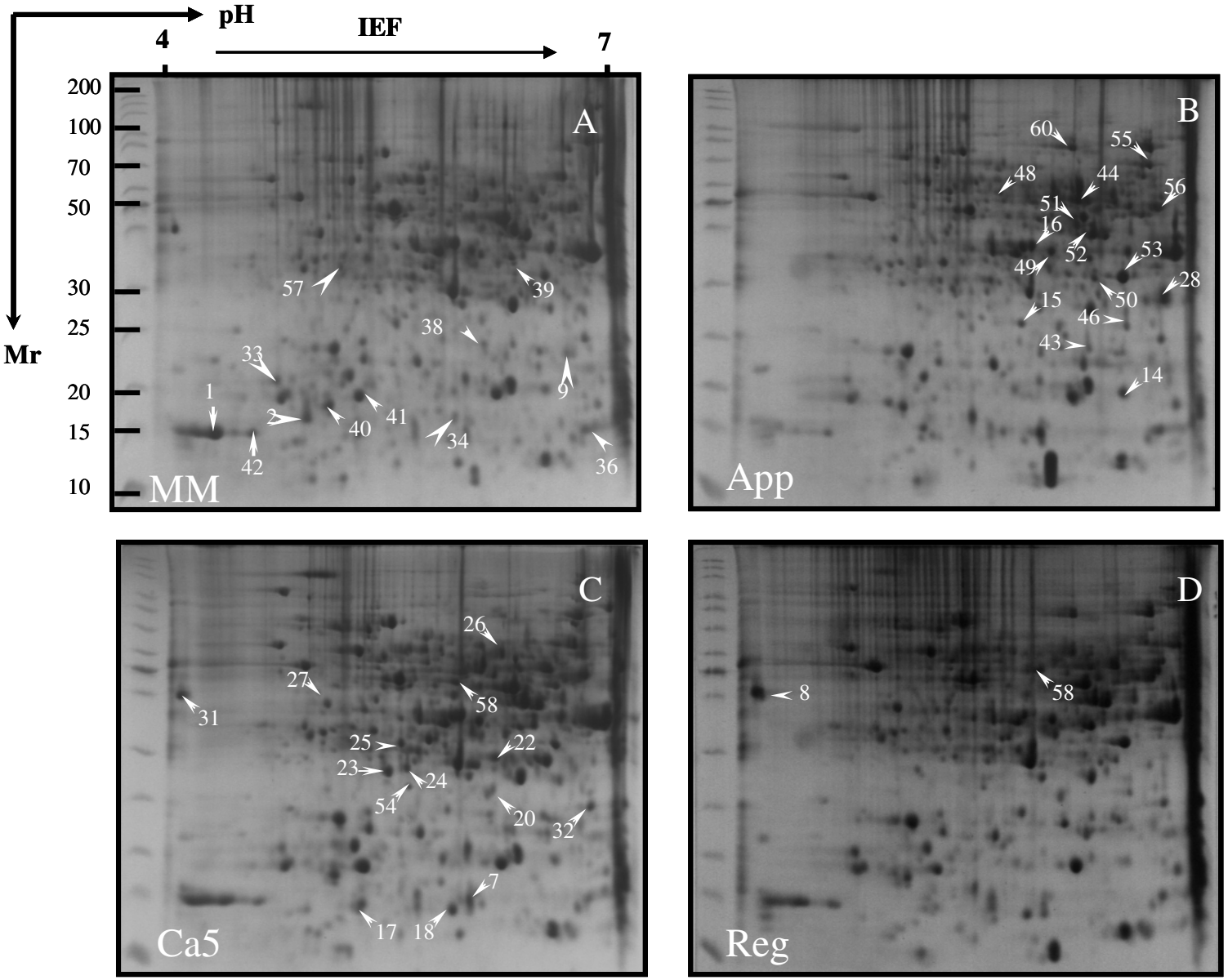

Figure 16. Representative 2-DE gels of *Colletotrichum acutatum* from. (A) MM, mycelia grown in minimal medium, (B) App, appressoria formation stage, (C) Ca5, *nir1* non-pathogenic mutant grown in minimal medium, (D) Reg, mycelia grown in complete nutrient supply. Proteins, 500 µg, were loaded and resolved on first dimension, pH 4-7 linear gradient and second dimension, SDS-PAGE on a 12% gel. Protein detection was achieved using colloidal CBB G-250 staining. Molecular mass (kDa) is denoted on the left, while the *pI* is given at the top of the figure. Significant differentially expressed protein spots are marked with arrows, and were analyzed (Table 6).

Table 6. Detection and quantification of differentially expressed protein spots[a]

Spots marked in images	Spot No.	Spot property[b]		Ratio[c]	Spots marked in images	Spot No.	Spot property[a]		Ratio[b]	Spots marked in images	Spot No.	Spot property[a]		Ratio[b]
		mm relative quantity	App relative quantity				App relative quantity	Ca5 relative quantity				App relative quantity	Reg relative quantity	
MM	1	1.984	0.206	0.104	Ca5	8	nd	0.653	∞	App	8	nd	1.9	∞
	2	0.810	0.005	0.007		27	0.232	0.201	1.15		60	0.617	0.298	2.07
	9	0.602	0.334	0.556		17	0.292	0.886	0.32		55	0.625	0.068	9.19
	33	0.308	0.228	0.739		18	0.156	0.575	0.27		56	0.462	0.007	65.7
	40	0.788	0.812	1.030		7	1.547	1.007	1.5		44	0.905	0.202	4.5
	41	0.872	0.136	0.156		32	0.368	0.393	0.93		53	0.778	0.372	2.09
	34	0.175	0.004	0.022		20	0.279	0.320	0.87		28	1.426	0.054	28.5
	36	0.499	0.816	1.639		54	0.066	0.112	0.58		50	0.369	nd	∞
	42	0.385	0.387	1.004		24	0.102	0.305	0.334		46	0.271	0.145	1.92
	38	0.106	0.053	0.505		23	0.002	0.804	0.005		15	0.312	0.007	44.5
	39	0.037	nd[d]	0.000		25	0.102	0.429	0.445		49	0.499	0.489	1.02
	4	1.253	1.068	0.852		22	0.055	0.334	0.164		52	0.906	0.203	4.46
	57	0.369	0.101	0.275		26	0.421	0.236	1.78		16	0.383	nd	∞
	5	0.358	0.008	0.022		58	0.129	0.197	0.65		51	0.407	0.023	20
App	60	0.119	0.617	5.205	MM	1	0.206	1.037	2		14	0.706	0.310	2.3
	55	0.041	0.625	15.1		2	nd[e]	nd			43	0.343	0.196	1.53
	56	0.445	0.462	1.038		9	0.334	0.266	1.25		48	0.163	0.062	2.66
	44	0.377	0.905	2.399		33	0.228	0.363	0.62					
	53	0.476	0.778	1.635		40	0.812	1.076	0.75	Ca5	31	0.012	1.056	0.011
	28	0.124	1.426	11.50		41	0.136	1.151	0.118		27	0.232	0.288	0.79
	50	0.085	0.369	4.337		34	0.004	0.003	1.33		17	0.292	0.150	1.94
	46	nd	0.271	∞		36	0.816	0.413	1.97		18	0.156	0.444	0.35
	15	0.015	0.312	21.00		42	0.387	0.473	0.818		7	1.547	0.729	2.08
	49	0.436	0.499	1.144		38	0.192	0.358	0.53		32	0.368	0.177	2.07
	52	0.636	0.906	1.425		39	0.255	nd	∞		20	0.279	0.315	0.88
	16	0.760	0.383	0.504		4	1.068	0.968	1.10		54	0.066	0.081	0.814
	51	nd	0.407	∞		57	0.101	0.420	0.24		24	0.102	0.021	4.85
	14	0.437	0.706	1.616		5	0.008	1.293	6.18		23	0.002	nd	∞
	43	0.203	0.343	1.493	App	60	0.617	0.073	8.45		25	0.102	0.062	1.64
	48	0.075	0.163	2.160		55	0.625	0.233	2.68		22	0.125	0.054	2.3
Ca5	31	0.988	nd	0.000		56	0.462	0.578	0.799		26	0.8	0.202	4
	27	0.526	0.232	0.441		44	0.905	0.099	0.80		58	0.129	0.160	0.8
	17	0.373	0.292	0.783		53	0.778	0.369	2.10	MM	1	0.206	0.901	0.22
	18	0.223	0.156	0.701		28	1.426	0.078	18.2		2	nd	nd	
	7	0.245	1.547	6.305		50	0.369	0.004	92.25		9	0.334	0.237	1.4
	32	0.306	0.368	1.202		46	0.271	0.233	1. 16		33	0.228	0.491	2.15
	20	0.146	0.279	1.910		15	0.312	0.008	39		40	0.491	0.128	3.83
	54	0.372	0.156	0.418		49	0.499	0.426	1.17		41	0.136	0.653	0.20
	24	0.068	0.063	0.935		52	0.906	0.514	1.76		34	0.004	0.009	0.44
	23	0.042	0.204	4.854		16	0.383	0.784	0.48		36	0.816	0.174	4.68
	25	0.136	0.102	0.748		51	0.407	0.626	0.65		42	0.387	0.489	0.79
	22	0.110	0.055	0.498		14	0.706	0.461	1.53		38	0.192	0.422	0.45
	26	0.404	0.421	1.04		43	0.343	0.235	1.45		39	0.255	0.627	0.40
	58	0.018	0.02	1.1		48	0.163	0.036	4.5		4	1.068	0.179	5.9
Reg	9	0.157	0.104	0.660		41	0.136	0.881	0.15		57	0.101	0.074	1.34
					Reg	9	0.104	0.205			5	0.008	0.172	0.04

[a] Comparison of relative quantity of the proteins spots among the treatments appressoria formation stage (App), w.t grown in minimal medium (MM), w.t grown in complete nutrient supply (Reg) and mutant grown in minimal medium (Ca5) (Fig. 16) were generated by the Delta2D software (version 3.2) (Decodon, Greifswald, Germany). Three images for each treatment were grouped to calculate the average relative quantity of each individual protein spot.
[b] Relative quantity of the spot, by setting total spot volume on a gel to 100%.
[c] The numerical expression ratio is the mean of the relative quantity of 'sample spot' divided by mean of the relative quantity of 'master spot', calculated for each gel and spot. These values were used to designate significantly differentially expressed proteins. Proteins were considered as differentially expressed when a relative fold change of > 2 or < 0.5 was measured.The numerical expression ratio was calculated by determining local background regions 17, average spot size is 5, weak spot sensitivity is 20% and noise cut off is 40%.
[d] n.d., not detectable spot.

4.3.2. Identification of differentially expressed proteins

All the 42 differentially regulated proteins were analyzed by LC-MS/MS and the most accurate database match for each protein is listed in Table 3. Most of the assemblies were derived from proteins of other fungi such as *Aspergillus* spp., *Neurospora crassa*, *Magnaporthe grisea*, *Colletotrichum* spp. and *Gibberella zeae*. These results suggest that there are many peptide sequences that are common between proteins in *C. acutatum* and other ascomycete fungi. In general, common variably-expressed proteins in all treatments belong to the following main functional categories: (i) Reactive oxygen species (ROS) scavenger machinery, (ii) carbohydrate and lipid metabolism, (iii) nitrogen metabolism, and (iv) protein synthesis and degradation. Their functional significance in relation to survival during environmental stresses and rapid activation of metabolic pathways is discussed.

Table 7. Proteins identified by LC-MS/MS from spots that differ in expressional ratio among growth of w.t and mutant Ca5 strains of *Colletotrichum acutatum* under nitrogen limiting conditions, w.t growth under complete nutrient supply and during the appressoria formation stage. Proteins were classified according to functional categories

Spot[a]	Putative identity[b]	Accession no[c]	Organism[d]	Mol.mass;pI[e]experimental/ theoretical	%Sequence coverage (peptides) [f]	Total score[g]	app reg mm ca5 regulation[h]
Stress responses and oxidative phosphorylation							
4	Cu-Zn superoxide dismutase	AAD19338	*Glomerella Cingulata*	20.3;6.3/16;5.9	29(8)	90	+ − + +
14	Hypothetical protein similar to gluthatione peroxidase	EAA74714	*Gibberella zeae*	18.8;6.5/19.1;6.3	15(5)	93.3	+ − + +
15	Hypothetical protein (CD: pfam00012 Hsp70 protein)	EAA57651	*Aspergillus nidulans*	26.3;5.8/72.3;5.7	8(4)	90	+ − − −
33	Hypothetical protein similar to putative stress response protein)	EAA77628	*Gibberella zeae*	20.6;4.7/28.4;4.33	9(2)	82	− + + +
40	Hypothetical protein (similar to cell death related)	XP_366662	*Magnaporthe grisea*	15.9;5.1/15;5.3	8(1)	93	+ − + +
60	Hypothetical protein: (similar to bifunctional catalase peroxidase Cat2)	AP007175.1	*Aspergillus oryzae*	80;6.2/82;5.8	4(4)	91	+ − − −
Main carbohydrates, glyoxylate and lipid metabolism							
1	Hypothetical protein (CD:Cytochrome coxidase subunit pfam02284)	EAA59740	*Aspergillus nidulans*	14.8;4.8/17.8.5;5.6	10(2)	93	− + + +
2	GTP-binding nuclear protein GSP1/Ran	Q7RVL0	*Neurospora crassa*	14.8;5/24.2;6.4	21(5)	93	− − + −
41	Hypothetical protein: similar to pyruvate carboxylase	EAA60227	*Aspergillus nidulans*	19.2;5.3/131;6.19	1(1)	85	− + + +

8	Putative isocitrate dehydrogenase	CAF31997	*Aspergillus fumigatus*	40.4;4.1/41.7;8.7	10(2)	97	− + − −
23	Phosphoglycerate kinase (PGK)	CAA38181	*Trichoderma viride*	29.5;6.1/44.9;6	11(3)	86	− − − +
25	Phosphoglycerate kinase	CAA38181	*Trichoderma viride*	31.5;5.53/44.9;6	11(3)	86	− − − +
36	Hypothetical protein Glutathione dependformaldehyde activating	XP_680863	*Aspergillus nidulans*	14;6.7/14.7;6.03	17(5)	92	+ − − −
37	Glyceraldehyde 3-phosphate dehydrogenase	P54117	*Glomerella lindemuthianum*	24.18;6.6/36.3;6.54	12(4)	85	− + + +
44	Hypothetical protein (CD: pfam00171 aldehyde dehydrogenas family)	EAA69530	*Gibberella zeae*	53.6;6.18/53.4;5.4	9(4)	92	+ − − −
52	Hypothetical protein CD: formate dehydrogenas NAD dependent	EAA75069	*Gibberella zeae*	38.6;6.2/40;6.1	16(8)	94	+ − − −
53	Hypothetical protein CD: malate dehydrogenase	EAA68236	*Gibberella zeae*	23.7;6.5/34;6.4	25(8)	93	+ − − −
56	Hypothetical protein CD: acetyl-CoA acetyltransferase	EAA73569	*Gibberella zeae*	49.6;6.6/43.6;7.5	6(3)	83	+ − + +
Purines and pyrimidine metabolism							
27	Hypothetical protein: (CD: adenosine kinase)	XP_457267	*Debaryomyces hansenii*	38;5/38;5.2	13(6)	89	− + + −
28	Orotate phosphoribosyl-transferas nucleotide transport	S30118	*Colletotrichum graminicola*	28.7;6.7/25.2;5.9	11(2)	89	+ − − −

Co-factor metabolism							
26	Hypothetical protein similar to protoporphyrinogen oxidase [coenzyme metabolism]	XP_328263	*Neurospora crassa*	60;6.17/57.9;5.8	8(3)	83	+ − + +
Amino acid metabolism, Protein synthesis and degradation, nitrogen metabolism							
2	GTP-binding nuclear protein GSP1/Ran	Q7RVL0	*Neurospora crassa*	14.8;5/24.2;6.4	21(5)	93	− − + −
5	Ketol-acid reductoisomerase precursore	XP_322910	*Neurospora crassa*	29.2;6.5/44.6;8.5	6(2)	95	nd + + +
16	Glutamine synthetase	AAB00322	*Glomerella cingulata*	39.9;5.7/39.9;5.7	23(11)	89	+ − + +
17	Hypothetical protein translation initiation inhibitor	EAA67634	*Gibberella zeae PH*-1	13.7;5.3/13.6;5.6	11(1)	87	+ − + +
18	Eukaryotic initiation factor 4a	XP_327706	*Neurospora crassa*	13.3;5.8/47.9;5.9	12(6)	84	+ − + +
22	Conserved hypothetical protein similar to proteasome alpha type 6	CAD70393	*Neurospora crassa*	29.5;6/27.7;5.6	13(4)	90	− − − +
24	Homocitrate synthase	AAF66618	*Neurospora crassa*	28.2;5.5/29.5;6	11(2)	96	− − − +
32	Hypothetical protein similar to 26s proteasome regulatory subunit mts4	BAE62886	*Aspergillus oryzae*	41.4;6.7/100;4.89	1(1)	89	+ − − −
34	Cobalamin-independent methionine synthase	AAF82115	*Aspergillus nidulans*	14.9;5.9/86.8;6.36	3(3)	93	+ − − +
39	EF2_NEUCR Elongation factor 2	EAA77131	*Gibberella zeae* PH-1	41.5;6.2/91.6;6.45	6(4)	95	+ − + +

	(EF-2)									
43	Hypothetical protein similar to prolidase	EAA68262	*Gibberella zeae*	21.4;6.2/26.1;5.8	6(2)	86	+	−	+	+
46	Glycine hydroxymethyl-transferase cytosolic	CAF05873	*Neurospora crassa*	23.7;6.5/52.9;6.93	6(2)	82	+	−	+	+
51	Hypothetical protein threonine dehydrogenase and related Zn-dependent dehydrogenases [Amino acid transport and metabolism]	XP_682383	*Aspergillus nidulans*	40.7;6.2/42.5;5.3	8(3)	89	+	−	−	−
54	Hypothetical protein pre-mRNA splicing factor Srp1	EAA67748	*Gibberella zeae*	23.9;5.4/32;10	18(5)	94	+	+	−	+
Cellular										
7	Hypothetical protein similar to tr|Q8NKC9 *Emericella nidulans* CipC protein	XP_457640	*Debaryomyces hansenii*	14;5.9/14.9;5.3	14(4)	86	+	−	−	−
48	Hypothetical protein CD: pfam00996 similar to secretory pathway gdp dissociation inhibitor	EAA74730	*Gibberella zeae*	54.5;5.6/51.4;5.37	9(4)	94	+	−	−	−
49	Hypothetical protein (CD: thiosulfate sulfurtransferase)	CAD60606	*Podospora anserine*	35.6;6/38.4;6.7	10(3)	91	+	−	+	+
55	Hypothetical protein CD:	EAA76084	*Gibberella zeae*	70.2;6.6/67.9;6	16(13)	88	+	−	−	−

	pfam0080 FAD binding domain, cytochrom e b5-like heme/stero id binding domain						
57	Tubulin β-chain	XP_323373	*Neurospora crassa*	38.5;5.2/49.4;4.84	8(3)	85	− + + +
Signal transduction							
42	Hypothetical protein CD: calmodulin related Ca2+-binding protein	XP_326472	*Neurospora crassa*	13.6;4.3/16.3;4.5	18(2)	89	− − + +
Unknown function							
38	Hypothetical protein	XP_328441	*Neurospora crassa*	26.5;6/36.2;6.3	8(2)	90	nd − + +

a) Assigned protein spot number as indicated in Fig. 16.
b) Identified protein of *Colletotrichum* spp. or homologous protein from other organisms.
c) Accession numbers in the NCBI databases.
d) Organism from which the identified protein originates.
e) Experimental and theoretical mass (kDa) and p*I* of identified protein. Experimental values were calculated with PD-Quest software and standard molecular weight markers. Theoretical values were retrieved from the protein database.
f) Percent of sequence coverage and the number of peptides identified in parentheses.
g) Average of peptides score, a peptide was considered as high quality if its Pep-Miner identification score was greater than 80 corresponding to the sequest Xcore of >1.5 for single charged peptides, >2.5 for double charged peptides and >3 for triple charged peptides.
h) Significantly up or down-regulated proteins (+/−respectively) among treatments.

4.3.2.1. Balancing the generation and elimination of Reactive Oxygen Species (ROS) during development and penetration into the host

4.3.2.1.1. Proteins associated with oxidative stress

Enzymes associated with the ROS scavenger machinery namely, Cu-Zn superoxide dismutase (SOD) and glutathione peroxidase (GPX) (spots 4, 14; Fig. 16), were detected as abundant proteins during the appressoria formation stage (5.9-fold and 2.3-fold changes, respectively), compared to growth under complete nutrient supply, but were not significantly different from the corresponding proteins under nitrogen limiting conditions (Table 7). The bifunctional catalase peroxidase (CAT2) (spot 60) was significantly up-regulated during appressorium formation compared to that in mycelium of w.t grown under nitrogen limiting or complete nutrient supply (5.2-and 2-fold change, respectively).

Quantitative real-time PCR was used to analyze transcription levels of the corresponding genes. During nitrogen limitation and the appressoria formation stage, 10-fold higher expression was observed for *sod1* compared to dormant conidia and growth under complete nutrient supply (Fig 17A). Furthermore, to investigate whether SOD activity would be enhanced during nitrogen limiting conditions and the appressoria formation stage, gel activity assays were performed. According to Dolashka-Angelova *et al*., (1999) the two apparent SODs which were detected are corresponding to MnSOD (upper band) and Cu/ZnSOD (lower band). SOD activity was markedly enhanced by nitrogen starvation and at the appressoria formation stage compared to activity observed in the presence of complete nutrient supply and in dormant conidia (Fig 17B). Expression of *gpx* during appressoria formation was up-regulated compared to complete nutrient supply, albeit only by 5.3-fold. Transcript levels of *cat2* were increased 2.5-fold during the appressorium formation stage in

comparison with mycelium under different nutritional conditions. Expression of *cat*2 in dormant conidia was 4-fold higher than mycelium grown under different nutritional conditions (Fig 17A).

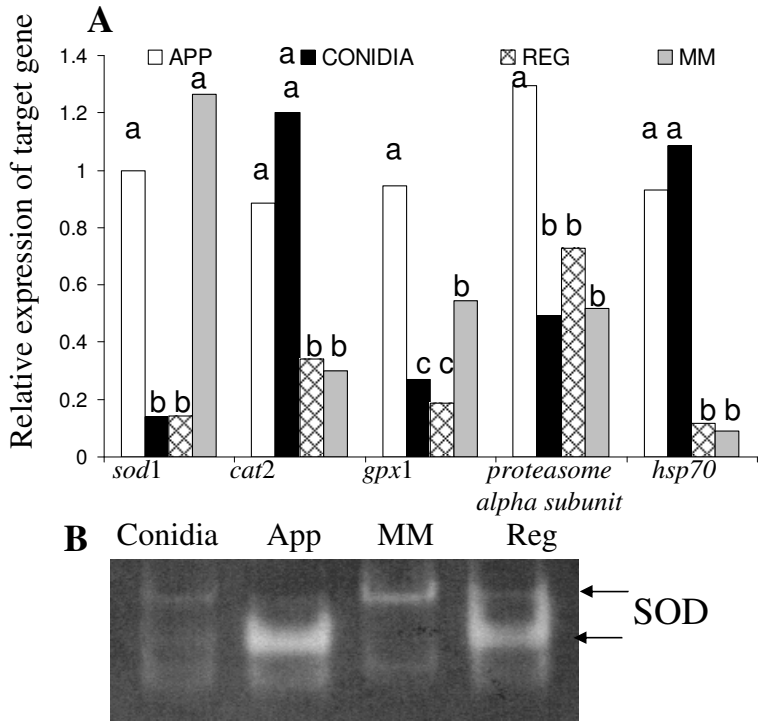

Figure 17. A. Quantitative real time-PCR (QRT-PCR) analysis of expression of *sod*1, *gpx*1, *cat*2, *hsp*70 and proteasome subunit alpha of *Colletotrichum acutatum* during the appressoria formation stage (app), dormant conidia (conidia), growth under nitrogen limiting conditions and complete nutrient supply. Values were calculated as fold change of expression of the corresponding gene among treatments compared to that of the appressoria formation stage. All samples were analyzed in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template). Averaged C_T values, the cycle at which the amplification curve reached threshold fluorescence, were then normalized to the endogenous control gene, β-tubulin. Fold change in target genes was determined by the $2^{-\Delta\Delta C}{}_T$ method, where $-\Delta\Delta C_T = (C_{TTarget} - C_{T\beta\text{-}tubulin})_{treatment} - (C_{TTarget} - C_{T\beta\text{-}tubulin})_{control}$. The appressoria-extracted sample was used as the control. Mean comparisons of the $2^{-\Delta\Delta C}{}_T$ values among treatments were calculated using LSD, according to the Tukey-Kramer multiple comparison test (treatments with different letters are significant difference, $P < 0.05$).
B. Relative SOD activity assay. SOD activity was determined by staining the native polyacrylamide gel with nitro blue tetrazolium and riboflavin. The following samples (20 µg) of protein extracted from dormant conidia (conidia), appressoria formation stage (app), complete nutrient supply (reg) and mycelia grown under nitrogen limiting conditions (mm), were loaded into the wells. The two apparent SODs are denoted with arrows.

Increased levels of ROS scavengers during the appressoria formation stage and nitrogen limitation prompted us to investigate the distribution of ROS products *in*

vitro under different nutrition supply and *in vivo* during pathogenesis. Additional proteins associated with stress and defense were identified: spot 15, which designates a protein similar to Hsp70 from *Aspergillus nidulans,* was up-regulated at the appressoria formation stage, compared to growth of w.t and the mutant under nitrogen limiting or the w.t grown under complete nutrient supply (21-, 39- and 44.5-fold change, respectively). Expression analysis of the corresponding *hsp*70 coding sequence gene by QRT-PCR during the appressorial formation stage and in dormant conidia was 10-fold higher when compared to that measured in mycelial samples from cultures grown under different nutritional conditions (Fig. 17A). An additional hypothetical protein, similar to a stress response protein from *Gibberella zeae* (spot 33), and a stress response protein, similar to a cell death-related protein from *M. grisea* (spot 40) were up-regulated under nitrogen limitation.

4.3.2.1.2. *In vitro* and *in vivo* intracellular ROS production

Intracellular ROS generation was investigated using 2',7'-dichlorofluorescein diacetate as a probe to detect and quantify intracellular-produced H_2O_2. ROS generation was observed within 3 hr after conidia were subjected to germination in water although ROS was not detected in dormant conidia (Fig 18A, B). Conidia germinated on a hard surface in the presence of water, formed an appressorium structure in which ROS accumulated, (Fig. 18C) an indication of changes in ROS levels during development. ROS was barely detectable in mycelium growing in the presence of complete nutrient supply, as opposed to enhanced ROS levels that accumulated under nitrogen limiting conditions (Fig. 18D, F). ROS intensity decreased when preferred nitrogen sources, such as ammonia were available in contrast to nitrate, a less preferred nitrogen source (Fig. 18E, G). Likewise, there was a significant increase in ROS accumulation

measured by fluorescence values in conidia germinating in the presence of water, within 3 hr, as compared to germination under complete nutrient supply or dormant conidia. In contrast, ROS decreased with addition of nitrogen sources such as ammonium, but increased with less preferred nitrogen sources such as nitrate (Fig. 19).

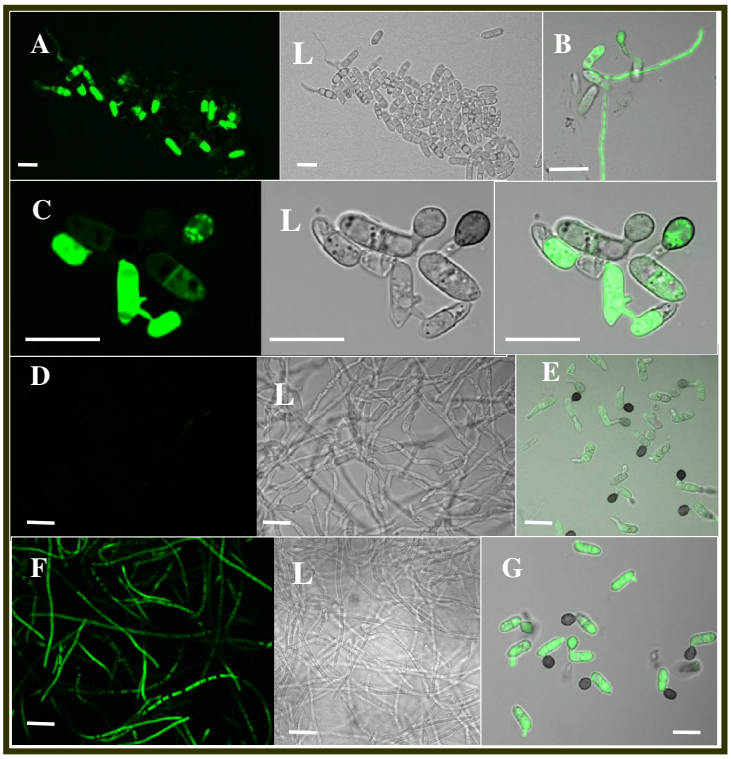

Figure 18. Assessment of *in vitro* Reactive oxygen species (ROS) production under different nutritional conditions and morphologies of *Colletotrichum acutatum*. *In vitro* ROS production in fungal cells was estimated by using the probe H_2DCFDA, and visualized with a laser-scanning confocal microscope. Rapid generation of ROS was detected within 3 hr after germination (A, B), 12 hr after germination at the appressoria formation stage (C). ROS was barely detected in mycelia grow in complete nutrient supply (D). Conidia germinating in presence of ammonium as a sole nitrogen source (E), High-levels of ROS within fungal hyphae during growth under nitrogen limiting conditions (F). Germination of conidia in the presence of nitrate as sole nitrogen source (G).
L-micrographs of corresponding images of A, C, D, and F as viewed under DIC microscopy. B, C (right), E and G are superimposition of DIC and fluorescence images. Scale bars in A-G =16µm.

The presence of glucose in the minimal medium acted as a substrate to the reaction driven by glucose oxidase which catalyzes the accumulation of H_2O_2 and gluconic acid, thus could not be compared among treatments lacking glucose. Following strawberry inoculation, rapid ROS accumulation was mainly observed beneath the

appressoria structures and appeared to be highly localized in the host apoplast at the host –appressoria interface. (Fig. 20A, B). Events of a halo formation in proximity to the appressoria structures were frequently observed, resulting in a decrease in host ROS accumulation (Fig. 20C). Generation and accumulation of ROS was observed within the appressoria structures, the emerged penetration peg and infection vesicles, 16 hr post inoculation, (Fig. 20D-F). ROS was observed in the intercellular and intracellular penetration hyphae (Fig. 20G-I), and accompanied all host colonization stages up to 4 days post inoculation (Fig. 20 J).

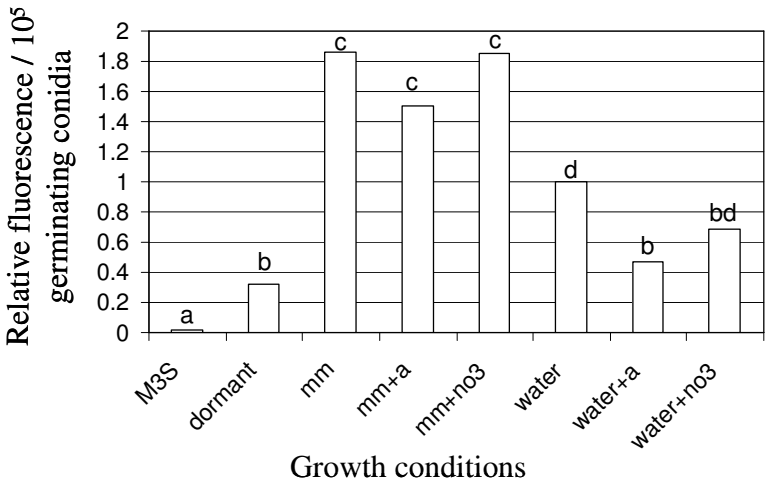

Figure 19. Quantitative relative fluorescence measurements. *Colletotrichum acutatum* conidia were allowed to germinate under different nutritional conditions: M3S rich medium, dormant conidia, minimal medium (mm) without nitrogen source or with ammonium (a) or nitrate (no_3), as a sole nitrogen source, and water without nitrogen source or with ammonium (a) or nitrate (no_3), as a sole nitrogen source. After the incubation periode conidia were immersed in 80 µl of 10 µM H_2DCFDA for 1 hr. Relative fluorescence of DCF was measured within 10 hr. Mean comparisons of fluorescence values were calculated using LSD, according to the Tukey-Kramer multiple comparison test at $P < 0.05$. Fluorescent values for treatments with different letters are significant difference.

4.3.2.1.3. Effect of ROS on appressoria development under nutrition limitation

To investigate whether ROS play a role in cell differentiation in *C. acutatum* during nitrogen limiting conditions the ability of proline, N-acetyl-L-cysteine (NAC) and mannitol as potent antioxidants and inhibitors of ROS production was tested. Unlike proline, addition of NAC (Fig. 21A) and mannitol (Fig. 21B) quenched ROS levels as

determined by fluorescence, characteristic for non-enzymatic defenses against oxidative stress.

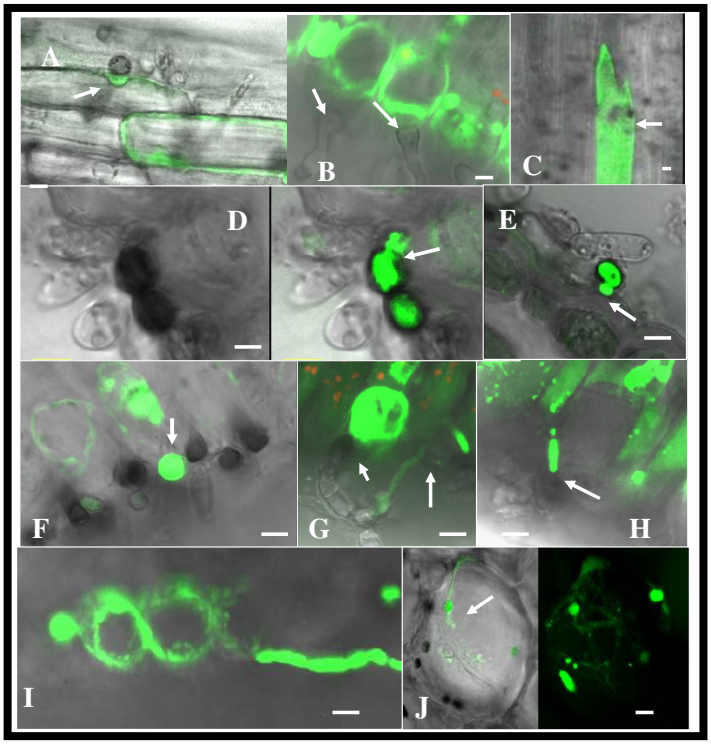

Figure 20. Assessment of *in vivo* fungal Reactive oxygen species (ROS) production during infection of strawberry by *Colletotrichum acutatum*. Cross-sections of infected tissues were prepared, 12hr and 72 hr post inoculation. Section samples were incubated with H_2DCFDA for 1 hr and examined by confocal microscopy. Superimposition of DIC and fluorescence images revealed rapid response of ROS accumulated beneath the appressorium depression (A, B) and directly adjacent to the host cell. Local accumulation of plant ROS response and halo formation close to the appressorium formation (C). Penetration of an appressorium germ tube is accompanied by fungal ROS production (D), left image analyses by DIC microscopy the right correspondent image and E are analyzed through superimposition of DIC and fluorescence microscopy. Formation of an infection vesicle and hyphae growing in the intercellular spaces of host cells accompanied by ROS production (F-I). Seventy-two hr after inoculation colonization of inner layer cells accompanied by fungal ROS production (J), left image analyzed by DIC microscopy, right correspondent image analyzed under fluorsence microscopy. Scale bars in A-G =10µm.

A significant decrease in ROS accumulation was correlated with a significant decrease in percent of appressoria formation with applications of threshold concentrations of 0.025 mM and 250 mM NAC, or mannitol, respectively (Fig 21A and B).

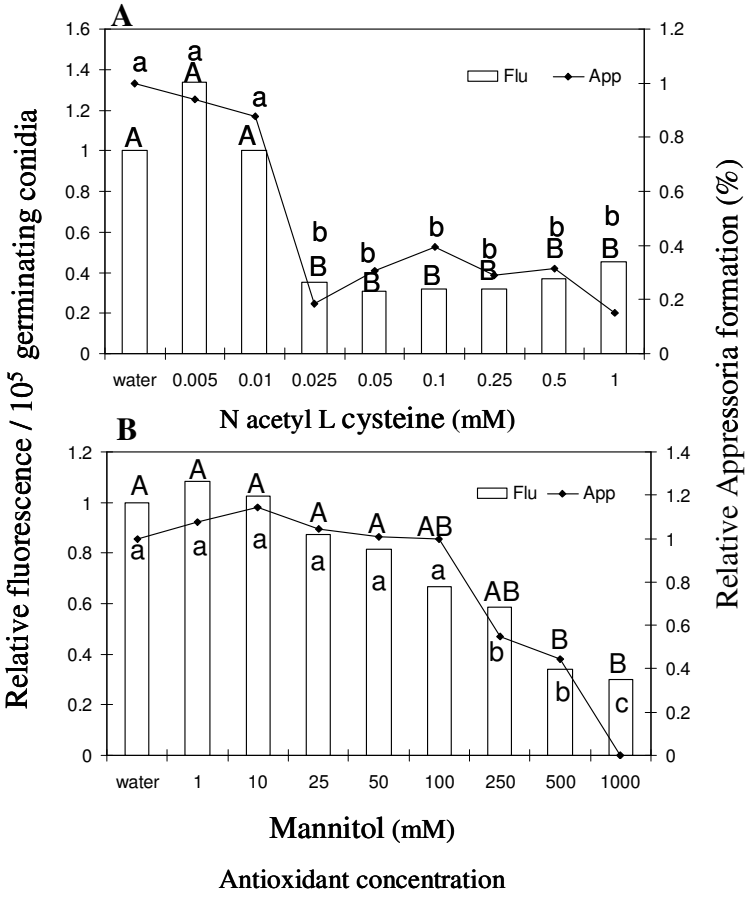

Figure 21. Effect of N-acetyl-L-cysteine (A) and mannitol (B) on appressoria formation and intracellular ROS production of *Colletotrichum acutatum*. Conidia were allowed to germinate in water in the presence of 0.005-1mM N-acetyl-L-cysteine or 1-1000 mM mannitol for 12 hr before ROS and percent appressoria formation measurements. Results indicate the mean from three independent experiments. Mean comparisons of germination and appressorium formation were calculated using LSD, according to the Tukey-Kramer multiple comparison test at $P < 0.05$. Mean values with different letters are significant.

4.3.2.2. Energy production and metabolic regulation shift

4.3.2.2.1. Proteins associated with energy and lipid metabolism in *Colletotrichum acutatum*

Several of the differentially regulated proteins detected in the 2D gels appeared to be involved in energy metabolism. Isocitrate dehydrogenase (IDH), a typical tricarboxylic acid cycle protein, was markedly up-regulated (protein spot 8; Table 6, 7) under growth in complete nutrient supply although it was down-regulated under nitrogen limiting conditions and hardly detected during the appressoria formation stage. Enzymes involved in the glyoxylate cycle, namely formate dehydrogenase and malate dehydrogenase or in fatty acid metabolism, namely aldehyde dehydrogenase

and acetyl-CoA acetyltransferase, (proteins spots 52, 53, 44, 56) were found to be up-regulated (4.46-, 2-, 4.5- and 65.7-fold change, respectively) during the appressoria formation stage compared to growth in complete nutrient supply (Fig. 16). Protein spot 2 corresponding to a GTP-binding nuclear protein GSP1/Ran belonging to Rab GTPases, was up-regulated under nitrogen limitation but was not detected in the *nir1* mutant nor during the appressorium formation stage or under complete nutrient supply (Tables 6 and 7). In relation to an increased catabolism, also worth noting was the identification of different key enzymes of the glycolysis/gluconeogenesis pathways, namely glyceraldehydes 3- phosphate dehydrogenase (GAPDH) (protein spot 37) phosphoglycerate kinase (protein spots 23 and 25), pyruvate carboxylase (protein spot 41), and glutathione dependent formaldehyde activating (protein spot 36), which were differentially expressed among treatments. Protein spots 23 and 25, corresponding to phosphoglycerate kinase which catalyzes the reversible reaction in the glycolysis process were up-regulated by 4-and 4.2-fold changes in Ca5 mutant compared to w.t growth under nitrogen limiting conditions. Likewise, homocitrate synthase (protein spot 24), which catalyzes the condensation of acetyl-CoA and alpha-ketoglutarate into homocitrate and is thought to be an important site of control of metabolic flow, was 4.8-fold up-regulated compared to w.t under the same conditions. This response suggests a shift in *C. acutatum* metabolism required for compensation during increased stress.

4.3.2.2.2. Occurrence and distribution of lipid bodies

To further study the potential role of lipid metabolism under nutrient deprivation and during appressorium function, the distribution of lipid bodies was examined under different growth conditions and developmental stages by Nile Red staining. Confocal

analysis of germinating conidia in water showed movement of lipid droplet reserves, mobilized from the conidium into the germ tube apex and to the incipient appressorium within 12 hr (Figs. 22A, B). Lipid bodies appeared inside secondary conidia upon production at the initial stages (Fig 22B). The appearance of lipid droplets was varied for conidia germinated in complete nutrient supply when compared with water. Under glucose or nitrogen deficiency, large lipid deposits were observed before being taken up into large vacuoles as observed in Fig 22C- E. Lipid bodies observed during growth in the presence of glucose and nitrate (Fig. 22F) or under complete nutrient supply (Fig. 22 G) were disseminated along the hyphae and were generally smaller and homogenous in size compared to growth under glucose deprivation (Fig. 22C). These observations suggest that fatty acid metabolism may be generally important under nutritional deprivation which occurs during appressoria formation.

4.3.2.3. Nitrogen metabolism, protein synthesis and degradation

Up-regulation of proteins involved in nitrogen metabolism, such as glutamine synthetase (GS) (protein spot 16) and enzymes involved in the biosynthesis of different amino-acids or protein synthesis, such as homocitrate synthase (protein spot 24), glycine hydroxymethyltransferase (protein spot 46), ketol acid reductoisomerase (protein spot 5) cobalamin independent methionine synthase (protein spot 34), threonine dehydrogenase (protein spot 51) and prolidase (protein spot 43), were significantly differentially expressed among the treatments (Tables 6 and 7).

Proteins involved in degradation via the proteasome were observed during the appressoria formation stage. The 26s proteasome regulatory subunit mts4 and proteasome subunit alpha type 6 (protein spots 32 and 22, respectively), were up-regulated during nitrogen limitation conditions.

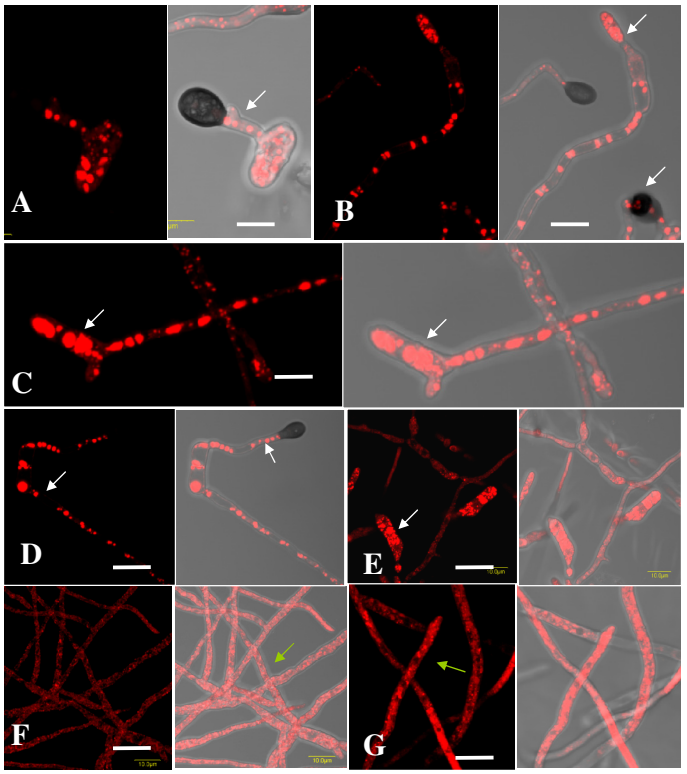

Figure 22. Cellular distribution of lipid droplets under nutrient deficiency conditions compared to growth in complete nutrient supply in germinated conidia and mycelia of *Colletotrichum acutatum*. Left images analyses by fluorescence microscope while right images are superimposition of DIC and fluorescence images. Conidia were allowed to germinate for 12 h in water drops (A-C) or in MM in the presence of nitrate and absence of glucose (D), in MM in presence of glucose and absence of nitrate (E), in complete nutrient supply, MM in the presence of glucose and nitrate (F), or Reg medium (G) and stained for lipid bodiesl by using Nile Red. Numerous small lipid droplets were seen under growth conditions with complete nutrient supply (indicated by green arrows), whereas under nitrogen or glucose limiting conditions larger lipid droplets were observed coalescing into vacuolar structures (indicated by white arrows). Scale bars in A-G =10µm.

Expression analysis of the corresponding proteasome alpha subunit revealed a relative increase in expression during appressoria formation compared to dormant conidia or mycelium growth under different conditions (Fig. 17). Up-regulation of these proteins might demonstrate a general activation of the degradation apparatus, but may also indicate specific functions in appressoria formation. Groups of translation regulators, translation initiation inhibitor, eukaryotic initiation factor 4a, EF2_NEUCR elongation factor which is associated with ribosomes during addition of amino acids to the polypeptide chain, and protein pre-mRNA splicing factor Srp1, (spots 17, 18, 39, and 54 respectively) were up- or down-regulated among treatments (Table 7).

DISCUSSION

Pathogenicity of *Colletotrichum* is dependent upon factors required for penetration, survival, and growth within the host plant. Upon entry and during establishment of a short biotrophic phase the shift to a rapid necrotrophic phase in the host is likely associated with a number of dramatic changes in host pathogen interface, including (i) developmental changes, such as the development of infection structures and the shift from large, primary to thin secondary hyphae, (ii) environmental changes, such as changes in the levels of active oxygen molecules produced by living plant cells versus degenerating plant cells, and (iii) nutritional changes, such as alteration in the types of nutrients released through membranes of living plant cells versus those released through membranes during plant cell degradation. In order to identify genes involved in the infection process, REMI mutagenesis and proteomic-based approaches were utilized. Some of the pros and cons of these approachs are discussed below.

5.1. REMI random mutagenesis as a tool for forward genetics study

Extensive research was conducted by performing REMI (restriction enzyme-mediated integration) mutagenesis to determine which genes play a role in pathogenicity of *Colletotrichum* on strawberry. By inserting DNA into the genome, a genome-wide series of random, tagged mutations can be created. Depending on the composition of of the construct, it is possible to disrupt genes, to tag promoters or enhancers, or to up-regulate genes. Thereafter, transformed isolates are selected and examined for phenotypic changes of interest. On the assumption that the phenotypic change is due to genetic disruption based on the inserted DNA, the genomic region contiguous with the inserted plasmid is retrieved by PCR-based methods such as in-PCR.

Limitations to the REMI approach include the generation of mutants in which the mutation may not be linked to the tagged site and thus lead to untagged mutation, all of which may rest from the REMI induces deletions and chromosomal rearrangement (Kahmann and Basse, 1999). In addition, meiotic instability of the integrated plasmid has also been reported (Epstein *et al*., 1998). Furthermore, a lethal mutation may not be detected (unless maintained in heterokaryon). Last, the degree of random coverage of the genome has yet to be proven. Therefore, it is important to confirm that mutations obtained from a REMI experiment are associated with integrated DNA. Even though this method has its shortcomings the ease of performing the procedure makes this approach very attractive and successful to tag pathogenicity related genes. REMI has already been used to tag genes of various other *Colletotrichum* spp.; *C. graminicola*. *C. magna* and *C. gloeosporioides* (Epstein *et al*., 1998; Redman *et al*., 1999; Thon *et al*., 2000). The genes tagged in *C. graminicola* were related to pathogenicity, conidial pigment, wall structure as well as hyphal growth and development. REMI was used to create non-pathogenic mutants of *C. magna* that can colonize the host but not produce visible anthracnose symptoms (Redman *et al*., 1999). REMI holds great promise for identifying key genes used by fungi to colonize plants while the technique has already shown great promise in identifying virulence genes of the mycoherbicide, *C. gloeosporioides* f. sp. *aeschynomene* (A. Sharon, personal communication). REMI mutagenesis was utilized in this project with the goal of identifying genes that play important roles in establishing and maintaining pathogenic infections of *Colletotrichum acutatum* on strawberry. Using a rapid and reliable inoculation method, five REMI transformants were identified that were reduced in their ability to cause anthracnose symptomes on strawberry plants. Flanking regions of the inserted plasmids in the reduced pathogenicity mutants were

recovered. Based on obtained sequences, the main focus of this work was on the Ca5 mutant, disrupted in *nir*1, a *nir*A-like transcription factor involved in nitrogen metabolism. Targeted gene disruption of the *nir*1 gene demonstrated that the pathogenicity defect in the Ca5 non-pathogenic mutant was attributed to the insertional mutation at the rescued genomic region. Evidence for the role of *nir1*, as well as the role of nitrogen availability in pathogenicity of *Colletotrichum acutatum* on strawberry was studied.

5.2. Development of a rapid and robust screening method

As a first step in evaluating pathogenicity of *Colletotrichum* REMI mutants on strawberry seedlings, it was necessary to develop a rapid, reliable screening protocol, by clearly defining the role of important factors affecting disease response. Young and older seedlings inoculated by foliar dip were similar in the time required to reach 50% mortality at 25°C (Table 2). Thus seedling transplanting, which is time and labor-consuming, is dispensable for screening pathogenicity by the foliar dip method. Recent studies have shown plant age to be an important consideration when screening strawberry seedlings for resistance. In general, seedlings become more resistant as they mature (Smith *et al*., 1990), as young immature, leaves are preferentially susceptible to infection by *Colletotrichum* spp. (Denoyes-Rothan and Guerin, 1996; Legard, 2000). In this study, older seedlings only, inoculated by the root soak method seemed to be more resistant at 25°C, compared with young seedlings (Fig. 5B). This may be the result of a more developed root system in the older seedlings. Traditionally, older seedlings are used to screen for cultivar resistance (Denoyes-Rothan *et al*., 1996; Smith *et al*., 1990; Smith and Spiers; 1982) while screening done at an early age is more rigorous, and may eliminate some mutants for

reduced pathogenicity. On the other hand, time, labor, space and material are the main advantages of the proposed method.

Both the root soak and foliar dip methods proved to be useful in pathogenicity assessment. However, inoculation of young and older seedlings by the foliar dip method allowed distinguishing increased virulence based on lesion size on leaves and petioles. Although only seedling mortality was monitored using this technique, it was important to assess mutant pathogenicity primarily during the second and third screening stages. The results presented here are in agreement with those obtained previously by Denoyes-Rothan and Guerin (1996) who found that in order to screen for cultivar resistance to *C. acutatum,* a dip inoculation technique in which entire plants were immersed in a conidial suspension, resulted in accurate disease response of susceptible cultivars.

Disease progress was delayed at 19°C compared to 25°C with all pathogenic isolates (Fig. 5), which was in agreement with previous studies showing that a latent infection period decreased as temperatures increased (King *et al*., 1997; Wilson *et al*., 1990). Although appearance of disease symptoms was delayed in seedlings inoculated by the leaf method at 19°C, disease incidence progressed and eventually reached similar levels to that at 25°C (Fig. 5C). An incubation temperature of 19°C allowed better discrimination between C.g 318 of *C. gloeosporioides* and C.a 149 of *C. acutatum* in seedlings inoculated by the root soaks method (Fig. 5D). Recent studies showed that at lower temperatures, *C. acutatum* produced more conidia than *C. gloeosporioides* (King *et al*., 1997; Ntahimpera *et al*., 1999). However at 25°C, there was little difference in disease incidence among control isolates; therefore, 25°C was chosen as the recommended incubation temperature.

Conidial concentration is an important factor in inducing the disease response in strawberry seedlings (Table 3). Significant differences in seedling mortality due to different concentrations of C.g 318 may be explained by lower sporulation of *C. gloeosporioides* compared to *C. acutatum* and a shorter latent period of *C. acutatum* compared to *C. gloeosporioides* (King *et al*., 1997). Initial inoculum concentration may have changed over time during incubation and may be important in such a short pathogenicity bioassay. A conidial suspension adjusted to 10^5 conidia/ml was effective in screening for reduced pathogenicity mutants, and well suited for the purpose of these screening tests.

The root soak and the foliar dip methods allowed for discrimination of nonspecific host-pathogen interaction, whereby the specific cucurbit pathogen, *C. magna*, did not cause disease in strawberry seedlings at the conidial concentrations tested (Fig. 6A). In order to screen for reduced pathogenicity mutants, the technique of foliage inoculation of young seedlings using inoculum concentration adjusted to 10^5 conidia/ml at an incubation temperature of 25ºC was selected. This method was consistent for evaluation of pathogenicity of *Colletotrichum* REMI mutants on strawberry seedlings. The developed technique was reliable and accurate since isolates that were reduced or impaired in pathogenicity on young seedlings during several screenings were consistently reduced or nonpathogenic on plants at all developmental stages including mature daughter plants (Fig 6A, Table 4). Therefore, this method is recommended for a preliminary screening of a large population of transformants. The proposed method requires considerably less space since Eppendorf tubes are used instead of pots. The rapid screening of potential mutants could not have been performed with such ease with the standard spray inoculation techniques (Denoyes-Rothan and Guerin; 1996; Smith *et al*., 1990, Smith and Spiers, 1982). Application of

this procedure may have potential for screening additional pathogens on strawberry, such as *Rhizoctonia* spp., and for assessing potential biocontrol agents.

The foliar dip method was devised for large scale screening and isolation of *Colletotrichum* REMI mutants with reduced pathogenicity. Five of the REMI mutants impaired in pathogenicity isolated by the rapid technique were characterized for initial parameters of germination, appressoial formation and growth rate. Molecular characterization of the reduced pathogenicity mutants allowed for the identification of specific genes involved in pathogenicity of *Colletotrichum* in strawberry. One of the five reduced pathogenicity mutants became the focus of this research. This mutant strain, designated Ca5, is mutated in a gene that encodes the NIR1 transcription factor involved in nitrogen metabolism.

5.3. Characterization of the Ca5 non-pathogenic mutant

Contrary to the w.t, which forms appressoria adjacent to conidia, the Ca5 non-pathogenic mutant germinates to form an elongated germ tube in the absence of any supplemental nitrogen source. An interesting aspect of the phenotype of this mutant is its ability to form necrotrophic lesions when preferred nitrogen sources are available. Even though Ca5 was non-pathogenic, it could be reisolated from strawberry plants up to two weeks following inoculation. This observation is consistent with previous reports on reisolation of *C. acutatum* from non-host plants (Freeman *et al*., 2001).

5.3.1. *nir*1, a *nir*A-like transcription factor is required during the early pre-penetration events

Several lines of evidence suggest that *nir1,* the gene which was mutated in the Ca5 strain, is a nitrogen regulatory gene of *C. acutatum*. Firstly, *nir1* shares homology

with the reported, positively acting nitrogen regulatory proteins NIRA in *Aspergillus nidulans* (25%) (Burger *et al*., 1991) and NIT4 in *N. crassa* (22%) (Fu *et al*., 1989) (Fig. 9). Secondly, the Ca5 mutant is defective in its ability to utilize nitrate and nitrite. Similarly, *nirA* loss-of-function mutations of *A. nidulans* lead to non-inducibility and consequent inability to utilize nitrate or nitrite (Burger *et al*., 1991). Gene-disruption experiments demonstrated that inactivation of *nir1* results in non-pathogenic phenotypes similar to the mutant strain Ca5, proving that *nir1* is required for pathogenicity. Expression of *nir1* increased when the w.t isolate was cultured in either minimal medium containing nitrate or glutamine or at the appressorium stage, compared to growth in rich medium and in dormant conidia. Many of the fungal transcriptional activators belonging to the zinc cluster family control catabolic pathways: NIT4 in *N. crassa* mediates nitrate induction (Yuan *et al*., 1991), FACB in *A. nidulans* controls the expression of acetate utilization (Todd *et al*., 1997), UaY in *A. nidulans* regulates purine utilization (Suarez *et al*., 1995) and QUTA in *A. fumigatus* regulates the utilization of quinic acid (Pain *et al*., 2004). Other examples of transcription factors containing a $Zn(II)_2Cys_6$ finger protein that are involved in the regulation of pathogenicity have been found in *Fusarium solani* f. sp. *pisi* (CTF1), which is involved in the transcriptional activation of a cutinase-encoding gene (Li and Kolattukudy, 1997) and in *C. lagenarium*, where CMR1 has been shown to be involved in the expression of the melanin biosynthesis genes *scd1* and *thr1* (Tsuji, *et al*., 2000). These findings, together with the observation regarding the Ca5 mutant phenotype which is impaired in the regulatory factor that affects appressorium formation, suggest the presence of a common mechanism that regulates transcription of appressorium-forming genes in a manner related to both development and nitrogen metabolism.

5.3.2. Nitrogen starvation as a perquisite for fungal appressoria morphogenesis

Formation of appressoria adjacent to conidia on strawberry leaves occurs in a nitrogen-limiting environment, a condition that prevails during this early infection stage. In the case of *C. acutatum* infection of strawberry, the biotrophic phase, in which the fungus maintains a symptomless existence within its host cells while removing nutrients from them, is followed by a transition to a necrotrophic phase, which involves major nutritional changes. Therefore, a mutation in the catabolic pathways required to utilize plant constituents could abolish or reduce pathogenicity. In *C. lindemuthianum*, targeted gene disruption of CLNR1, the AREA/NIT2-like global nitrogen regulator, resulted in reduced pathogenicity (Pellier *et al*., 2003). Similar to that, disruption of *Fusarium oxysporum* f. sp. *lycopersici* (the causal agent of vascular wilt in tomato) global nitrogen regulator FNR1, abolished *in vitro* expression of nutrition genes normally induced during the early phase of infection, and significantly delayed infection rate of tomato seedlings (Divon *et al*., 2006). On the other hand, disruption of the *M. grisea* NUT1 gene, a homolog of *areA* and *nit2*, had only a slight effect on infection efficiency on rice (Froeliger and Carpenter, 1996). Gene disruption of *areA*-GF of *Gibberella fujikuroi*, a homolog of the major nitrogen regulatory gene *areA*, conferred a significant reduction in gibberelin production (Tudzynski *et al*., 1999). On the other hand, examination of the transcriptional adaptation of *Fusarium oxysporum* f. sp. *lycopersici* during nutritional stress and plant colonization indicated that nitrogen starvation partially mimics *in planta* growth conditions (Divon *et al*., 2005).

Nitrogen starvation was assessed in the *C. acutatum*-strawberry system following expression of nitrogen metabolism genes during different phases of fungal development and plant colonization. Unlike *nit1*, expression of *gln1* was not

modulated by availability of nitrogen sources *in vitro* and thus could not be used as an indicator for nitrogen starvation. In contrast, *C. gloeosporioides* glutamine synthetase transcription is induced by nitrogen starvation in culture and its expression is elevated during pathogenesis on *Stylosanthes guianensis* (Stephenson *et al*., 1997). The results presented in this study demonstrate that *nit1* expression is induced by contact with hard surfaces during appressorium formation and during colonization of the plant. The reduction in pathogenicity of *C. acutatum nit* mutants and expression of *nit1* in all the infection stages supports the notion that partial nitrogen-starvation conditions prevail during colonization.

It has been shown in this study that the effect of nitrogen availability on virulence is stage specific. The early stages of infection are biotrophic in nature and are accompanied by nitrogen nutrition limitations. Later stages, which are necrotrophic in nature, include hyphal biomass accumulation and subsequent appressoria produced from mature hyphae. As the Ca5 *nir1* mutant lacks the ability to react to lack of nitrogen that is characteristic of the early infection stages, the morphogenetic response is that of hyphal formation. However, lack of the nitrogen source does not enable the mutant to accumulate biomass (unless an exogenous nitrogen source is supplied). Thus, in the w.t, at the early stage of development, nitrogen limitation induces immediate appressoria formation (required for survival and plant penetration) and at the later, necrotrophic stage, nitrogen is no longer a limiting factor. The early developmental block imposed by the lack of NIR1 in the mutant renders it incapable of completing the biotrophic phase, required for virulence. However, addition of external glutamine or urea complemented the non-pathogenic Ca5 phenotype.

It was also shown that PKA signaling plays a role in the germination phenotype of *C. acutatum*. Since PKA is reportedly involved in pathogenicity of other *Colletotrichum* species (Lee *et al*., 2003), it is conceivable that this is also the case for *C. acutatum*. Yang and Dickman (1999) as well as Yamauchi *et al* (2004) demonstrated that inactivation of PKA did not abolish appressoria formation. The suggestion that PKA is a negative regulator of appressorium formation is in agreement with the mentioned observations. Thus, increasing, rather than inhibiting, PKA activity resulted in impaired appressoria formation. This study demonstrated that exposing the *nir1* mutant to PKA affectors did not suppress the non-appressorium forming phenotype. This observation indicates that both *nir1* and PKA are involved in the normal appressorium formation process. However, whether they function in parallel or *nir1* is downstream of PKA has yet to be determined. The report of the negative cAMP-dependent expression regulation of a GATA-binding protein in rat (Zhang *et al*., 2002) may support the possibility of a similar hierarchal positioning of PKA and NIR1 in *C. acutatum.* Nevertheless, it is also possible that cAMP may directly affect additional, alternative, cellular components (Kopperud *et al*., 2003), which may prove to be involved in NIR1 function.

It is concluded that the C6 transcription factor NIR1 may play a role in appressorium development under nitrogen starvation, and may act in concert with PKA. The results also suggest that *nir1* is not essential for the necrotrophic phase of pathogen development in the host and supports the hypothesis that limitation of preferred nitrogen sources is stage specific and may lead to the induction of specific pathogenicity genes, resulting in disease development.

While REMI is still being used successfully for random mutagenesis, there is a clear, major trend of harnessing high throughput strategies for gene discovery, for

example all genome analysis are being performed using microarray experiments to analyze gene expression during nitrogen starvation, appressoria formation or specific stages during the course of infection (Teichert *et al*., 2004; Divon *et al*., 2005; Donoforio *et al*., 2006). With the significant number of fungal genomes being sequenced, genome-based analysis promises to uncover a great deal of new information in coming years. Since none of the *Colletotrichum* spp. have been sequenced thus far, further analysis of downstream targets of the NIR1 transcription factor or differentially regulated genes by genome-based analysis could not yet be considered. Whereas future studies which will rely on the potential of partial transcriptome analysis, suppressive subtractive hybridization (SSH), or investigating the functions and interactions of the NIR1 itself by using recombinant DNA approaches to fluorescently label endogenous proteins (e.g. GFP fusion proteins) may be used to analyse potential targets of the NIR1 transcription factor or differentially regulated genes related to pathogenicity.

Proteomics has a number of key advantages over other techniques such as microarrays. Since, the protein is the actual functional unit in the cell, the true picture of what is happening during specific developmental stages is being deciphered. Another fundamentally important advantage is that it is possible to identify proteins that have been post-translationally modified. Modification of proteins plays a key role in their function, therefore, the ability to identify changes in these proteins can be of key importance. Many proteins are activated by phosphorylation, or are expressed in an inactive form (proproteins, preproteins or preproproteins) and must be cleaved before they can be actived. These modifications will produce changes in the size, the pI or both, and can be identified on 2-DE by the anomalous migration of the protein under investigation.

5.4. A proteomics approach towards understanding global changes occuring under nitrogen starvation and implications on pathogenicity

The present proteomic study focused on quantitative changes of differentially regulated proteins. Work focused on functional properties of proteins which may play a role in encountering nutritional deprivation.

5.4.1. Antioxidant enzymes as indicators of ROS during appressoria development

ROS accumulation is balanced by means of a cellular antioxidant system, among which major players are catalase, superoxide dismutase and components of the ascorbate-glutathione cycle (Aguirre *et al*., 2005; Rodriguez and Redman, 2005). Up-regulation of SOD, GPX and CAT2 during the appressoria formation phase and partially, under nitrogen limitation prompted examination of the spatial and temporal distribution of ROS *in vitro*, under different morphologies and growth conditions, and during strawberry colonization. The increase in ROS production under nitrogen limitation suggested that it might play a role in cellular signaling of nitrogen deprivation, occuring during early stages of infection. Similar to findings in this study, several reports in different eukaryotic systems have shown a correlation between developmental processes and the up-regulation of specific antioxidant enzymes, such as superoxide dismutases, catalases, catalase peroxidases and peroxiredoxins (Aguirre *et al*., 2005). Induction of antioxidant enzymes in structures that are undergoing development such as appressoria, suggests that ROS are produced initially and during cell differentiation. Application of N-acetyl L cysteine and mannitol quenched ROS levels and decreased appressoria formation (Fig. 21). The ability of these antioxidants to inhibit appressoria formation is consistent with the idea that ROS are needed for the developmental processes to procede.

Rapid accumulation of ROS by the host which was observed beneath the appressoria structures is apparently caused by local mechanic pressure generated by the developing appressoria. Further evidence for this was found in *C. lindemuthianum* in which it was shown that appressorium maturation, but not appressorium function, was sufficient to induce most plant defense responses (Veneault-Fourrey *et al*., 2005). The presence of abundant ROS produced by the host following inoculation has been observed also for *C. coccodes* causing anthracnose disease in tomato (Mellersh *et al*., 2002). As is well known, H_2O_2 generation plays a role in restricting fungal penetration and inhibition of fungal invasion leading to the hypersensitive response, and triggering rapid necrosis at infection sites, or activating defense related genes (Garre *et al*., 1998; Apel *et al*., 2004). While in some of the observed penetration events the plant ROS response was not observed, which may indicate that *C. acutatum* evades recognition by the host, detoxifies the compounds (as indicated by halo formations in proximity to the appressoria), or is able to suppress other key defenses capable of generating ROS. Early results in this study regarding the induction and avoidance of strawberry defense responses upon inoculation with *C. acutatum* demonstrated that rapid induction of chitinase is correlated with germination and appressoria formation– chitin rich structures. The rapid induction of the detected chitinase may inhibit germination, although not arresting the infection process. Likewise, resistance toward *C. acutatum* was not enhanced in transgenic plants expressing the chitinase gene chB5 in strawberry (Vellicce *et al*., 2006). Factors affecting induction of strawberry PR proteins such as the role of fungal ROS have to be studied.

Up-regulation of fungal ROS scavengers during the appressoria formation stage could be an indication for involvement of enzymatic ROS scavenging in protecting the fungus from general stress generated by host defense mechanisms. This is in

agreement with the requirement of Cu/ZnSOD for the protection of *Candida albicans* against oxidative stresses and expression of its full virulence (Hwang *et al*., 2002). The possibility of involvement of ROS scavengers in detoxification of the prospective ROS produced by plants in defence against pathogens has been raised also for *Phytophthora infestans* in potato (Ebstrup *et al*., 2005).

As indicated, ROS have a number of potential biochemical functions such as biochemical signaling, regulation of gene expression and protein inhibition (Rodriguez and Redman, 2005). Results from this study suggest that ROS may play an important role in developmental processes occurring under nitrogen limitation and during early pre-penetration events.

Induction of additional stress-related factors such as the Hsp70 protein during the appressoria formation seems to be in fairly good accordance with the intensive catabolism that occurs at this developmental stage. Upon germination, conidia are able to produce proteins very rapidly, while peptide chains in the ribosome are protected by heat shock proteins during adverse conformation changes (Rocco *et al*., 2006). These findings support the evidence that stress response proteins, in addition to a protective effect against stresses, may play a pivotal role in development under adverse physiological conditions.

5.4.2. Metabolic regulation required during appressoria morphogenesis

A consequence of appressorium lipolysis is likely to be the generation of fatty acid β-oxidation and subsequent activation of the glyoxylate shunt and gluconeogenesis. The glyoxylate cycle provides a means for cells to assimilate two-carbon compounds into the tricarboxylic acid (TCA) cycle and channel them via gluconeogenesis to generate glucose and fuel secondary metabolic pathways, such as

melanin biosynthesis, which are critical for appressorium function (Wang *et al*., 2003). Induction of the glyoxylate cycle indicates that a cell is utilizing lipid metabolism as its predominant source for ATP generation, involving β-oxidation of fatty acids and the production of acetyl-CoA (Wang *et al*., 2003; 2005). Lipid droplet deposits observed during nutrition deprivation indicate that utilization of lipid bodies occur during appressoria development (Fig. 22). Isocitrate dehydrogenase (IDH), an important branch point between catabolic and anabolic processes in the cell was clearly up-regulated under growth in complete nutrient supply (Fig. 16). Thus the catabolic process that occurred during appressoria formation of *C. acutatum* induced the synthesis of a series of proteins involved in the glyoxylate cycle namely, formate dehydrogenase and malate dehydrogenase, or in fatty acid metabolism, namely aldehyde dehydrogenase and acetyl-CoA acetyltransferase. Up-regulation of these enzymes during appressoria formation and partially under nutritional limitation, indicates that cells prepare for lipid-based energy supply which confers β-oxidation of fatty acids. Observations in *C. acutatum* support recent findings in *Magnaporthe grisea* which demonstrated that the glyoxylate cycle may be fundamental in allowing conidial germination and appressoria formation before host invasion, and associate lipid bodies as main storage reserves (Wang *et al*., 2005). Two key studies which have examined the role of the glyoxylate cycle in the fungal phytopathogens, *M. grisea* and *Leptosphaeria maculans,* demonstated that mutants lacking isocitrate lyase activity, a key enzyme of the pathway, were unable to germinate and cause disease (Idnurm *et al*., 2002; Zhang *et al*., 2003; Solomon *et al*., 2004). Recent work in *C. lagenarium* suggests that peroxisomal metabolic pathways play functional roles in appressorial and subsequent host invasion steps (Asakura *et al.*, 2006). Likewise, in this study it is

suggested that *C. acutatum* uses lipid metabolism extensively during appressoria formation and under nutrient deprivation conditions (Fig. 22).

5.4.3. Enhancement of nitrogen and protein metabolism during early developmental events

Increased abundance of glutamine synthetase (GS), which allows assimilation of nitrogen and biosynthesis of glutamine when prefferential primary nitrogen sources are lacking, is consistent with the previous findings in *C. acutatum*, which showed that nitrogen starvation prevails during appressoria formation (Horowitz *et al*., 2006). Similar enhanced expression of GS occurred during pathogenesis of *C. gloeosporioides* on *Stylosanthes guianensis* (Stephenson *et al*., 1997). A recent study in *Gibberella fujikuroi* showed that GS had a significant impact on transcriptional control of primary and secondary metabolism (Teichert *et al*., 2004), which may indicate that a metabolic shift occurred during appressoria formation and under nitrogen starvation. Germinating conidia must produce proteins very rapidly to accommodate the infection process. As a consequence, an increase in differentially regulated proteins during appesssoria formation and under nitrogen limitation condition, was represented by proteins involved in amino acid metabolism, protein synthesis and degradation (i.e. translation initiation factor and elongation factors). Likewise, up-regulation of proteins involved in protein synthesis has been found in *Phytophthora infestans*. Up-regulation of these proteins might demonstrate enhanced cell metabolism by general activation of the transcription/translation apparatus and may also indicate specific functions of importance in appressoria formation of *P. infestans* (Ebstrub *et al*., 2005). Up-regulation of the mts4 subunit of the proteasome 26s during appressoria formation has been demonstrated previously in *M. grisea* by Kim *et al*. (2004) with 20S proteasome subunits. These proteins take part in

degradation processes of targeted proteins during a number of cellular processes and play important roles in protein turnover, and may be involved in the complex cellular events that lead to appressorium formation. Furthermore, Kim *et al*., (2004) suggested that the proteasome may be involved in mobilizing storage proteins during appressorium formation. However, the function of the proteasome subunit in appressoria formation deserves further study.

Additional proteins spots, involved in more general functions, were clearly up-regulated during the appressoria formation stage and their role in pathogenicity should be defined in future. Among them, is a protein which is similar in function to the secretory pathway GDP dissociation inhibitor of the yeast *Saccharomyces cerevisiae*, which regulates distinct vesicular traffic events in eukaryotic cells with dissociation of GDP from the Sec4/Ypt/rab family of GTP binding proteins. An additional appressorium induced protein is a hypothetical protein similar to the *A. nidulans* CipC protein. The CipC protein was originally identified in *A. nidulans* as a protein that is produced in high amounts in the presence of the antifungal agent concanamycin A. Target mutation of the CipC gene in *Cryptococcus neoformans* implicates this gene as serving an important role during growth *in vivo* (Steen *et al*., 2003). A protein involved in electron transport, cytochrome b5-like heme/steroid binding domain, was detected only at the appressoria formation stage. Many members of this subfamily are membrane anchored by an N-terminal transmembrane alpha helix. This family also includes a domain in some chitin synthases. The function of these proteins in appressoria viability will require further studies.

5.4.4. Proteins associated with the mutation of the Ca5 non-pathogenic mutant

Analysis of the differentially- expressed proteins in the Ca5 mutant compared to w.t growth both under nitrogen limiting conditions, revealed several proteins which

were differentially regulated. Whether their induction is attributed to the Ca5 mutation is still unknown. Increased abundance of homocitrate synthase, the protein pre-mRNA splicing factor and phosphoglycerate kinase, might be an indication of enhanced metabolic activity. Similarly, deletion of the *Gibberella fujikuroi* glutamine synthetase, a key enzyme of nitrogen assimilation, caused dramatic changes in carbon metabolism, cross-pathway and general transcriptional and translation control (Teichert *et al*., 2004). Besides proteins which were up-regulated in the Ca5 mutant one of the down-regulated proteins was a GTP-binding nuclear protein, GSP1/Ran belonging to Rab GTPases which are implicated in vesicle trafficking and vesicle formation. Since the fungus prepared for lipid-based energy supply, during germination and appressoria formation which is based on vesicle trafficking, this protein may have an important functions in appressoria formation. Further studies need to consider down-regulated proteins as potential targets for the NIR1 transcription factor.

5.5. Concluding remarks

To conclude from this study, findings in the field of *Colletotrichum* biology in relation to nitrogen starvation, development and pathogenicity, are as follows:

- A large scale screening technique was developed for pathogenicity assessment of *Colletotrichum* isolates and its benefit for selection of reduced-virulence mutants was verified.
- In depth characterization of the Ca5 non-pathogenic mutant has demonstrated fundamental factors affecting *Colletotrichum* colonization as:

    * Early developmental block imposed by the lack of NIR1 in the mutant renders it incapable of completing the biotrophic phase.

* Regulation of development and morphogenesis processes under nitrogen starvation is mediated by NIR1 transcription factor.

* Nitrogen limitation is a stage specific condition, predominant during the pre-penetration stage. The reduced pathogenicity of *C. acutatum nit* mutants and expression of *nit1* in all the infection stages suggest that partial nitrogen-starvation conditions prevail during colonization.

* Nitrogen nutrition has a significant impact on disease progress. Thus, application of nitrogenous fertilizers to strawberry growth must be applied in a controlled manner.

- Products of ROS accompany nitrogen starvation and appressoria formation in *C. acutatum.*

  * Increase in abundance of fungal ROS scavenger enzymes during appressoria development and under nitrogen starvation are correlated with fungal ROS generation detected under corresponding conditions.

  * Quenching ROS levels which resulted in decreased appressoria formation may indicate that ROS and nitrogen deficiency play an important role in appressoria morphogenesis.

  * Modulating the occurrence and distribution of ROS may be fundamental in permitting pathogenicity to proceed before host invasion.

  * Increase in abundance of enzymes belonging to the glyoxylate cycle during appressorium morphogenesis suggests a lipid based energy supply.

5.6. Control of pathogenicity genes of *Colletotrichum acutatum* by an elaborate sensory network: a proposed model

Many key fungal virulence and pathogenicity factors are transcriptionally controlled by an extensive network of distinct, interacting signal transduction pathways. To summarize the findings of the current study-sophisticated sensory apparatus, signal transduction mechanisms, regulated targets, and cross-talk occur between metabolic systems function in disease establishment.

Much of the success of *C. acutatum* as a pathogen is attributed to its ability to adapt to the diverse microbial habitats in the strawberry host. Indeed, various environmental cues induce appressoria morphogenesis that is critical for virulence. It was demonstrated that nitrogen deprivation induced appressoria morphogenesis in *C. acutatum* by direct or indirect stimulation. Current research indicates that cAMP-PKA signal transduction is involved in the appressorium formation process, since increasing rather than inhibiting PKA activity resulted in impaired appressoria formation. Increased expression of the *nir1,* a *nirA*-like transcription factor during appressoria formation, suggests that this gene plays a role in a nitrogen-regulated signaling pathway. Assuming that *Colletotrichum* harbors similar nitrogen metabolism as *Aspergillus nidulans,* two main pathways regulating nitrogen metabolism are suggested. (i) Nitrogen catabolite repression (NCR) regulates the ability of the fungus to discriminate between rich and poor nitrogen sources. (ii) Nitrogen catabolite derepression, in the presence of a rich nitrogen source, such as ammonium, NCR prevents transcription of genes necessary for the utilization of poor nitrogen sources and induces filamentous growth. In the presence of a poor nitrogen source, NCR is abolished and subsequent primary and secondary metabolic changes

occur. *C. acutatum* regulation of utilization and sensing of nitrogen sources have been assessed with respect to pathogenicity. Mutational analyses of the Ca5 non-pathogenic mutant indicate that the NIR1, a NIRA-like transcriptional regulatory protein functions independently in promoting appressoria development. The role of *nir1* in appressoria development was deduced from the observations that a mutation in the *nir1* transcription factor induced filamentous growth. Thus, common regulatory transcription factors involved in nitrogen metabolism induce the expression of genes necessary for appressoria development. Furthermore, it was now shown that equilibrium in reactive oxygen species (ROS) is essential for appressoria morphogenesis of *C. acutatum* in niches where the available nitrogen is limited. The induction of antioxidant enzymes in structures that are undergoing development and fully differentiated structures accompanied by ROS production, suggest that ROS might be involved in cell signaling and development under nitrogen limitation. These data demonstrate that under nitrogen deprivation NIR1, PKA and ROS-signaling are important mediators of fungal morphogenesis and pathogenesis. However, whether they function in parallel or NIR1 is downstream of PKA or ROS, signaling has yet to be determined.

Given that *C. acutatum's* germinating conidia respond to a variety of different growth and environmental signals, it is not surprising that multiple signaling pathways concert together to induce fungal morphogenesis. Once conidia germinate on a strawberry plant surface, different signaling pathways leading to a primary metabolic shift provide energy reserves that are relevant for metabolic adaptation and protection against stress. Abundance of proteins corresponding to enzymes involved in primary metabolism show that there is a striking degree of coordinate regulation of some of these proteins in the same pathway. These results suggested a very complex and well-

balanced regulation that makes possible the shift from anabolic to catabolic pathways in *C. acutatum* during different stages of colonization. Nonetheless, additional metabolic characteristics as well as regulatory instruments that compensate environmental challenges need to be addressed in future research with the aim to assess the significance of fungal primary metabolism for pathogenicity of *Colletotrichum* species.

We anticipate that results obtained here will further our understanding of the unique regulatory system related to the morphogenesis of this fungal pathogen.

6. BIBILIOGRAPHY

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APPENDIX

תקציר

הסוג *Colletotrichum* גורם מחלת האנתרקנוז הינו בין הפתוגנים החשובים של תות שדה בעולם. הסוג גורם לרקבון בכל חלקי הצמח ומופיע כנקרוזות שקועות הגורמות לחיגור, ומובילות להתמוטטות הצמח כולו. שלבי ההדבקה לפני החדירה דומים בעיקרם בכל המינים השייכים לסוג, בעוד שההבדלים העיקריים בדרכי ההדבקה ניכרים רק לאחר החדירה לפונדקאי. בפטריה *Colletotrichum acutatum* התוקפת תות שדה, מזוהה שלב ביוטרופי קצר מיד עם החדירה לצמח, בו הפטריה גדלה מתחת לקוטיקולה ובין דפנות תאי האפידרמיס תוך הסתעפות בין התאים. בשלב הבא, מזוהה חדירה לתוך תאי הפונדקאי, הסתעפות קורים תוך תאיים וכניסה מהירה לשלב הנקרוטרופי בהדבקה המלווה במוות תאי הפונדקאי.

הבנת הבסיס המולקולרי של תהליך הפתוגניות של הפטריה *C. acutatum*, הינה דרישה הכרחית למחקר מעמיק של האינטרקציה הפתוגנית של הפטריה בתות שדה. אחת הגישות לאיתור גנים החיוניים למהלך ההדבקה וההתבססות של הפתוגן, הינה יצירה ובידוד של תבדידים מוטנטים בלתי פתוגנים (Forward genetic approach). יישום מוצלח של גישה זו מחייב את קיומה של מערכת סריקה הדירה ומהירה להערכת פתוגניות בהיקף גדול של התבדידים המוטנטים על תות שדה. כצעד ראשון בבניית מערכת זו לאיתור מוטנטים פחותי פתוגניות כויילה מערכת סריקה המבוססת על נבטי תות שדה. לכיול השיטה נבחנו השפעתם של גיל הנבטים, טמפ׳ ההדגרה וריכוז המידבק על התקדמות המחלה של שני מיני ה *Colletotrichum* : *C. gloeosporioides* ו *C. acutatum* לשם קביעת תנאים אחידים שישמשו לקביעת פתוגניות התבדידים. תגובתם של נבטים בשני שלבי התפתחות 12 שבועות (צעירים) ו 15 שבועות (בוגרים) לאילוח נבחנה בשתי שיטות אילוח, אילוח שורשים או אילוח נוף ובשתי טמפ׳ הדגרה 19°C ו 25°C. במעקב אחר התקדמות המחלה נצפתה תמותה של נבטים צעירים תוך ארבעה ימים מהאילוח בשני מיני *Colletotrichum* בשתי שיטות ההדבקה, שהובילה ל 50% תמותה תוך 10 ימים ב 25°C. לעומת זאת תמותת נבטים בוגרים עוכבה ב 4 ימים לעומת נבטים צעירים שאולחו בשיטת אילוח השורשים. התקדמות המחלה הואטה הן בנבטים הצעירים והן בבוגרים בטמפ׳ ההדגרה הנמוכה יותר. השיטה שפותחה הוכיחה את אמינותה כשהמין *Colletotrichum magna* פתוגן דלועיים לא גרם לסימני מחלה בשתי שיטות ההדבקה וכן בבידוד חמישה תבדידים פחותי פתוגניות אשר המשיכו להציג פנוטיפ זה גם בשתילי בת של תות שדה.

מערכת טרנספורמציה שפותחה עבור *C. acutatum* ו *C. gloeosporioides* מתות שדה שימשה ליצירת אוסף תבדידים מותמרים על פי שיטת ה REMI (restriction enzyme mediated integration). מערכת הסריקה המוצעת שימשה לסריקת מעל 1000 תבדידי REMI והניבה חמישה תבדידים פחותי פתוגניות. איפיון ראשוני של התבדידים פחותי הפתוגניות כלל איפיון מורפולוגי, זיהוי מספר עותקי המחדר בגנום ובידוד הגן ששובש התוחם את המחדר. מחקר מעמיק של תבדידים אלו יכול לסייע בזיהוי ולימוד לעומק של גנים החיוניים להשלמת תהליך הפתוגניות בסוג *Colletotrichum*. בעבודה זו, מוטנט REMI בלתי פתוגני על תות שדה המכונה Ca5 היווה את לב המחקר. ממצאים אודותיו היוו פתח למחקר מעמיק בסוגיית רעב לחנקן כן או לא ? והשפעת היכולת לנצל מקורות חנקן על הפתוגניות בסוג *Colletotrichum*.

תבדיד Ca5 בלתי פתוגני על תות שדה, אופיין בנביטת קורים ארוכים על פני תות שדה ועל משטח קשיח בתנאי רעב לחנקן. לעומת זאת, באותם התנאים יוצר תבדיד הבר כרית הדבקה סמוך לנבג הנובט. הרצף הגנומי התוחם את המחדר (flanking region) במוטנט Ca-5 בודד בריאקצית inPCR והראה דמיון במעקובת חומצות האמיניות לפקטור שעתוק המעורב במטבוליזם של חנקן *nir-A* מהפטריה *Aspergillus nidulans*. שיבוש מכוון של הגן *nir1* בתבדיד הבר גרם לירידה בפתוגניות ולפגיעה ביצירת כריות ההדבקה. במבחני נביטה *in vitro* ו *in vivo* נצפה כי חוסר זמינות במקורות חנקן, הינו בעל השפעה ישירה על מורפולוגיית הפטריה *C. acutatum* התוקפת תות שדה. אילוח איברי תות בתבדיד הבר או ב Ca-5 בתוספת מקורות חנקן, השרו גדילת תפטיר אפיפיטי שלוותה ביצירת כריות הדבקה ותוצאתה ביצירת נגע אופייני. בנוסף, באילוח עם המוטנט Ca-5 בשילוב פציעה התפתח נגע נקרוטי אופייני. כדי לאמוד את המצב התזונתי של *C. acutatum* במהלך המורפולוגיות השונות של הפטריה ובשלבי האכלוס, נבחן ביטוים של שני גנים המעורבים במטבוליזם של חנקן Nitrate reductase (*nit1*) ו- Glutamine synthetase (*gln1*), תחילה במצעי מזון שונים. בעוד שבביטוי *gln1* לא נצפה שינוי בגידול במצע עשיר לעומת מצע ללא מקורות חנקן, בביטוי *nit1* נצפה הבדל מובהק. ביטוי *nit1* מושרה במצע ללא מקורות חנקן ובנוכחות ניטרט ומעוכב במצע עשיר, מכאן *nit1* יכול להוות סמן לזמינות מקורות חנקן לפטריה. בנוסף, נמצא כי ביטוי *nit1* גבוה בשלב יצירת כריות ההדבקה. ממצאים אלו מדגישים שתנאי רעב לחנקן מהווים אינדוקציה ליצירה מיידית של כריות

הדבקה. עיכוב cAMP-dependent protein kinase A (PKA) לא השפיע על יצירת כריות ההדבקה במוטנט Ca5, מכאן מוצע ש PKA הינו רגולטור שלילי ליצירה מיידית של כריות הדבקה, וכן שפקטור השעתוק NIR1 פועל במקביל או במורד הזרם במסלול ה PKA. ביטוי *nit1* זוהה בשלבי האכלוס המוקדמים בתות שדה ומקביל לביטוי β-tubulin הפטריתי. פתוגניות מוטנטים הפגועים ביכולת לנצל ניטרט (*nit* mutants) הופחתה באופן מובהק, אם כי עדיין נבחנו שיעורי אכלוס בצמח. ממצאים אלו יחדיו מוכיחים שהפטריה *C. acutatum* נתונה במצב רעב חלקי לחנקן במשך שלבי ההדבקה בתוך הצמח. חקר איפיון המוטנט Ca5 מצביע על כך שפקטור השעתוק *nir1* תחת תנאי רעב לחנקן מבקר התפתחות כריות הדבקה וכן מוכיח שהרעב לחנקן הינו דומיננטי בשלב לפני החדירה.

ממצאים אלו המסתמכים על המוטנט Ca5 ומייחסים ליכולת החישה והתגובה למחסור במקורות החנקן את היכולת להשלים את תהליך ההדבקה עודדו לבחון את הקשר בין זמינות מקורות חנקן, מורפולוגיה ופתוגניות בסוג *Colletotrichum*, תוך ניצול הגישה הפרוטאומית. התגובה התאית לרעב לחנקן בהקשר של התפתחות והתמיינות מבני הדבקה במין *C. acutatum* נלמדה.

השוואת החלבונים המסונתזים בתפטיר הגדל תחת תנאי רעב לחנקן, תפטיר הגדל במצע עשיר, תפטיר מוטנט Ca5 הפגוע ביכולת ניצול החנקן ושלב יצירת כריות ההדבקה חשפה סידרת חלבונים שביטויים נבדל באופן מובהק בין הטיפולים. סך החלבונים המבוטאים בצורה נבדלת בין הטיפולים השונים סווגו לפי תפקידם בתא לקטגוריות עיקריות: מטבוליזם של פחמימות וחומצות שומן, מטבוליזם של חנקן וחומצות אמיניות, סינתיזה ופירוק של חלבונים, תגובה לעקה ולוכדי רדיקלים חופשיים של חמצן. מבין החלבונים הנבדלים נעשה ניסיון להתמקד באותם החלבונים המעורבים במסלולים מטבוליים או במעבר סיגנלים המובילים לשינויים המהותיים הדרושים בשלב המכריע בהדבקה עם החדירה.

ביטוי חלבונים השייכים לקבוצת לוכדי רדיקלים חופשיים (ROS reactive oxygen species) הפועלים במערך ההגנה כנגד רדיקלים חופשיים הוגבר תחת תנאי רעב לחנקן ובשלב יצירת כריות ההדבקה ובשמותיהם, CuZn superoxide dismutase (SOD) ו glutathione peroxidase (gpx). האנזים bifunctional catalase (CAT2) נצפה בעיקר בשלב כריות

ההדבקה. נוכחותם של אנזימים אנטיאוקסידנטים אלו יכולה לנבוע מהצטברות ROS ולכן יצור כמותי ואיכותי של ROS תחת תנאי גידול ומורפולוגיות שונות נבדק. ייצור ROS הוגבר באופן ניכר תחת תנאים של רעב לחנקן, המהווה תנאי מחייב ליצירת כריות הדבקה, זאת לעומת זיהוי רמות נמוכות של ROS שזוהו במשך גידול במצע עשיר. בשלבי ההדבקה בצמח זוהתה הצטברות ROS צמחי כתגובת הגנה מיידית, המצטבר במרווח האפופלסטי בעיקר בנקודת המגע בין כרית ההדבקה והדופן הצמחית. ייצור ROS פטריתי זוהה בנבגים הנובטים, במשך כל השלבים טרום החדירה עם החדירה ולאחריה. תפקיד ROS כמבקר התפתחות מבני הדבקה נחקר באמצעות גישה פרמקולוגית. יישום אנטיאוקסידנטים לדוגמת N-acetyl-L-cysteine או mannitol גרמו להפחתת ייצור ROS וירידה באחוז יצירת כריות הדבקה. ממצאים אלו מצביעים שייצור ROS יכול לבקר תגובה מיידית לתנאי רעב לחנקן ולווסת שלבי התמיינות והתפתחות מוקדמים של הפתוגן.

ביטוי נבדל של קבוצת אנזימים השייכת למסלול ניצול חומצות שומן מצביעה על העתקת פעילות מטבולית בשלב יצירת כריות ההדבקה ובשלב ההרעבה. ביטוי חלבונים ספיציפים השייכים למעגל ה glyoxylate ולמטבוליזם של חומצות שומן כדוגמת malate dehydrogenase (MDH), formate dehydrogenase (FDH) ו acetyl coA acetyltransferase הוגבר בשלב יצירת כריות ההדבקה. לעומת זאת האנזים isocitrate dehydrogenase (IDH) נצפה בקושי בשלב יצירת כריות ההדבקה. ממצאים אלו מצביעים שבעת הרעבה עיקר האנרגיה לנביטה ויצירת הכרית מסופקת ממאגר חומצות השומן. בעבודה זו הוכח שהתפתחות מבני הדבקה תחת תנאי רעב לחנקן מקודמים על ידי שינויים מטבוליים.

ממצאים אודות המוטנט הבלתי פתוגני Ca5 מסקרנים ומציגים את הצעד הראשוני בהגדרת גורמי פתוגניות ברמה המולקולרית בסוג *Colletotrichum* התוקף תות שדה. מחקר זה מנצל את איפיון המוטנט Ca5 ומוכיח שתהליכים פיזיולוגיים ומורפולוגיים מגוונים של הפטריה מבוקרים על ידי גנים החיוניים לפתוגנזה. מחקר זה מדגיש את התפישה של הבקרה של רעב לחנקן על התפתחות ופתוגניות בסוג *Colletotrichum*.

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זיהוי ואפיון גורמים גנטיים חיוניים לביטוי פתוגנזה של *Colletotrichum* spp. בתות שדה באמצעות בידוד תבדידים פחותי פתוגניות

חיבור לשם קבלת תואר דוקטור לפילוסופיה

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