Expression of defence-related genes in sugar beet plants infected with *Rhizoctonia solani* and treated with benzo- (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH)

By

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ABSTRACT

The chemicals inducers SA, BABA, and BTH were tested as seed treatment and soil drench on a partial-resistant cultivar of sugar beet grown in sand infested with the *Rhizoctonia solani* AG 2-2IIIB. In another series of experiments, BTH was applied as soil drench on sugar beet plants inoculated with *R. solani*. The chemical inducers were ineffective in reducing pre-emergence damping-off and post-emergence plant mortality. Despite these results, treatment with BTH altered the levels of expression ratios of four defence encoding genes associated with systemic resistance: chitinase, peroxidase, chalcone isomerase, and chalcone synthase. BTH sensitised sugar beet plants without the necessity of *R. solani* infection to up-regulate substantially the transcript level ratios of *chalcS* and *chit3*, while levels of *chalcI* were down-regulated levels below 1. Of interest, was the significant increase of transcript levels of *chit3* in sugar beet plants infected with *R. solani* and treated with BTH. In conclusion, sugar beet plants were capable of over expressing selected genes in response to a chemical inducer, but contrary to what had been reported, gene activation in sugar beet as a result of BTH treatment does not confer disease resistance against *R. solani*.

Résumé

Les inducteurs chimiques SA, BABA et BTH ont été utilisés pour humidifier le sol et comme traitement sur les graines d'un cultivar partiellement résistant de betterave à sucre dans un sol infecté avec *Rhizoctonia solani* AG 2-2IIIB (en pré-émergence). Du BTH a aussi été appliqué pour humidifier le sol lorsque des plantules de betterave à sucre étaient inoculé avec *R. solani* (en post-émergence). Les inducteurs chimiques se sont montrés inefficaces à réduire la mortalité aussi bien avant qu'après l'émergence de la plante. En dépit de ces résultats, le traitement avec le BTH a modifié les niveaux d'expression de quatre gènes associé à la résistance systémique: chitinase, peroxidase, chalcone isomerase et chalcone synthase. En pré-émergence, le BTH a induit l'expression de *chalcS* et de *chit3,* alors que le niveau de *chalcI* a diminué par rapport aux plantes non traitées et non infectées. En post-émergence, seulement l'expression de *chit3* a été induite par le BTH. En conclusion, les plantules de betterave à sucre sont capables de sur exprimer certains gènes en réponse à un inducteur chimique, mais contrairement à ce qui a été précédemment rapporté, l'activation des gènes dans cette plante lors d'un traitement au BTH ne confère pas de résistance contre *R. solani.*

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List of Abbreviations

act	Actin
AG	Anastomosis group
a.i	Active ingredient
ANOVA	Analysis of variance
BABA	β-amino butyric acid
BTH	Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
chalcI	Chalcone isomerase
chalcs	Chalcone synthase
chit3	Chitinase 3
ef	Elongation factor
g3pdh	Glyceraldehydes 3-phosphate dehydrogenase
per	Peroxidase
PR	Pathogenesis-related protein
RS	*Rhizoctonia solani*
SA	Salicylic acid
SAR	Systemic acquired resistance
QRT-PCR	Quantitative real-time reverse transcription- polymerase chain reaction

CHAPTER I

General Introduction

1.1. Introduction

The fungus *Rhizoctonia solani* anastomosis group 2, type 2 (AG-2-2) is one of the most common soil-borne pathogens of sugar beet (*Beta vulgaris* L.) plants causing damping-off and root rot of plants just before or after emergence of the plant from the soil (Cattanach et al. 1991), leading to major economical loss (Whitney and Duffus 1986). Disease control measures against sugar beet root rot and stem canker include the use of resistant or tolerant cultivars, fungicides (Panella 1998; Kataria 1991), and biological control agents (El-Tarabily 2004; Bargabus et al. 2002). In addition to these measures, reduction of disease can be achieved by the plant's own inducible defence mechanisms. Typically, this inducible resistance system is known as systemic acquired resistance or SAR (Ryals et al. 1996) which develops either locally or systemically in response to necrotizing pathogens or to the exogenous application of salicylic acid (SA) or synthetic compounds such as benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH or ASM commercialized as Bion, or Actigard, 2,6-dichloroisonicotinic acid (INA), β-amino butyric acid (BABA) and potassium salts (Oostendorp et al. 2001; Vallad and Goodman 2004). These compounds have been developed as a SAR activator with no antimicrobial properties, but with increased crop resistance to a wide range of diseases. These activators trigger the SAR signal transduction pathway in several plant species (Lawton et al. 1996; Kessmann et al. 1994) by switching on a wide range of well characterised SAR genes (Hammerschmidt et al. 2001), primarily those encoding PR-proteins (Durrant and Dong 2004; Ton et al. 2005), cell wall hydroxyproline-rich glycoprotein (HRGP), and

production of peroxidases and phytoalexins (Van Loon 1997; Kuc, 1995). Such plant defence activators are usually applied as a foliar spray treatment (Bokshi et al. 2003; Benhamou and Belanger 1998), but can also be applied to seeds (Geetha and Shetty 2002; Latunde-Dada and Lucas 2001) or as soil drench (Si-Amour et al. 2003; Tosi et al. 1999).

In plants, defence mechanisms include both constitutive and inducible systems. Pathogen and chemical–induced defences in plant include cell wall degrading proteins (pathogen-related PR-proteins). In sugar beet, the biosynthesis of PR-proteins namely chitinase and β-1,3-glucanase has been intensively studied in plants infected by pathogens (Nielsen et al. 1993; Nielsen et al. 1996; Gottschalk et al. 1998) and/or treated with the synthetic inducers of SA pathway: SA and its function derivative INA (Nielsen et al. 1994), and BTH (Burketova et al. 1999, 2003a,b). These studies dealt with increased induced resistance to *Cercospora* leaf spot and *Rhizomania* disease of sugar beet and monitored the activities of PR proteins or their transcription level mRNA with northern analysis.

The recent development of real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR) allows accurate expression profiling of RNA transcripts and has become the most useful method for characterizing gene expression in plant-microbe interaction studies (McMaugh and Lyon 2003; Wen et al. 2005; Restrepo et al. 2005). Studies aimed at measuring transcript levels of defence genes in plants in response to chemical inducers using PCR methodologies are limited to very few reports (Borges et al. 2003; Cools and Ishii 2002).

As far as we know there is no literature report on the effect of synthetic inducers on the induction of resistance genes in sugar beet following infections with *R. solani*. The present study investigated the development of *R. solani* disease in inoculated plants

following treatments with SA, BABA and BTH, and monitored the expression of resistance genes in sugar beet plants following BTH treatment. The effects of treatment with BTH were studied by measuring transcript levels with quantitative real-time RT-PCR of genes encoding PR proteins and key-encoding genes in the phenylpropanoid pathway that lead to phytoalexin production.

1.2. Hypotheses

I. Chemical inducers such as SA, BABA and BTH effectively protect sugar beet plants against Rhizoctonia root rot when it is applied as soil drench.

II. Protection of sugar beet plants treated with BTH is correlated with the activation of induced resistance.

III. Sugar beet plants primed with BTH, elicit resistance at the molecular level that is associated with the transcriptional activation of selected defence-related genes, increasing the resistance to the fungal pathogen *R. solani* AG2-2IIIB.

1.3. Objectives

I. To investigate the ability of BTH, SA, and BABA to protect sugar beet plants from pre- and post-emergence damping off caused by *R. solani* .

II. To investigate whether application of BTH alters the transcription levels of selected defence genes encoding chalcone isomerase, chalcone synthase, chitinase III, and peroxidase in *R. solani* infected sugar beet plants and compare the levels with the transcripts of non-treated but infected plants.

CHAPTER II

Literature Review

2.1. Sugar beet *(B. vulgaris L.)*

Beta vulgaris L. is a biennial diploid (2n=18) plant with a small genome (750 Mbp) (Schmidt et al. 1998), which belongs to the Chenopodiaceae family. Sugar beet is a specialized type of *Beta vulgaris* L. that has the ability to convert sunlight into sugar, a vital source of high-pure energy used as food for mankind (Mukhopadhay 1987). Climate influences the biology of sugar beet greatly, so the plants are biennial in colder climates, developing a large and succulent taproot in the first year and seeds in the second year, in comparison with warmer places where sugar beet develop as an annual crop. Geographically, sugar beet and other cultivars such as fodder beet or beetroot are best suited to grow in moderate climates like Europe, parts of U.S.A., Canada, the Ukraine and Russia (Plant Biosafety Office, Ontario, Canada, 2001). In Canada, sugar beet is grown in Alberta and Ontario, and the estimated surface area that is cultivated with sugar beet is continuously increasing in Canada. (For example, in 1998, 42,000 and 6,500 acres were planted in Alberta and Ontario, respectively. Sugar beet production in Canada represents about 10 to 15 percent of total domestic sugar consumption (Canadian Federation of Agriculture, 1998).

Sugar beet is exposed to many diseases caused by fungi, viruses, bacteria, mycoplasma, and nematodes which can reduce the quantity and quality of the yield (van Dijken 2001). Among them, root and foliar diseases caused by fungal pathogens such as

rhizomania caused by a virus transmitted by the soil-born fungus *Polymyxa betae*, damping-off and root rot caused by *Rhizoctonia solani* Kuhn, and leaf spot caused by *Cercospora beticola* are the most widespread diseases of sugar beet.

2.2. *Rhizoctonia solani L.*

Rhizoctonia solani Kühn belongs to the kingdom Fungi, subkingdom Basidiomycetia, Phylum Basidiomycota. The teleomorph or the sexual stage of *R. solani* belongs to *Thanathephorus cucumeris* (Frank) Donk. In nature, the teleomorph occasionally produces basidiospores while the anamorph or the asexual stage is predominantly found in the form of vegetative mycelia and/or over-wintering resistant structures, called sclerotia. Parmeter et al. (1969) reintroduced the concept of "hyphal anastomosis" for a better classification of the species complex *Rhizoctonia solani*. This concept is based on the occurrence of hyphal fusion or anastomosis between isolates belonging to the same group, called anastomosis groups (AGs). Isolates of *Rhizoctonia* that are not able to fuse are considered genetically unrelated. There are now 13 anastomosis groups (AGs), divided in subgroups identified on the basis of molecular characteristics (Carling et al. 2002). Knowledge related to the genetics and sexuality of *R. solani* species complex is limited to only a few AGs. It has been shown that isolates belonging to AG 1 and AG 4 differ in their genetics compared to AG 2 and AG 3 (Adams 1988; Julian et al. 1999).

R. solani isolates belonging to AG 2 subgroup 2IIIB cause several types of damage, including seedling damping-off, crown and root rot, as well as dry rot canker in older plants of sugar beet (Herr 1996; Scholten 2001) and survive as sclerotia in infested

plant tissues (Herr 1996). Young plants affected by damping-off have the collar region water-soaked and can be broken easily at/or near the soil line (Mukhopadhyay 1987), while infected mature sugar beet plants display crown and root rot cankers (Herr 1996) that are characterized by the production of black lesions that develop into spongy decay at the surface of the beet root and crown, and appear as concentric dark and light coloured rings (Scholten 2001). When the infected plants die, *R. solani* persists in soil as sclerotia on plant debris or other organic matter that has not completely decomposed in soil. The over-wintering vegetative sclerotia represent the major source of inoculum. The teleomorph stage is rarely found on sugar beet plants (Mukhopadhay 1987).

Sugar beet is not the only plant affected by *Rhizoctonia solani*. Rhizoctonia disease is a source of severe damage for plants such as: rice (Paulitz and Schroeder 2005), wheat and barley (Strausbaugh et al. 2004), cotton (Delgado et al. 2005), potato (Brewer and Larkin 2005; Yanar et al. 2005), chickpea (Chang et al. 2004), soybean (Ciampi et al. 2005; Zhao et al. 2005), lettuce (Grosch et al. 2004), turf grass (Blazier et al. 2004), forest trees (Stepniewska-Jarosz et al. 2006), and many others plants.

2.3. Control management of *R. solani* in sugar beet

Tremendous efforts have been undertaken towards an efficient control of Rhizoctonia disease. The disease is difficult to be controlled by only one method. Thus, an extensive program including methods such as: cultural practices, organic amendments, pesticides, use of chemical fertilizers and resistant varieties as well as biological and chemical inducers could help diminish the impact of the fungal pathogen on crop production.

2.3.1. Cultural practices

The most recommended and utilised measure in the field to reduce disease incidence are cultural management practices (Cattanach et al. 1991). These include: (i) crop rotation with less susceptible crops such as barely or wheat, (ii) field sanitation by elimination of diseased sugar beet plants, (iii) avoidance of tilling and hilling in the field, (iii) moderate irrigation, (iv) weed control during the first 4 weeks of sugar beet emergence, and (v) the addition of organic amendments to enhance the activity of indigenous fauna combined with the addition of biological control agents such as *Trichoderma or Gliocladium* species (Baby and Manibushanrao 1996; van Bruggen et al. 1996).

2.3.2. Resistant varieties

Resistance of sugar beet to *R. solani* can be augmented by classical breeding methods. Sugar beet is an out-crossing crop species. The genetic base of the resistance is not yet fully known, but there are at least four genes involved, which have more additive than dominant basis (Hecker and Ruppel, 1976). This type of resistance is a field tolerance rather than an absolute resistance meaning that under heavy infections resistant hybrids can be affected as well (Hecker and Ruppel, 1976). The first sugar beet breeding lines with resistance to Rhizoctonia were developed in the USA (Panella et al. 1995). Methods used for breeding are usually mass or recurrent selection, and visual evaluation in the field under different environmental conditions (Ruppel et al. 1979; Scholten et al. 2001). Selection pressure in the field is difficult to be adjusted and the tests with these lines generated variable data (Scholten et al. 2001; Buttner et al. 2004). Currently, there

are few varieties with partial resistance to *R. solani* available on the market such as: HI0062, Heracles, Premiere, and Laetitia, but their resistance against Rhizoctonia crown rot is not effective at the seedling stage, and is environmentally influenced.

2.3.3. Fungicides

There are no chemicals registered to control *R. solani* in sugar beet, and chemical control for root rot diseases is often expensive and not stable in field. Commercial sugar beet seed is usually pre-treated with one or more protective fungicides used against root rot pathogens (Cattanach et al. 1991). The use of selective fungicides has been found to increase the resistance to *Rhizoctonia solani* but, on the other side, sugar beet becomes more susceptible to other soil-borne fungal diseases such as *Pythium* or *Fusarium* spp. (Rodriguez-Kabana and Curl 1980).

Research priorities call for novel protection methods that are compatible with sustainable agriculture, thus favouring the use of alternative methods such as the application of chemical inducers of resistance (Kuc 2001).

2.3.4. Induced Resistance with biological and chemical activators

A new promising alternative to control *Rhizoctonia* diseases of sugar beet is to make use of the plant's own defence response, which can confer a high level of durable protection for the plants through systemic acquired resistance (SAR). This can be achieved by the use of biological and chemical inducers that in turn can elicit substantial increases in the expression of defence genes in the plants (Hammerschmidt et al. 2001; Conrath 2001).

Agrios (1988) defined disease resistance as the impossibility of the pathogen or other damaging factor to develop in plants. Plants have evolved two different types of resistance that can be either constitutive or inducible. Induced resistance develops during plant-pathogen interactions (Ryals et al. 1996; Sticher et al. 1997) and can be defined as systemic acquired resistance (SAR) (Ross 1961), or induced systemic resistance (ISR) (Kuc 2001). During the first International Symposium on Induced Resistance to Plant Diseases held at Corfu, Greece in 2000, scientists reached a consensus that SAR and ISR can be used synonymously (Hammerschmidt 2001).

2.3.4.1. Biological inducers

Beneficial organisms such as rhizobacteria, arbuscular mycorrhizal fungi and non-pathogenic fungi have been reported to protect plants from various pathogens by ISR. Direct evidence that the plant growth promoting rhizobacteria (PGPR) and strains of non-pathogenic binucleate *Rhizoctonia* that remain in plant roots, can induce resistance in plants to foliar or systemic pathogens has been demonstrated (Xue et al. 1998; Wen et al. 2005; Siddiqui et al. 2002; Tsror et al. 2000; Hwang 2003).

In sugar beet production, few antagonistic organisms including yeast (El-Tarabily 2004), *Pythium oligandrum* (Takenaka et al. 2003), *Pseudomonas fluorescens* (Nielsen et al. 1998; Thrane et al. 2001), were reported to protect sugar beet against *R. solani* AG-2-2 but their efficiency was not tested under field conditions. This protection was mediated through induced systemic resistance resulting in enhanced activities of pheneylalanine ammonia lyase and chitinase (Takenaka et al. 2003).

2.3.4.2. Chemical inducers (activators)

Chemical compounds such as 2,6-dichloro isonicotinic acid (INA) (Metraux et al. 1991), benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (Kunz et al. 1997), 3-(Allyloxy)-1,2,benzothiadiazole-1,1-dioxile (probenazole) (Watanabe 1979), and β-amino butyric acid (BABA) (Jakab 2001) have the capacity to induce defence response in plants and increase the general host systemic resistance. The resistance observed in plants after treatment with these compounds is not due to direct action of the compounds on the pathogen, because neither these compounds nor their derivatives exhibit in vitro antibiotic activity (Metraux et al. 1991).

2.3.4.2.1. BTH or ASM and INA

Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH or ASM), and commercialized as Bion™, Actigard®, or BOOST® is the first product of the new generation of crop protectants (benzothiadiazoles) that has been shown to be effective through resistance activation (Gorlach et al. 1996; Brisset et al. 2000). INA and BTH and their derivatives are known to mimic the activation of systemic resistance by necrotizing pathogens, resulting in cross protection against pathogens. At the molecular level, they stimulate the activation of defence-related genes without requiring salicylic acid (SA) production (Metraux et al. 1991; Oostendrop 2001), and/or jasmonic acid (Benhamou and Belanger 1998), and they act as a secondary messenger that activates signal transduction pathways (Metraux 2001).

Different types of application of BTH in the form of soil drench, seed treatment or foliar sprays have been effective to protect plants from several types of fungi causing

powdery mildew in wheat (Gorlach et al. 1996), *R. solani* in *Brassica* ssp. (Jensen et al 1998), rhizoctonia sheath blight in rice (Sing and Rohilla 2001), *Fusarium* crown and root rot in tomato (Benhamou and Belanger 1998), dry rot (*Fusarium semitectum)* and early blight (*Alternaria solani*) in potato (Bokshi et al. 2003). Phytophthora root and crown root (*Phytophthora capsici*) in peppers (Matheron and Porchas 2002), late blight (*Phytophthora infestans*) on potato (Si Amour 2003), downy mildew (*Plasmopara helianthi* Novot.) (Tosi 1999), anthracnose (*Colletotrichum lagenarium*) in cucumber (Kauss 2003), downy mildew of pearl millet (Geetha and Shetty 2002), and anthracnose (*Colletotrichum destructivum*) in cowpea (Latunde-Dada and Lucas 2001).

2.3.4.2.2. β-amino butyric acid (BABA)

Several experiments have been carried out with the aim to understand the mechanism of action of BABA, but the interpretation of the results was made difficult by the diverse effects observed depending on the plant and pathogen species, as well as the on the mode of application (Gozzo 2003). When applied as foliar spray, it rapidly enhanced the accumulation of the PR-1 pathogenesis related protein, in infected tomato plants with *Phytophthora infestans* (Cohen et al. 1994). When injected into tobacco stems, no accumulation of soluble PR proteins was observed, although necrotic lesions were not observed as a result of *Peronospora tabacina* infection (Cohen 1994). In spite of these observations, BABA had been successfully reported to be effective against *Phytophthora capsici* in pepper (Sunwoo et al. 1996), and *Phytophthora infestans* in tomatoes and potatoes (Cohen 2002). Applied as soil drench, BABA was effective in controlling *Phytopthora* species (Si-Amour 2003), *Botrytis cynerea* (Zimmerli et al. 2001) or *Alternaria brassicicola* (Ton et al. 2004), *Phytophthora infestans* in tomato

(Tian et al. 2004), and *Heterodera latipons* and *H. avenae* (Oka and Cohen 2001), and *Plasmopara vinicola* in grapes (Hamiduzzaman et al. 2005).

2.3.4.2.3. Salicylic acid (SA)

The plant signalling molecule salicylic acid (SA) plays an important role in induced disease resistance pathways. When applied exogenously, SA induction of SAR occurs followed by the subsequent expression of different PR encoding genes (Van Loon 1997). SA applied as soil drench has been shown to induce systemic resistance against several fungal diseases: *Phytophtora palmivora* in cacao (Okey et al. 1996), powdery mildew (*Blumeria graminis f.sp hordei*) in barley (Beßer et al. 2000), and anthracnose (*Colletotrichum gloeosporioides*) in cashew (Lopez and Lucas 2002).

2.4. Systemic resistance

Systemic and induced resistance are similar, both of them referring to the capacity of the plant to trigger a defence response upon primary infection with a pathogen or the addition of a synthetic inducer. The inducers stimulate assimilation of secondary metabolites with a role in plant defence, which reduce the action of the pathogen and suppress the disease symptom development compared with non-induced plants (Hammerschmidt 1999). It was also demonstrated that plant resistance against pathogens is more efficient and rapid if plants are pre-treated with a necrotizing pathogen or a chemical inducer that can lead to "conditioning", "priming", or "sensitization" (similar terms) of the plants (Sticher et al. 1997; Conrath 2001; Heil 2002).

When necrotic pathogens or chemical inducers are involved, the resistance response is induced via salicylic acid (SA) pathway followed by the expression of a set of

genes encoding pathogenesis-related proteins known as PR proteins (Van Loon 1997; Gozzo 2003) among which chitinases and β-1,3-glucanases are the most studied. When plants are inoculated with biological inducers such as non-pathogenic rhizobacteria, resistance functions via the jasmonate (JA)-ethylene (ET)-dependent pathway is associated with the production of defensins, which are antimicrobial peptides (Pieterse et al. 1998).

2.4.1. Pathogen-related proteins

The damage caused by the pathogen in incompatible interactions remains restricted as a result of the plant's defensive response. This response is associated with a coordinated and integrated set of metabolic alterations that are instrumental in limiting the ingress of the pathogen (Van Loon 1997). Among these alterations, PR proteins are induced not only at the place of the infection but also in distal tissues (Gozzo 2003). Currently, fourteen PR families are recognized as reliable markers for SAR and are classified according to their structure and activity (Table 1).

Table 1. The families of pathogenesis-related proteins*

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	unknown	*ypr1*
PR-2	Tobacco PR-2	b-1,3-glucanase	*ypr2,* [*Gns2('Glb,)*]
PR-3	Tobacco P, Q	chitinase type I-VII	*ypr3, Chia*
PR-4	Tobacco `R'	chitinase type I, II	*ypr4, Chia*
PR-5	Tobacco S	thaumatin-like	*ypr5*
PR-6	Tomato Inhibitor I	proteinase-inhibitor	*ypr6*
PR-7	Tomato	endoproteinase	*ypr7*
PR-8	'Cucumber chitinase	chitinase type III	*ypr8, Chib*
PR-9	Tobacco `lignin-forming peroxidase	peroxidase	*ypr9, Prx*
PR-10	Parsley `PR1'	`ribonuclease-like'	*ypr10*
PR-11	Tobacco class V chitinase	chitinase, type I	*ypr11*
PR-12	Radish Rs-AFP3	defensin	*ypr12*
PR-13	*Arabidopsis THI2.1*	thionin	*ypr13, Thi*
PR-14	Barley LTP4	lipid-transfer protein	*ypr14, Lip*

*Taken from van Loon and van Strien 1999.

PR-1 to -11 (Van Loon et al 1994), PR-12 (Terras et al 1992), PR-13 (Epple et al 1995), PR-14 (Garcia-Olmedo et al 1995).

2.4.1.1. Chitinases

Classes of acidic and basic chitinase catalyze the hydrolysis of chitin, the predominant constituent of fungus cell wall and have lysosomal activity (Tuzun 2000). They play an important role in plant development and growth, and participate in legume nodulation, in programmed cell death and pathogenesis (Kasprzewska 2003). Chitinase expression is induced during plant-pathogen interaction, elicitor treatment, or exposure to ethylene (Graham and Sticklen 1994; Kombrink and Somssich 1995) releasing an oligosaccharide elicitor that will determine the induction of phytoalexins (Li et al 2003). All plants analyzed to date (Table 2), contain multiple forms of chitinases which have been divided in to several different classes on the basis of their structural and functional properties (Neuhaus 1995; Cornelissen 1996):

Table 2. Classification of plant chitinase genes as proposed by Meins (1994) and modified by Neuhaus (1995) and Cornelissen (1996).

Family	Subfamily	Gene product known as	Other characteristics of gene product
Chi-a	*Chi-a1*	Class I chitinase	
	Chi-a2	Class II chitinase	
	Chi-a4	Class IV chitinase	
	Chi-a5	UDA	Long proline-rich domain present
	Chi-a6		
Chi-b		Class III chitinase	
Chi-c			Homologous to bacterial chitinase
Chi-d	*Chi-d1*	Class I PR-4 proteins, win proteins, hevein	Chitin-binding or hevein domain present
	Chi-d2	Class II PR-4 proteins	Chitin-binding or hevein domain not present

Chitinases belonging to class III and IV are the most widely studied with respect to the defence response of plants and the induction of systemic resistance. Generally, transcripts of chitinase III are constitutively expressed in guard cells, hydathodes and vascular bundles and their expression increases with the age of the plants (Kaszprzewska 2003; Lawton et al. 1994). Since chitinase activity in plants drastically increases after infection, it is assumed that chitinases are involved in protecting plants against plant pathogens (Bowles et al. 1990). In line with this, several reports have shown that enhanced resistance to certain pathogens is correlated with increased activities and expression of chitinases in plant tissues of the following pathosystems: cucumber mosaic virus (Anfoka 2000), pepper-*Colletotrichum coccodes* (Hong et al. 1999), potato-*Phytopthora infestans* (Buchter et al. 1997).

In sugar beet, chitinases (class III and IV) accumulated in leaves during infection with *Cercospora beticola* leading to increased resistance to new infections (Nielsen et al. 1993; 1994). Although the majority of chitinases are induced by infection or other types of stress, they are also expressed as a result of exogenous applications of chemical inducers. For example, the application of BTH and SA to healthy sugar beet elicited the accumulation of acidic chitinases (class III) in intercellular space of sugar beet leaves as well as in epidermis and parenchyma cells (Burketova et al. 1999; Burketova et al. 2003). The exogenous application of salicylic acid (SA) was found to induce chitinase accumulation in cotton, and this accumulation was correlated with increased resistance to *Verticillium daliae* toxin (Li et al 2003).

2.4.1.2. Peroxidase

Peroxidases are reactive oxygen species (ROS) which catalyses the oxidation of phenolic compounds and aromatic amines and contribute to plant cell wall lignification and strengthening which can explain their role in plant resistance (Van Loon 1997). Generally, they are related to local defence responses but have been associated with systemic resistance in different plant species and their role in plant defence has been extensively reviewed (Bowles et al. 1990; Lamb and Dixon 1997). Increased peroxide activity has been observed in a number of resistant interactions involving plant-pathogenic fungal and bacterial interactions and their increase has been associated with decreases in the rate of multiplication and spread of the pathogen (Lagrimini et al. 1987; Wu et al. 1995; Chittoor et al. 1997; Benhamou et al.1998; Brisset et al. 2000; Trognitz et al. 2002). In the pathosystem apple-*Erwinia amylovora,* local and systemic activation of peroxidases was associated with protection against fire blight as a result of apple treatment with the chemical inducer ASM (Brisset et al. 2000). All of the above studies demonstrate that peroxidases are markers of defence in various pathosystems.

2.4.1.3. Phenylpropanoid pathway enzymes

Chalcones are core enzymes of the phenylpropanoid pathway and precursors of isoflavonoid phytoalexins. They have an important role in the normal development and growth of plants, and are involved in local defence response (Durango et al. 2002). *R. solani* infection in mycorrhizal bean plants induced the accumulation of chalcone synthase and chalcone isomerase in leaves (Guillon et al. 2002). High amounts of chalcone isomerases were detected in tomato plants as result of treatment with chemical inducers (TOGE-1 and TOGE-2) in the absence of infection (Flors et al. 2003). These

results demonstrate that chalcones are induced systemically in plants as a result of stress or treatment with chemical inducers.

In sugar beet infection with *Cercospora beticola* suppressed the expression of two genes involved in phenylpropanoid metabolism: phenylalanine ammonia lyase (BvPAL) and cinnamic acid 4-hydroxylase (BvC4H) (Schmidt et al. 2004). These two genes encode enzymes that play an important role as precursors in phenylpropanoid mechanism with an important role in phytoalexin biosynthesis, chalcone synthesis, and plant defence response (Bowles 1990).

2.5. Detection and quantification of defence associate genes

2.5.1. Molecular methods

Quantification at the transcriptional level of defence-related genes primed with chemical inducers can be performed using (i) Northern blot analysis, (ii) cDNA arrays, and (iii) real-time RT PCR technologies. (i) Northern blot was used to estimate transcript accumulation of peroxidase in barley induced with dichloro-isonicotinic acid (DCINA) against powdery mildew fungus (Kogel et al. 1994) or to detect the level of defence response in *Arabidopsis* treated with probenazole (Yoshioka et al. 2001). The high amount of RNA (more than 10 g) was necessary for the analysis but the low accuracy and sensitivity limits its utilization. Alteration of the expression of PR mRNA in *Arabidopsis* plants conditioned with BABA was analyzed by RNA blot analysis (Zimmerli et al. 2000). (ii) Defence-related pathways and global changes in gene expression can be studied more in detail using cDNA arrays, which allow detailed studies of different metabolic pathways. Alteration of gene expression in *Arabidopsis* plants infected with *Alternaria. brassicicola* was examined using micoarrays and validated with reverse

transcription polymerase chain reaction quantitative QRT-PCR experiments (Schenk et al. 2003). This method is very expensive and not affordable for many laboratories. (iii) Real time reverse transcription (QRT-PCR) analysis, a reliable and precise method is becoming the current method of choice for gene expression studies replacing northern analyses. Using real time RT-PCR, expression analysis of defence encoding genes in plant-microbe interaction studies or in studies dealing with chemical and/or biological resistance inducers has been recently demonstrated (Borges et al. 2003; Wen et al. 2005).

In RT-PCR reactions, total RNA extracted from cells or tissues exposed to different treatments is reverse-transcribed into cDNA, and amplified using specific primers through PCR. Real-time RT-PCR differs from classical or conventional RT-PCR by the quantification of the amplified product at each cycle throughout the PCR reaction. In real-time PCR fluorescent chemistries such as molecular beacons, Taqman probe, and SYBR green are used to detect the amplification of target genes and to assess their expression levels using a calibration curve (Bustin and Nolan 2004).

Thus, real-time RT-PCR allows the amplification of a biological sample together with fluorochrome present in the reaction to be followed in real-time during the exponential phase of the run allowing the amount of the starting material to be determined. During the exponential phase in real-time PCR experiments, a fluorescence signal threshold is determined at which all samples can be compared. This threshold is calculated as a function of the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than the background fluorescence. The number of PCR cycles required to generate sufficient fluorescent signal to reach this threshold is defined as the cycle threshold, or CT. These CT values are directly proportionate to the amount of starting template and are the basis

for calculating mRNA expression levels (Bustin 2002). Since this methodology has the ability to measure small changes in gene expression and quantify low amounts of copy number, it becomes attractive to be incorporated in different applications including gene expression quantification (Smidt et al. 2005), mRNA expression (Schmid 2003), SNP (single nucleotide polymorphism) analysis and allelic discrimination assays (Afonina 2002), confirmation of microarray data (Zou et al. 2002), GMO (genetically modified organisms) testing and pathogen detection (Watzinger et al. 2006).

2.5.2. Primers

In comparison with other methods which require the hybridization of long nucleic acid base pairs such as Northern blot and microarray, real-time PCR requires short specific primers (Gachon et al. 2004). Ideally, primers should be between 70-300 base-pairs (bp) when using the fluorochrome chemistry SYBRgreen®. Also, primers should be designed to avoid primer-dimer formation which can induce signal contamination and influence the fluorescence measurement leading in inaccurate representation of the true target concentration. Melting curve analysis of each product is used to differentiate between specific and non-specific products. In order to avoid false results, amplicon sequencing and screening with the genome database is recommended.

2.5.3. Nucleic acid purification and cDNA synthesis

The quality of RNA is critical for gene expression analysis using real-time RT-PCR methodologies (Bustin 2002). Commercially available kits such as: RNeasy mini kit (Qiagen), Illustra RNAspin mini isolation kit (Amersham Pharmacia), Tri®Reagent RNA isolation reagent (Sigma), and RNA isolation kit (Stratagene) are good enough to produce

clean and high quality RNA. But often, the RNA is contaminated with DNA, and therefore DNAse treatment is recommended as a measure to ensure purity of RNA. This step is always required before reverse transcription in order to avoid false results. Accurate expression analysis depends very much on reverse transcription because it can be a source of variability.

2.5.4. Housekeeping genes

Housekeeping genes (HKGs) or sometimes referred to as normalizer genes, are internal controls used to normalize data in order to remove sampling differences in real time RT-PCR (Vandesompele et al. 2002). Actins, tubulins, glyceraldehyde-3-phosphate dehydrogenase (g3pdh or gapdh), 18S rRNA or 28S rRNA are ubiquitously expressed in all cells and can be used as HKGs if their expression is stable under various experimental conditions (Pffafl 2001; Thellin et al. 1999). Normalizer gene used as internal control should be quantified at the same time with the target gene. Because different studies demonstrate that housekeeping genes become variable under different treatment conditions and in different tissues (Rubie et al. 2005; Glare et al. 2002), the use of more than one housekeeping gene is recommended in order to achieve reliable results (Thellin et al. 1999; Vandesompele et al. 2002; Nicot et al. 2005). Softwares such as Bestkeeper (Pffafl et al 2004) and GeNorm (Vandesompele et al. 2002) are tools to compare, rank different housekeeping genes and permits the researcher to determine the most stable gene.

2.5.5. Methods of quantification

There are two basic quantification methods of analyzing data from real time: (i) absolute quantification and (ii) relative quantification (Livak and Schmitgen 2001; Liu and Saint 2002; Pffafl 2002).

(i) Absolute quantification requires a standard curve or calibration curve (Pffafl 2002) constructed from dilution series of control template of known concentration to measure the exact level of the template in the samples (e.g., the precise copy numbers of mRNA transcripts per cell or unit mass of tissue) (Liu and Saint 2002). Usually, the standard curve is a straight line generated by plotting the log of initial template copy number against the CT generated for each dilution. Problems related to measurements accuracy can determine errors in the efficiency of the amplification and finally reduce the reproducibility. A standard curve should be constructed at least of four different dilutions concentrations and run in duplicate. Linearity of standard curve is defined by Pearson correlation coefficient (R^2) and should be close to 1. Ideally the efficiency should be close to 100% for standards and target. Error in good laboratory practices such as: probe degradation, pippeting, PCR inhibitors, primer-dimer formation can lead in false results and low efficiency.

(ii) Relative quantification is based on the expression of the target gene relative to a reference gene. Two relative quantification methods are currently available: 1-The comparative CT method (threshold cycle) is generally applied when the amplification efficiency of a reference gene is equal to that of the target gene (Liu and Saint 2002; Livak 1997; Pfaffl 2002). This method detects the relative gene expression with the formula: $2^{-\Delta\Delta CT}$.

The second method developed by Pfaffl (2001) is denoted by:

$$\text{Ratio} = (E_{target})^{\Delta CPtarget(control\text{-}sample)} / (E_{ref})^{\Delta CPref(control\text{-}sample)},$$

where E is the real-time RT-PCR efficiency calculated with formula: $E=10^{[-1/slope]}$, and ΔCP is the deviation of crossing points or CT (threshold cycle) of control-unknown sample for target or reference (HKG) gene. Differences in CT value between an unknown sample and control sample are expressed as fold changes relative to the control.

CHAPTER III

Material and Methods

3.1. Fungal material and inoculum preparation

A highly pathogenic *Rhizoctonia solani* Kühn isolate 225 (IRS code) belonging to AG 2-2IIIB was produced on oat kernels according to the method of Cardoso and Echandi (1987). Starter cultures for inoculum production were produced by placing one colonized oat kernels on potato dextrose agar (PDA™; Difco Laboratories, Detroit, Michigan) culture plates at 24^0C for one week. For experiments in which sugar beet seeds were grown in infested sand, inoculum consisted of 3-week-old colonized sand ground oatmeal (20:1) prepared as follows: a mixture of sieved sand (2 mm) and ground oatmeal (20:1) were placed in plastic polypropylene jars (Fisher®) with screw caps (1000 ml). The mixture was autoclaved three times on three consecutive days with 24-36 hours interval at 121^0 C for 45 minutes. One-cm pieces of one-week-old starter culture of *Rhizoctonia solani* isolate 225, were added to the soil-oatmeal mixture, and incubated for 3 weeks at room temperature, and to obtain a uniform and even distribution of *R. solani* growth in the containers the mixture was shaken once a week. Prepared sand-oatmeal inoculum was stored at 4^0 C for a maximum period of two or three months. For experiments employing six-week-old sugar beet plants, the inoculum consisted of four-day-old *R. solani* colonized millet seeds prepared as follows and according to Nagendran and McGrath (personal communication): de-hulled seeds of millet, sterilized on three consecutive days at 120^0 C for 20 minutes each day, were placed as single layer on the

surface of an actively growing 3-day-old PDA fungal culture and were then incubated at room temperature in the light for four days.

3.2. Plant material and culture conditions

Seeds of sugar beet (*Beta vulgaris* L.) cv. HI0062 diploid, partial resistant hybrid to *R. solani,* were supplied by Hilleshøg (a branch of Syngenta), Landskrona, Sweden and stored at 4^0 C. Seeds were surface-sterilized for five minutes in 30 % H_2O_2, rinsed four times in sterile distilled water, and then dried on filter papers. The percent seed germination of the cultivar was tested to be 100 %.

Custom made PVC tubes (15 cm x 1.5 cm outer diameter) were used to grow the plants. Prior to use, the PVC tubes were surface sterilized for 1 hr in 2.5 % (w/v) NaOCl, rinsed with distilled water. For water retention and to prevent the sand from drying out, one end of the tubes was plugged with 2 cm x 2 cm wet foam, and filled with 70 g of wet sand previously pasteurized for 1 hour at 121^0 C. One sugar beet seed/PVC tube was placed at 1 cm depth in the sand.

Plants were grown in a controlled environment chamber (Conviron®) calibrated to 23^0 C and 10/14 h day/night, with moderate humidity and light intensity of 350 μmoles/m^2/s. Plants were watered daily at the same time, with 2.5 ml sterile water. For two weeks old plants in post-inoculation experiments the water was replaced once per week with 2.5 ml of modified (1:1 v/water) Hoagland's solution containing 0.5 M K_2SO_4, 1 M $MgSO_4$, 0.05 M $Ca(H_2PO_4)$, 0.01 M $CaSO_4$, FeEDTA at 134 g/100ml, and micronutrient solution at 1 ml/liter (2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4H_2O$, 0.22 g of $ZnSO_4 \cdot 7H_2O$, 0.08 g of $CuSO_4 \cdot 5H_2O$, and 0.02 g of H_2MoO_4 in 1 liter of water).

3.3. Chemical inducers

BTH [1,2,3- benzothiadiazole-7-thiocarboxilic acid S-methyl ester] (Actigard) was obtained from Syngenta Crop Protection Canada Inc. (Guelph, ON, Canada) as a 50 % water dispersible granule (WG 50). It was dissolved in water and solutions of 10, 30, and 50 mg active ingredient (a.i)/L in deionised distilled water were prepared. Salicylic acid SA (Sigma Chemicals Canada, Ltd.) was dissolved in deionised distilled water and prepared at three different concentrations: 1.0, 2.5 and 5.0 mM of SA respectively. BABA [beta-aminobutyric acid] was obtained from Sigma-Aldrich, Canada, Ltd., and was prepared at two different concentrations: 0.5 and 1.0 g /L of deionised distilled water. All the above solutions of the inducers were prepared on the day of application.

3.4. Experimental series

3.4.1. Experiment 1. Phytotoxicity of chemical inducers

To choose the optimum concentration of the chemical inducers that does not cause phytotoxic effect, 60 seeds of sugar beet for each treatment were soaked in 200 ml of different concentrations of SA, BTH, BABA for 24 h at room temperature with constant stirring. Control seeds were soaked in sterile distilled water. Treated and control seeds were then separately sown in PVC tube containing wet sterile sand. After 10 days, the sand was drenched with 2.5 ml of different concentrations of SA, BTH, BABA dissolved in distilled water. Control treatments were drenched with 2.5 ml of water. Treated and untreated plants were placed in a controlled growth chamber in a complete randomized block design (CRBD) with three replicates for each combination of 20 plants per

treatment. At 12 days, all the plants were harvested and assessed for % emergence and seedling vigour.

3.4.2. Experiment 2: Effect of BTH, BABA, and SA on disease severity caused by *R. solani* on sugar beet grown in *R. solani* infested sand.

Sugar beet seeds were soaked in BTH (50 mg a.i./L), BABA (1 g/L), or SA (1 mM) solution with constant stirring for 24 h at room temperature and then planted at 1 cm depth in PVC tubes filled with sterile sieved sand containing 0.075 % concentration of sand-oatmeal *R. solani* inoculum or in PVC tubes containing sand-oatmeal alone (control treatment). At planting and 7 days after planting, each PVC tube received 2.5 ml of BTH solution (50 mg/L), BABA (1 g/L), or SA (1 mM) as soil drench of each chemical inducer, and the plants were harvested 14 days after seeding.

There were four treatments for each inducer: a) healthy plants grown in non-infested sand and not treated with BTH, BABA, or salicylic acid (-BTH-RS;-BABA-RS;-SA-RS); b) plants treated with inducer only (+BTH-RS; +BABA-RS;+SA-RS); c) plants grown in infested *R. solani* sand only (-BTH+RS; -BABA+RS; -SA+RS); d) plants grown in *R. solani* infested sand and treated with inducers (+BTH+RS; +BABA+RS; +SA+RS). There were three replicates and each replicate of each treatment consisted of 25 seeds. The PVC tubes for each replicate were placed in racks and were arranged in a split plot randomized complete block design with time as main plot and treatment as subplot. The plants were harvested 14 days after seeding. The entire experiment for BTH was repeated twice, while that for BABA and salicylic acid only one trial was conducted. Plants were observed daily after they had emerged and rated for crown lesions and damping-off

symptoms at 7 and 14 days after seeding. Emerged plants represent all those that emerged, including diseased and/or damped-off. All emerged plants were scored for disease using the following scale: 0 = no symptoms; 1= nearly healthy with small lesions; 2 = plants damped-off but with recognizable tissue; 3 = plants completely damped-off unrecognizable tissue At 14 days, all plants with no symptoms were pulled out carefully and roots were immediately washed in distilled cold water to remove sand debris. The entire plant was flash-frozen in liquid nitrogen and stored at -80^{0}C.

3.4.3. Experiment 3: Effect of BTH on disease severity caused by *R. solani* on sugar beet inoculated plants .

Six-week-old sugar beet plants grown in sterile sand in PVC tubes were inoculated with one millet seed completely covered with *R. solani* mycelia or with sterile millet seeds depending on the treatment. Preliminary experiments using two or three millet seeds caused high percentage of mortality due to severe *R. solani* infections. The millet seeds were placed at the surface of the sand and at a distance of 0.5 cm away from the sugar beet plant. Each tube received 2.5 ml of Hoagland's solution once per week starting from the third week until harvesting.

Two applications of BTH (50 mg a.i./L) were applied as soil drench mixed with the fertilizer (2.5 ml/tube). One was applied one week before inoculation (5 weeks old plants) and the second application was at the time of inoculation (6 week old plants). There were four treatments: a) healthy plants grown in non-infested sand and not treated with inducer(-BTH-RS); b) plants treated with inducer only(+BTH-RS;); c) plants grown in infested *R. solani* sand only (-BTH+RS); d) plants grown in *R. solani* infested sand and treated with BTH (+BTH+RS). There were three replicates and each replicate of each

treatment consisted of 17 plants. PVC tubes were arranged in a split plot randomized complete block design with time as main plot and treatment as subplot. The entire experiment was repeated twice.

Plants were observed daily after their inoculation and rated for crown lesions and damping-off symptoms at 7 and 14 days after infection. Disease severity scale for plant lesions 7 days after infection ranged from 0 = no disease to 3 = damping-off or dead. Disease severity scale for plant lesions 14 days after infection ranged from 0 = no disease; 1 = 25 % lesions on hypocotyls; 2 = 25-50 % lesions; 3 = 51-75 % lesions; 4 = 76-100 % lesions. At 8 weeks old (14 days after infection) plants with no disease symptoms were carefully pulled out and roots were immediately washed in distilled cold water to remove sand debris. The entire plant was flash-frozen in liquid nitrogen and stored at -80^0 C.

3.5. Primer design

Primers were designed for defence-related genes (chalcone isomerase, chalcone synthase, chitinase class III, and peroxides) and for three housekeeping genes (actin, *g3pdh*, and alpha elongation factor. Description of the PCR amplification product size, accession no., and sequence are listed in Table 3. All the primers, except for *g3pdh*, and alpha elongation factor, were manually designed using nucleotide sequences specific deposited at Genbank for each of the genes (http://www.ncbi.nlm.nih.gov/entrez/query.fgi?db=nucleotide). The sequences were aligned using ClustalW program (http://workbench.sdsc.edu/), and primers were designed from the most conserved region. The primers obtained were checked with DNAman and verified with BLASTN in NCBI (http://www.ncbi.nlm.nih.gov) for homology. Primers

for *g3pdh* and alpha elongation factor were designed by M. McGrath, University of Michigan. All primers were custom synthesized by Alpha DNA (Montreal, Quebec).

Virtually all RNA samples may have traces of contaminating DNA, and primers spanning intron-exon boundaries can amplify this DNA, which can result in inaccurate quantification during real-time RT-PCR runs. Thus, DNase I treatment was carried out to eliminate DNA contamination that may lead to false result. cDNA, and DNA extracted from sugar beet plants were amplified using the designed primers in conventional reverse transcription (RT)-PCR and PCR assays, respectively. The identity of amplification products was confirmed on 1.2 % agarose gel electrophoresis followed by ethidium bromide staining. Along with them, negative controls (no template) and positive controls (DNA extracted from sugar beet) were also included.

Table 3. Primers used in RT-PCR for amplifying defence-related genes of *Beta vulgaris*.

Target gene*	Primer sequences (5'→3')	Size (bp)	Gene location (accession no)	Reference
act BV	AAGTACCCAATTGAGCACGGT AATCAGTGAGATCACGACCAG	340	AW063023	1
ef BV	ACTCCCAAATAAGAATGCCTAT CCAATGTAACACTGTCTCCAA	451	BI073178	2
g3pdh BV	GATGCCCCTATGTTTGTTG TGGGTAGCAGTGATGGAGT	175	BI095886	2
chalcS BV	CACAAGCACTAGACATGTTAC ATGCCTTCACTCCACTTGG	183	BI643106	3
chalcI BV	GTGCCATTGAGCTAGCAGATT TCTGAACTGCCTTGGCTTCTT	192	BI073245	2
per BV	CAGTGGGCGGAAGACAGGGTC CTCTCTGGAGGCCACACCTTG	144	AW063022	1
chit3 BV	TAGGAGGTGGTGCCGGAGGC GCTTGTACTGTGGTCCACTGG	354	S66038	4

* *act* BV =actin; *ef* BV =alpha elongation factor; *g3pdh* BV = glyceraldehydes 3-phosphate dehydrogenase; *chalcS* BV =chalcone synthase; *chalcI* BV = chalcone isomerase; *per* BV = peroxidase; *chit3* BV =chitinase 3.

1. McGrath, J.M., de los Reyes, B.G., Myers, S., and Derrico, C. 1999. Mapping the sugar beet (*Beta vulgaris*) genome with EST markers. Unpublished; 2. de los Reyes, B.G., McGrath, J.M., and Myers, S. 2001. Differential gene expression in sugar beet plants (*Beta vulgaris*) germinated under stress conditions.Unpublished; 3. de los Reyes, B.G., McGrath, J.M., Myers, S., and Derrico, C. 2000. Differential gene expression in sugar beet plants (*Beta vulgaris*) germinated under stress conditions. Unpublished; 4. Nielsen, K.K., Mikkelsen, J.D., Kragh, K.M., and Bojsen, K. 1993. An acidic class-III chitinase in sugar-beet induction by *Cercospora beticola*, characterization, and expression in transgenic tobacco plants. Mol. Plant-Microbe Interact. 6(4): 495-506.

3.6. RNA isolation and cDNA preparation

100 mg of frozen tissue of the pooled healthy plants for each treatment of experiments with sugar beets sown in infested sand and of inoculation experiments was ground into a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted under sterile conditions using the Qiagen RNeasy Plant mini Kit™ (Qiagen Inc., Mississauga, ON, Canada) and treated DNAse with RNase-Free DNase kit™ (Qiagen) according to the manufacturer's recommendations. Residual contamination of RNA with genomic DNA was verified by conventional PCR using the primers listed in Table 1. RNA samples contaminated with DNA were purified using RNeasy columns (Qiagen) until no DNA was detected. Nucleic acid concentration and quality of extracted total RNA was determined by the UV absorption measurement at 260 using NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, USA) and by formaldehyde gel electrophoresis. Total RNA (500 ng) was reverse transcribed at 37^0C for 1 h using the Omniscript RT kit™ (Qiagen) according to the manufacturer's recommendations.

3.7. cDNA amplification of defence-associated genes by conventional RT-PCR

Conventional RT-PCR assays (GeneAmp PCR system 9700 thermocycler (Perkin-Elmer) [PE]) were conducted on cDNA templates (1:50 dilution) using the designed primers listed in Table 1 in order to verify the identity of amplified PCR products. Amplification was carried out in a final volume of 25 µl containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM of each primer, 0.5 U *Taq* polymerase (Invitrogen Life Technologies Burlington, ON, Canada) and 2 µl of cDNA template. The PCR conditions were an initial denaturation at 94^0C for 1 min, 35 cycles of the following temperature

conditions were used: 94^0 C for 30 s, 55^0 C for 30 s, and 72^0 C for 45 s. A final extension at 72^0 C for 1:30 min was performed. For the target gene *chalI*, the amplification conditions were: 1min for the initial denaturation at 94^0 C followed by 35 cycles of amplification at 94^0 C for 30 s, 60^0 C for 30 s, and 72^0 C for 45 s. An extension step at 72^0 C for 1:30 min was added at the end of each run. In all RT-PCR runs, negative controls containing no cDNA templates were run under the same conditions.

The amplified products for all target genes were purified using QIAquick PCR Purification kit™ (Qiagen), quantified using Nanodrop spectrophotometer, and sequenced to confirm the specificity of the RT-PCR products (McGill University and Genome Quebec Innovation Centre Sequencing). Serial dilutions in the range of concentration from 10^6 to 10^{13} copies/µl of purified PCR products and over 7 orders of magnitude were used to construct standard curves for each target gene.

3.8. Gene expression analysis by real-time QRT-PCR

Real-time QRT-PCR was performed for each of the defence-related genes, and for the three housekeeping genes on cDNA templates prepared from total RNA that was extracted from sugar beet plants using the Thermal Cycler Stratagene Mx3000P machine. 4 µl of each concentration of standard solution prepared from purified RT-PCR products was included in each run. Reactions were prepared in optical strip tubes Mx3000p® (Stratagene, Tx, USA) using the following master mix: 10 µl of Brilliant SYBR Green™ PCR master mix (Qiagen), 0.30 µl (30 nM) of ROX™ (passive reference dye used to normalize SybrGreen fluorescent signal) and 4 µl of cDNA (diluted 1:50). Different amounts of primers and water were used depending on the primer concentration. For *act,*

ef, chit3, chalI, and *per*, the primer final concentration was 75 nM; 100 nM for *gapdh;* and 125 nM for *chalS*. Water was added to a final volume of reaction (20 µl). The optimum reaction conditions for *chalI*, *chit3*, and *act* were: initial denaturation step at 95^0 C, 40 cycles of amplification at 95^0 C for 30 s, 63^0 C for 1 min and 72^0 C for 30 s. The optimum reaction condition for *per* was: initial denaturation step at 95^0 C, 40 cycles of amplification at 95^0 C for 30 s, 67^0 C for 1 min and 72^0 C for 30 s. Following amplification, a dissociation melting curve program (55^0 C to 95^0 C), with a heating rate of 0.5^0 C/s. For *chalS*, the reaction conditions were: initial denaturation step at 95^0 C, 45 cycles of amplification 95^0 C for 30 s, 63^0 C for 1 min and 72^0 C for 15 s. For *gapdh* the reaction conditions were: initial denaturation step at 95^0 C, 45 cycles of amplification 95^0 C for 30 s, 61^0 C for 1 min and 72^0 C for 15 s. For *ef* the reaction conditions were: initial denaturation step at 95^0 C, 45 cycles of amplification 95^0 C for 30 s, 58^0 C for 1 min and 72^0 C for 15 s. Similar dissociation (melting curve) analysis was performed at the end of the real-time RT-PCR.

Fluorescence threshold was set by default method at 32.5 % with Stratagene software for Mx3000P Real-Time PCR instrument. Standard curve was generated by plotting the log of the initial template copy number against the CT (threshold cycles numbers) generated for each serial dilution. Standard curve quantification was performed in duplicate to check for PCR reaction efficiency, precision and sensitivity. The equation of the line that best fits the data was determined by minimizing error for regression analysis. The R^2 value was calculated to estimate the accuracy of the real-time RT-PCR as a quantification method. The slope of the standard curve was used to calculate PCR efficiency according to the formula:

[1] $[10^{(-1/slope)}]-1$ (Radonic et al. 2004).

Sample CT values were used to estimate template quantity by comparing them to standard curve.

Reactions were performed in technical duplicates and averages were used. The CT value for each QRT-PCR reaction was normalized using the CT corresponding to actin (housekeeping) sugar beet gene and used to calculate fold differences of gene expression in different treatments compared to a control sample according to the formula of Pfaffl (2001) and modified by McMaugh and Lyon (2003):

[2] $$\text{fold change} = [E_{gene})^{\Delta CT_{gene}\,(control\text{-}sample)}]/(E_{actin})^{\Delta CT_{actin}\,(control\text{-}sample)}].$$

E_{gene} is the real-time RT-PCR efficiency of a defence gene transcript, E_{actin} is the real-time RT-PCR efficiency of gene actin transcript, ΔCT_{gene} is the CT deviation of control-sample of the target gene transcripts, and ΔCT_{actin} =CT deviation of control-treatment of actin transcripts.

3.9. Statistics

Non-parametric Kruskal-Wallis test (PROC NPAR1WAY in SAS) was used to compare disease severity scores among the four treatments (significance at $P < 0.05$). The emergence (%) of plants was evaluated using two-way analysis of variance (ANOVA PROC GLM SAS). Statistical analysis to calculate fold change of defence gene expression in real-time RT-PCR was done using one-way analysis of variance (ANOVA PROC GLM in SAS) (SAS Institute Inc, Cary, NC, USA). Homogeneity between two repeated trials was tested using χ^2 test ($P < 0.05$) (Gomez and Gomez 1983). Where applicable, data from the two repeated trials were pooled and analyzed as six blocks in

order to increase the power of analysis. Comparisons between means were made using least significant differences (LSD) at a 0.05 probability when ANOVA indicated model and treatment significances.

The BestKeeper software (http://www.gene-quantification.info/) was used to determine the most stable housekeeping gene (HKG) using repeated pair-wise correlation analysis ($P < 0.001$) and to determine the "best" one using geometric means. Highly correlated HKG were combined into an index. Relation between the index and HKG is described by Pearson correlation coefficient (r), coefficient of determination (r^2) and the p-values. The weighted index is then correlated with target genes using the same pair-wise correlation analysis (Pffafl et al. 2004).

CHAPTER IV

Results

4.1. Phytotoxicity of chemical inducers

At all rates of BTH and BABA, none were phytotoxic when applied as seed soak and soil drench to sugar beet plants. The compounds did not produce chlorosis on the cotyledons and first true leaves (Data not shown). Although the highest percent emergence of sugar beet plants was observed for control plants, there was no significant difference in percent seedling emergence between BTH and BABA inducer-treated plants and the control (Table 4). At high rates (2.5 mM, and 5 mM) of SA, sugar beet plants showed a substantial reduction in percent emergence. Concentrations of BTH at 50 mg a.i./L, BABA at 1 g/L, and SA at 1 mM were selected to be used in future experiments.

4.2. Effect of chemical inducers on disease supression caused by *R. solani* on sugar beet grown in *R. solani* infested sand.

Plants grown in *R. solani* infested sand and treated with BTH showed similar level of disease rates compared to the other two treatments and the control (Table 5), but had a significantly ($P < 0.05$) lower percent emergence compared to *R. solani* infected or BTH treated seedling and to the control plants (Fig. 1). Plants grown in sand infested with *R. solani* and/or treated with BABA and SA showed no disease symptoms and their percent emergence was comparable to sugar beet plants that were non-infected and not treated (Control treatment) 7 and 14 days after seeding ($P > 0.05$; data not shown).

Table 4. Effect of different concentrations of chemical inducers applied as seeds treatment and soil drench on percent emergence of sugar beet seedling.

Inducer	Concentration	% emergence*
Salicylic acid (SA)	0 mM (Control)	91.7 a
	1.0 mM (138.1 mg/L)	80.0 a
	2.5 mM (345.3 mg/L)	20.0 b
	5.0 mM (690.0 mg/L)	18.3 b
BTH	0 mg (Control)	90.0 a
	50 mg a.i./L	85.0 a
	30 mg a.i./L	83.0 a
	10 mg a.i./L	90.0 a
BABA®	0 mg (Control)	100.0 a
	500 mg/L	91.7 a
	1000 mg/L	88.3 a

* Means represent the average values of three replicates. Means followed by different letters within a column are significantly different according to LSD test ($P < 0.05$).

Table 5. Effect of BTH on disease severity of sugar beet caused by *Rhizoctonia solani* (plants grown in infested *R. solani* soil and plant inoculation experiments).

	Disease scale*			
	Sugar beets grown in *R. solani* infested sand†		Plants inoculated with *R. solani*‡	
	Time (days)			
	7	14	7	14
-BTH-RS	0a	0a	0a	0a
+BTH-RS	0a	0a	0a	0a
-BTH+RS	0a	0a	0a	2b
+BTH+RS	0a	1a	0a	2b

*Values in a column with different letters are significantly different according to Kruskall-Wallis test ($P < 0.05$).

†All emerged plants were scored for disease using the following scale: 0 = no symptoms; 1 = standing up with small lesions; 2 = plants damped-off but with recognizable tissue; 3 = completely damped-off plants with brown tissue unrecognizable. Values represent median of six replicates.

‡Disease severity scale for plant lesions 7 days after infection ranged from 0 = no disease to 3 = damping-off or dead. Disease severity scale for plant lesions 14 days after infection ranged from 0 = no disease; 1 = 25 % lesions on hypocotyls; 2 = 25-50 % lesions; 3 = 51-75 % lesions; 4 = 76-100 % lesions. Values represent median of six replicates.

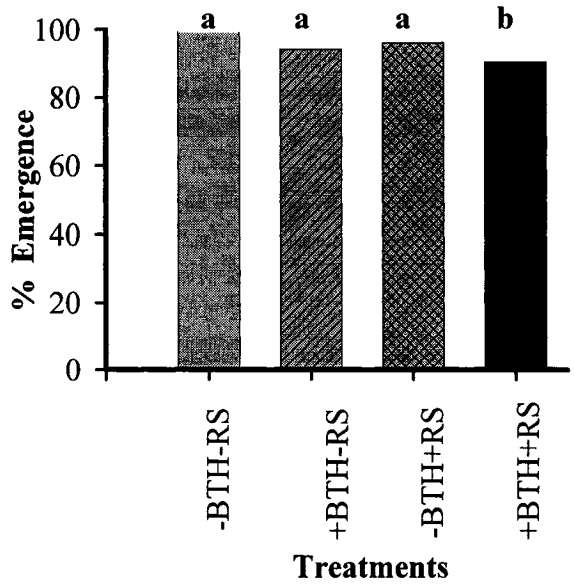


Figure 1. Effect of chemical inducer BTH on % emergence (sugar beets sown in *R. solani* infested sand experiment).

Means represent the average values of 12 observations. Values in a graph with different letters are significantly different according to LSD test ($P < 0.05$).

4.3. Experiment 3: Effect of BTH on disease severity caused by *R. solani* on sugar beet inoculated plants.

Because the percent emergence of beet plants with BTH was generally slightly higher although not significant than those observed with the other inducers, post-inoculation experiments were conducted with BTH only.

Table 5 demonstrates that irrespective of the treatment, sugar beet plants showed no disease symptoms 7 days after applying BTH as soil drench ($P > 0.05$). Soil drench with BTH (50 mg a.i./L) did not protect sugar beet plants from *R. solani* infections harvested 2 weeks after infection (+BTH+RS). These plants had similar disease index score as those that were infected but not treated ($P < 0.05$). Significantly more disease was observed after two weeks of post infection compared to one week.

4.4. Quantification of defence-related genes expression in sugar beet plants

In this study we monitored the expression of four defence related genes that are known to be activated in sugar beet plants upon elicitation, wounding, or infection (Table 6). The designed primer sets successfully amplified the expected size of amplified product when cDNA was used a template (Fig.2). The specificity of the amplified products in pre- and post-inoculation experiments was verified by melting curve analysis and gel electrophoresis (Fig. 2). In pre-inoculation experiments, RT-PCR products for each primer set showed a single peak with the following specific melting temperature: *chalcI*, 78.16 ± 0.74^0 C, *chalcS*, 78.7 ± 0.25^0 C, *chit3*, 82.012 ± 0.2^0 C, *per* 80.95 ± 0.29^0 C, and *act* 80.55 ± 0.55^0 C. Similarly, in post-inoculation, RT-PCR products for each primer set showed a single peak with the following specific melting temperature: *chalcI*, 79.58 ± 0.23^0 C, *chalcS*, 79.6 ± 0.25^0 C, *chit3*, 82.125 ± 0.275^0 C, *per* 82.04 ± 0.32^0 C, and

act 82.05 ± 0.7^0 C (Fig 2 A, C, E, G, and I) and a unique putative product when cDNA was used as a template (Figs. 2 B, D, F, H, and J). No signal or PCR product were detected when RNA was used a template (Data not shown) or when template was omitted from the reaction (Figs. 2 B, D, F, H, and J)

4.4.1. Standard curves and sensitivity of real-time QRT-PCR.

Different concentrations of amplified cDNA purified products that ranged over seven fold magnitude were used to construct standard curves for each of the five genes analyzed in this study. A linear regression between logarithms of known gene cDNA copy number and real-time CTs over the range of cDNA concentrations was established (Fig. 3). The largest amount of target cDNA resulted in lowest CT number. The square regression correlation coefficient (R^2) of detection ranged between 0.992 and 1 for all genes studied in pre-inoculation experiment (Data not shown). In post-inoculation experiments, the R^2 ranged between 0.957 and 0.999 (Fig 3). Amplification plots were highly reproducible between triplicate samples of the same treatment and between technical replicates, and fluorescence data from negative controls containing no templates remained well below the threshold level (Figs 4 A to E).

4.4.2. Validation of reference gene for normalization.

In order to choose the best housekeeping gene, the Bestkeeper software was used. Based on the results, the expression level of the housekeeping gene actin (*act*) was found most stable exhibiting insignificant variation between treatments ($P > 0.05$) as compared to the expression of *g3pdh* and *ef.* Thus, the relative expression ratio of defence–

associated genes in the treated plants versus the control (-BTH-RS) was estimated by normalizing against *act* (Table 6).

4.5. Expression of defence-related gene transcript levels.

Genes encoding for *per*, *chit3*, *chalcS* and *chalcI* were expressed at varying transcript levels in all treatments in experiments where sugar beet seeds were grown in *R. solani* infested sand, and in sugar beet plants inoculated with *R. solani* (Table 6). In experiments where sugar beet seeds were grown in *R. solani* infested sand-oat mixture, a 9 fold increase in transcript levels of *chalcS* was observed in plants that received BTH applications only (+BTH-RS) compared to levels observed in infected plants treated or not with BTH. Seedlings that were infected only (-BTH+RS), or infected and BTH treated (+BTH+RS) had similar but higher *chalcS* transcript levels relative to control seedlings (Table 6). In the case of chalcone isomerase (*chalcI*), seedlings that were treated with BTH or grown in infested sand mixture (+BTH+RS) had lower transcript levels than the control with a substantial reduction observed in seedlings that were infected but not treated with BTH (-BTH+RS). Regardless of the treatment, the transcript levels of *per* and *chit3* were similar to those estimated in non-infected and non-treated sugar beet seedlings ($P > 0.05$).

In experiments where sugar beet plants were infected with *R. solani* and treated with BTH (Table 4), transcript levels of *chit3* and *chalcI* significantly varied ($P < 0.05$). Relative to control plants (-RS-BTH), the transcript levels of *chit3* increased by 15 fold in *R. solani* infected and protected plants with BTH (+BTH+RS), followed by 6-8 fold increases in BTH treated sugar beet plants only (+BTH-RS) and *R. solani* inoculated plants (-BTH+RS), respectively (Table 4). In the case of *chalcI, R. solani* inoculations (-

BTH+RS) induced the transcript levels by almost 2 fold ($P < 0.05$), while treatments with BTH (+BTH+RS) did not alter the transcript levels (Table 4). The expression ratio levels of *per* and *chalcS* were unaffected by treatments ($P > 0.05$).

Table 6. Effect of BTH on defence gene transcript levels in sugar beet seedlings grown in *R. solani* infested sand and in plants inoculated with *Rhizoctonia solani* *

	Fold change†			
	per	*chit3*	*chalcS*	*chalcI*
Seedlings in infested sand				
+BTH-RS	1.89 a	1.87 a	9.41 b	0.95 ab
-BTH+RS	0.93 a	1.84 a	1.84 a	0.19 a
+BTH+RS	1.60 a	1.09 a	2.96 a	1.23 b
Inoculated plants				
+BTH-RS	1.17 a	5.73 a	1.40 a	0.95 a
-BTH+RS	0.93 a	7.53 a	1.83 a	1.76 b
+BTH+RS	0.73 a	15.1 b	1.16 a	0.90 a

* Transcripts levels were estimated using real-time quantitative-reverse transcription-polymerase chain reaction (QRT-PCR). Fold change was calculated from the real-time QRT-PCR efficiencies and the threshold cycle (CT) deviation of an inoculation treatment versus control. Note: the relative expression ratio of defence–associated genes in the treated plants versus the control (-BTH-RS) was estimated by normalizing against actin.

† The fold change expression represents the means of 6 replicates. Significance was determined among treatments using least significance difference (LSD) ($P < 0.05$). The fold change ratio of control was always 1 and was not included in the statistical analyses.

Fig. 2. Example of analysis of real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) products amplified for the following defence-related genes: A and B, *chalI*; C and D, *chalS*; E and F, *chit3*; G and H, *per*; and I and J, housekeeping gene actin, using the designed primers listed in Table 1. A, C, E, G, I Melting peak profiles. A, *chalcI*; C, *chalcS*; E, *chit3*; G, *per*; I, *act* transcripts.

Symbols: ● = -BTH-RS; ○= +BTH-RS; ▼= -BTH+RS; Δ= +BTH+RS; ■ = negative control. B, D, F, H, J, corresponding agarose gel (1%) electrophoresis.

Lanes: L = 100 bp DNA ladder, lane 1 to 4 amplification of cDNA from 1 ng of RNA of the four treatments: -RS-BTH; -RS+BTH; +RS-BTH; +RS+BTH; lane 5 = negative control.

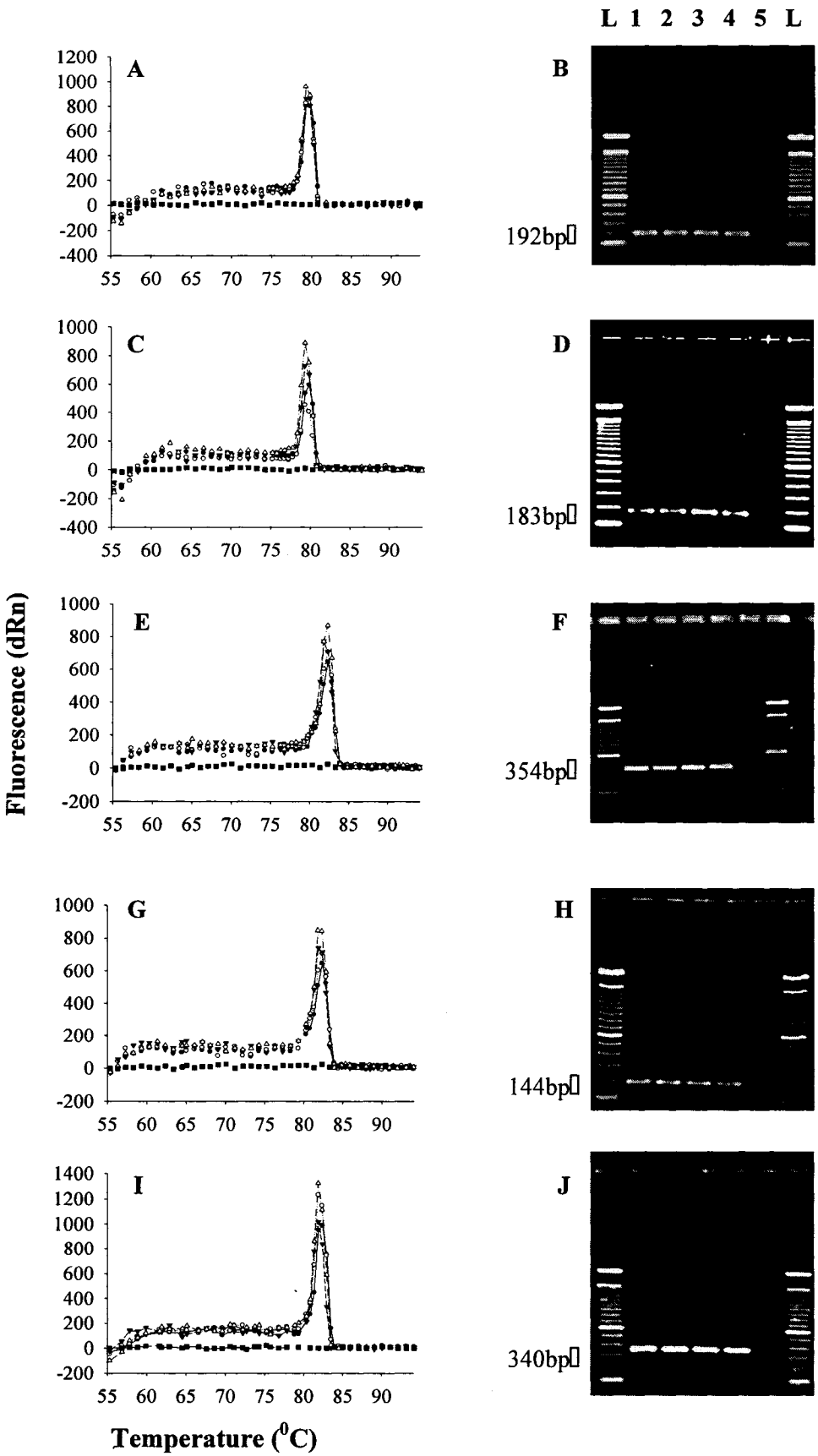

L 1 2 3 4 5 L
A
B
C
D
E
F
G
H
I
J
Fluorescence (dRn)
Temperature (0C)
192bp
183bp
354bp
144bp
340bp

Fig. 3. Example of real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) for the defence-related genes *chalI, chalS, chit3, per*, and one housekeeping gene *act*. Depending on the gene, purified RT-PCR products were diluted serially in the range of concentration from 10^6 to 10^{13} copies/µl and over 7 orders of magnitude to be amplified in QRT-PCR assay. A linear relationship was obtained for each run by plotting the threshold cycle number (CT) against the logarithm of the known amount of starting template in the dilution series. The equation of the line that best fit the data was determined by minimizing error for regression analysis. The R^2 value was calculated to estimate the accuracy of the real-time RT-PCR as a quantification method. The slope of the standard curve was used to calculate the efficiency of the real-time QRT-PCR according to the formula: $E = 10^{(-1/slope)}$. A, *chalcI*; B, *chalcS*; C, *chit3*; D, *per*; E, *act* homolog transcripts.

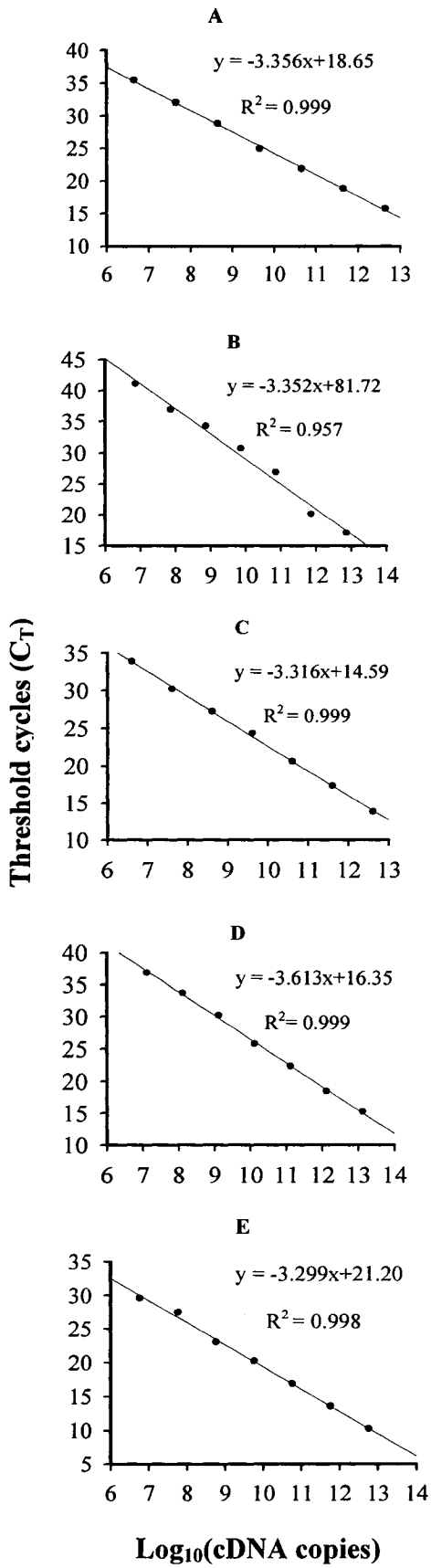
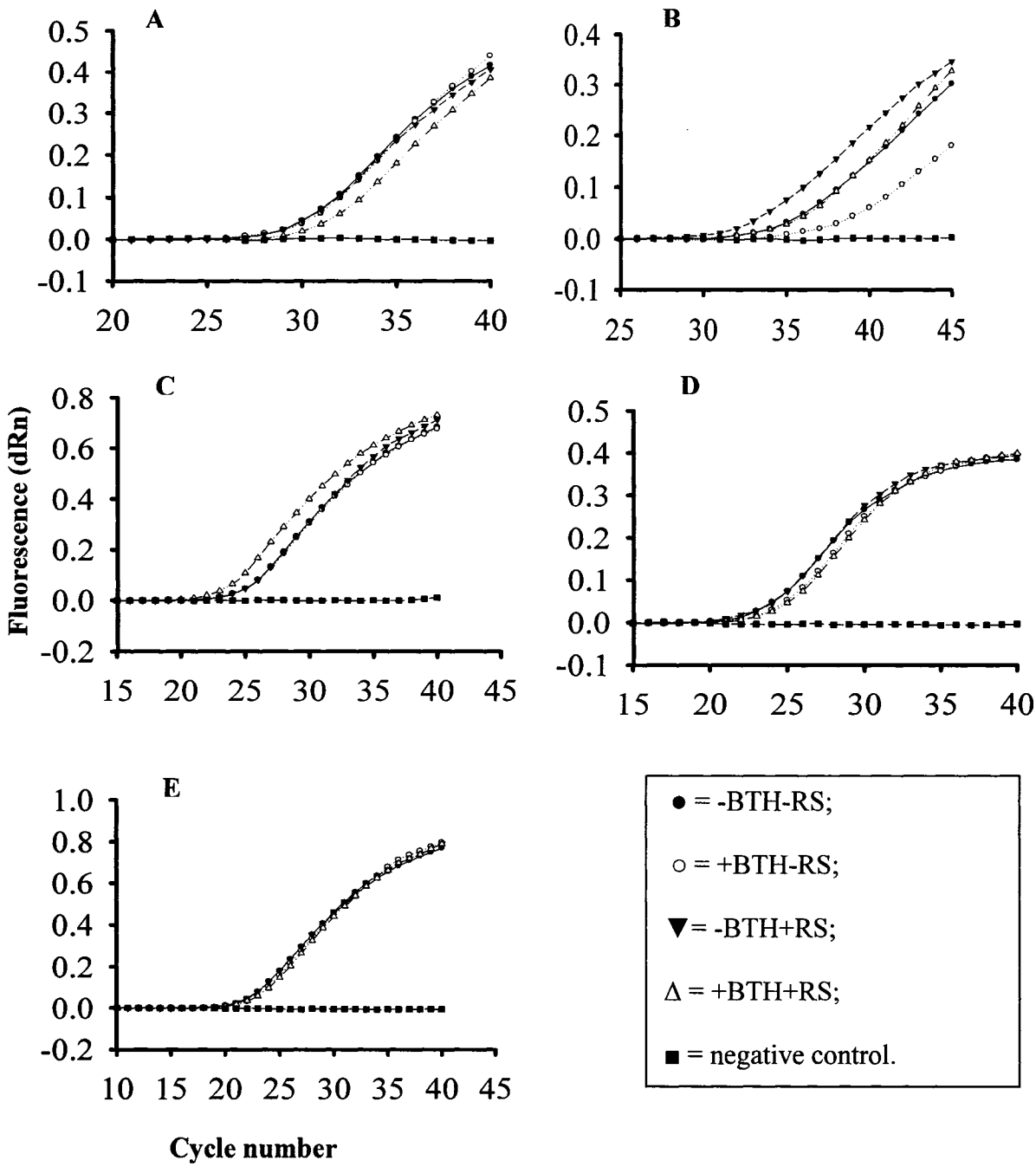

A
y = -3.356x+18.65
R2 = 0.999
B
y = -3.352x+81.72
R2 = 0.957
C
y = -3.316x+14.59
R2 = 0.999
D
y = -3.613x+16.35
R2= 0.999
E
y = -3.299x+21.20
R2 = 0.998
Threshold cycles (CT)
Log10(cDNA copies)

Fig. 4. Kinetics of fluorescence signal versus cycle numbers during amplification of defence-related genes: *chalcI, chalcS, chit3, per*, and one housekeeping gene actin cDNA subjected to four treatments. A, *chalcI*; B, *chalcS*; C, *chit3*; D, *per*; E, actin homolog transcripts.


A
B
C
D
E
Fluorescence (dRn)
Cycle number
● = -BTH-RS;
○ = +BTH-RS;
▼= -BTH+RS;
Δ = +BTH+RS;
■ = negative control.

CHAPTER V

Discussion

In this study, treatment of BTH, BABA, and SA did not enhance resistance to pre-emergence seedling mortality caused by *R. solani*. Furthermore, post-emergence seedling mortality was not reduced due to applications of BTH. Rather, applications of BTH at 50 mg a.i./L as seed treatment followed by soil drench to plants had no significant effect on disease severity. These results are in contrast to what has been recently reported on BTH capability of inducing SAR in sugar beet against another important soilborne disease *Rhizomania* (Burketova et al. 2003a) and against *Cercospora* leaf spot (Bargabus et al. 2002). It is possible that BTH induce resistance to some but not all diseases on sugar beet as in the case of cucumber diseases. Ishii et al. (1999) reported that BTH treatments were effective against anthracnose and scab of cucumber but not on Fusarium wilt of cucumbers.

Timing and mode of application, dosage per plant and concentration of the applied defence activator should be considered when comparing results from different studies. We have used concentrations of BTH applications as soaked seeds and soil drench that did not cause sugar beet toxicity and similar to those that have been previously shown to induce disease resistance in several plants (Tosi 1999; Latunde-Dada and Lucas 2001; Geetha and Shetty 2002; Kauss 2003). We soaked sugar beet seeds with BTH and drenched the soil. A different method of application or multiple applications could have resulted in differences in response to BTH. Induced disease resistance to rhizomania in sugar beet has been found after BTH was applied as root dip (Burketova et al. 2003a),

while induced disease resistance against rice sheath blight caused by *R. solani* was reported after BTH was applied as foliar and soil drench (Rohilla et al. 2002). Multiple applications of BTH on pepper and tomato were necessary to induce resistance against phytopthora root and crown rot of pepper (Buonario et al. 2002; Matheron and Porchas 2002) and for bacterial wilt of tomato (Pradhanang et al. 2005).

The failure of BTH inducing resistance in sugar beet plants against *R. solani* could be that the plant did not respond to the chemical inducer treatment. This is consistent with several reports in which BTH treatments did not result in reduced incidence of tobacco blackshank incited by *Phytopthora parasitica* var. *nicotianae* (Csinos et al. 2001), pink snow mould in ryegrass caused by *Microdoochium nivale* (Hofgaard et al. 2005), Fusarium wilt of cucumber (Ishii et al.1999), nor enhanced resistance to grapevine downy mildew (Hamiduzamman et al. 2005) or to root knot and cyst nematodes of cereals (Oka and Cohen 2001).

To the best of our knowledge, we are the first to perform real-time QRT-PCR assays to quantify the expression levels of several defence-related genes in sugar beet in response to *R. solani* infections and treatments of BTH under controlled growth conditions. Several technical factors that could have affected the accuracy of the target gene quantification were carefully controlled when conducting the experiments. (i) All of the primer's specificities and efficiencies were established and rigorously validated for the 4 different targets and one housekeeping gene. (ii) Calibration experiments with external standards demonstrated a strong dependency of the CT numbers on the logarithm of starting DNA quantities over 7 orders of magnitude. Linearity of standard curves with correlation coefficient (R^2) ranging between 0.9572 and 1.00 proved essential for accurate calculation of relative level of gene transcripts. (iii) Statistical validation of the

housekeeping gene, actin whose expression remained constant among treatments ($P < 0.05$) as compared to the other two housekeeping genes, alpha-elongation factor or gapdh. (iv) Individual QRT-PCR efficiencies for target and house-keeping genes were estimated on a sample per sample basis (Pfaffl 2001, http://www.gene-quantification.info/) in order to take into account inherent sample and gene PCR efficiency variations, and (vi) The application of a mathematical model combined with statistical analysis that accounts for both differences in amplification efficiencies and threshold cycle number (McMaugh and Lyon 2003, Pfaffl et al. 2001). Taken all this together, we demonstrate, as did other recent studies (Bezier et al. 2002; Wen et al. 2005; de Souza et al. 2005), the utility of real-time QRT-PCR for reliable and accurate assessment of gene expression of sugar beet RNA in response to pathogen infection and treatment with a chemical inducer.

In this study, we addressed the question whether BTH treatment induces the transcript levels of four defence genes: *chit3, per, chalcI* and *chalcS* all of which are associated one way or another with systemic resistance in several plant species (Gozzo 2003; Durrant and Dong 2004). Our findings indicate that although BTH applications did not reduce disease incidence caused by *R. solani*, they did however compared to the control, sensitize the plants to substantially up-regulate the transcript levels ratios of two out of four defence encoding genes; chalcone synthase and chitinase 3. Contrary to what has been reported on BTH treatment of sugar beet inoculated with Cercospora leaf spot (Nielsen et al. 1996; Bargabus et al. 2002) or of rhizomania diseased sugar beets (Burketova et al. 2003a), the results of this study suggest that gene activation in sugar beet as a result of BTH treatments does not confer disease resistance against *R. solani.*

The ability of BTH to increase the expression of PR proteins including glucanases and chitinases without the necessity of pathogen infection (Burtekova et al. 1999,

Burketova et al. 2003b) in sugar beet or in other crops such as roses (Suo and Leung 2001) is well documented. The present study on expression chitinase and chalcone synthase encoding genes supports these data. Low, albeit insignificant, levels of peroxidase in both types of experiments were similarly expressed in all treatments, indicating that BTH has no effect on peroxidase expression.

The significant increase in the expression of transcript levels of *chit3* in sugar beet plants inoculated with *R. solani* is a strong indication that *R. solani* stimulated a systemic accumulation of chitinase, a PR-3 protein (Nielsen et al 1993, 1994) and chalcone isomerase one of the encoding genes of key enzymes of the phenylpropanoid pathway that lead to phytoalexin accumulation (Cramer et al. 1989). This is not surprising since the sugar beet cultivar we have used in this study is considered a partial resistant hybrid to *R. solani*. In line with this observation, Nielsen et al. (1993) reported on the substantial increase of mRNA encoding class III chitinase in tolerant sugar beet cultivars infected with Cercospora leaf spot.

On the other hand, we also showed that inducible plant defences are repressed due to *R. solani*. A decrease of transcripts of *chalcI* to levels below the constitutive levels of control plants, and almost to undetectable levels was observed in sugar beet seedling infected with *R. solani*. This is in agreement with the recent study of Schmidt et al. (2004) who reported on the suppression of transcript accumulation of BvPAL (phenylalanine ammonia lyase), another gene encoding a key enzyme of the phenlypropanoid pathway, due to the development of Cercospora leaf spot of sugar beet. Because of the key role played by the above genes, some pathogens have evolved mechanisms for reducing the activation of some of these genes and therefore to increase the chances of successfully infecting the plants. Suppressor molecules such as supprescine A and B, secreted by the

pathogen *Mycosphaerella pinodes* have been identified in peas and led to the delay in phytoalexin accumulation (Yamada et al. 1994; Yamada et al.1989). Whether *R. solani* and *Cercospora beticola* secrete a suppressor molecule in order to reduce sugar beet defence remains unknown.

In summary, the results of the present study suggest that sugar beet was capable of expressing selected defence genes in response to a chemical inducer, but the expression although substantial with some genes was not sufficient to stop *R. solani* infections.

CHAPTER VI

Concluding comments

Rhizoctonia solani, the casual agent of crown and root rot in sugar beet, causes significant economic losses world-wide, and is considered a destructive pathogen of many vegetables, flowers and fruits. An emerging alternative strategy in plant protection that could replace or be combined with fungicides is the stimulation of the natural plant's defence response by chemical inducers or resistance enhancers. Positive correlations have been established between chemical induction of systemic acquired resistance (SAR) associated genes and increased tolerance to pathogens (Vallad and Goodman 2004). The molecular mechanism by which chemical inducers operates to trigger SAR is still under investigation but there is consensus that SAR response is probably based on multiple mechanisms including cell wall reinforcement and the systemic accumulation of pathogenesis-related genes.

Only a few reports have been published on the cellular events occurring between the inducing stimulus and the onset resistance in sugar beet. In this perspective, it is essential to explore the molecular mechanism of natural defence responses of sugar beet, study the effect of these inducers on sugar beet, and identify the most effective scenario of defence against *Rhizoctonia solani.* Previous studies dealing with BTH treatment of sugar beet demonstrated that protection to the foliar pathogen *Cercospora beticola* and the soilborne disease Rhizomania was positively correlated with enhanced activities of PR proteins namely chitinase and glucanase. These results prompted us to investigate whether the application of potential chemical activator of SAR such as BTH can protect

sugar beet from pre- and post-emergence damping –off caused by the soilborne pathogen *Rhizoctonia solani*, and whether this protection is related to enhanced expression of genes associated with plant defence.

The question whether BTH induces transcript levels of four defence genes was addressed. It was clearly demonstrated that BTH treatment elicited alteration of transcript levels of the genes. However, expression of genes was not correlated with reduction in disease severity caused by *R. solani*. Since chemical inducers are known to increase resistance systemically through out the plant, it would be worthwhile in the future to study the spatial distribution of defence-associated genes in sugar beet. We have used disease score index to rate disease severity. This method is the most accepted method for quantification of *R. solani* infections. Measurements of lesion surface area, which is another method used for disease severity could not be applied on sugar beet young plants because of their small size.

In this study, only one concentration of the chemical inducer was used and applied as soil drench as method of application. It is possible that different modes of applications of BTH including foliar and seed treatment or the combination of both could have generated different results. Thus, future studies should be aimed to test whether different timing periods and mode of application as well as various concentrations BTH could play a role in the reduction of disease severity. Another factor that requires further study is to test whether combination of chemical inducers with ISR biotic elicitors such as yeast, *Pythium oligandrum*, or *Pseudomonas* spp may elicit SAR in sugar beet as has been previously reported in other crops (Zhang et al. 2005; Srivastava et al. 2001)

We have chosen to apply real-time quantitative reverse transcription (QRT)-PCR technologies instead of northern analysis to estimate accurately the mRNA expression of

the genes. This study demonstrated the utility of real-time QRT-PCR for reliable and accurate assessment of gene expression of sugar beet RNA in response to pathogen infection and treatment with a chemical inducer.

Future studies targeted at assessing the accumulation of proteins corresponding to the encoded genes should be attempted and correlated with levels of gene expression as a result of BTH treatment.

In conclusion, this study had shown that sugar beet is able to express varying amounts of gene transcript levels of selected defence genes in response to a chemical inducer, but it was clearly demonstrated that up-regulation of some of these genes were not correlated with reduction of disease caused by *R. solani.*

REFERENCES

Adams, G.C. 1988. *Thanatephorus cucumeris* (*Rhizoctonia solani*), a species complex of wide host range. *In*: Advances in Plant Pathology, vol. 6: Genetics of Plant Pathogenic Fungi. *Edited by* Ingram, D.S., Williams, P.H., Academic Press, London, pp. 535–552.

Afonina, I.A., Reed, M.W., Lusby, E., Shishkina, I.G., and Belousov, Y.S. 2002. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence: BioTechniques 32: 940-944; 946-949.

Agrios, G.N. 1998. Plant Pathology. 3rd Ed.Academic Press Inc., San Diego, USA. 952 pp.

Anfoka, G.H. 2000. Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester induces systemic resistance in tomato (*Lycopersicon esculentum.* Mill cv. Vollendung) to Cucumber mosaic virus. Crop Prot. 19: 401-405.

Baby, U.I. and Manibhushanrao, K. 1996. Influence of organic amendments on arbuscular mycorrhizal in relation to rice sheath blight disease. Mycorrhiza 6: 201-206.

Bargabus, R.L., Zidack, N.K., Sherwood, J.E., and Jacobsen, B.J. 2002. Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol. Mol. Plant Pathol. 61: 289-298.

Beßer, K., Jarosch, B., Langen, G., and Kogel, K.H. 2000.Expression analysis of genes induced in barley after chemical activation reveals distinct disease resistance pathways. Mol. Plant Pathol. 1: 277-286.

Benhamou, N., and Belanger, R.R. 1998. Induction of systemic resistance to Pythium damping-off in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response. Plant J. 14: 13-21.

Bezier, A., Lambert, B., and Baillieul, F. 2002. Study of defence-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea.* Eur.J.Plant Pathol. 108: 111-120.

Blazier, S.R. and Conway, K.E. 2004. Characterization of *Rhizoctonia solani* isolates associated with patch disease on turfgrass. Proc.Okla.Acad.Sci. 84: 41-51.

Bokshi, A.I., Morris, S.C., and Deverall, B.J. 2003. Effects of benzothiadiazole and acetylsalicylic acid on beta-1,3-glucanase activity and disease resistance in potato. Plant Pathol. 52: 22-27.

Borges, A.A., Cools, H.J., and Lucas, J.A. 2003. Menadione sodium bisulphite: a novel plant defence activator which enhance local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape. Plant Pathol. 52: 429-436.

Bowles, D.J. 1990. Defence-Related Proteins in Higher-Plants. Annu. Rev. Biochem. 59: 873-907.

Brewer, M.T., and Larkin, R.P. 2005. Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. Crop Prot. 24: 939-950.

Brisset, M.N., Cesbron, S, Thompson, S.V., and Paulin, J.P. 2000. Acibenzolar-S-methyl induces the accumulation of defence-related enzymes in apple and protects from fire blight. Eur. J. Plant Pathol. 106: 526-536.

Büchter, R. Strömberg, A., Schmelzer, E., and Kombrink, E. 1997. Primary structure of acidic (class II) chitinase in potato. Plant Mol. Biol. 35:749-761.

Buonario, R., Scarponi, L., Ferrara, M., Sidoti, P., and Bertona, A. 2002. Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. Eur. J. Plant Pathol. 108: 41-49.

Burketova, L., Sindelarova, M., and Sindelar, L. 1999. Benzothiadiazole as an inducer of beta-1,3-glucanase and chitinase isozymes in sugar beet. Biol. Plant. 42: 279-287.

Burketova, L., Stillerova, K., and Feltlova, M. 2003a. Immunohistological localization of chitinase and beta-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar beet roots. Physiol Mol. Plant Pathol. 63: 47-54.

Burketova, L., Stillerova, K., Feltlova, M., and Sindelarova, M. 2003b. Immunohistological analysis of chemically induced proteins in sugar beet. Biol. Plant. 47: 243-251.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29: 23-39.

Bustin, S.A., and Nolan, T. 2004. Template handling, preparation, and quantification. *In*: A-Z of quantitative PCR. *Edited by* Stephen A. Bustin. International University Line, La Jolla, California, pp: 143-191.

Büttner, G., Pfaähler, B., and B. Maärlaänder. 2004. Greenhouse and field techniques for testing sugar beet for resistance to Rhizoctonia root and crown rot. Plant Breeding 123: 158-166.

Canadian Federation of Agriculture, 1998. Agriculture in Canada. http://www.inspection.gc.ca/english/plaveg/bio/dir/bio0201e.pdf.

Cardoso, J.E., and Echandi, E. 1987. Biological control of Rhizoctonia root rot of snap bean with binucleate *Rhizoctonia*-like fungi. Plant Dis. 71: 167-170.

Carling, D.E., Baird, R.E., Gitaitis, R.D., Brainard, K.A., and Kuninaga, S. 2002. Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*: Phytopathology 92: 893-899.

Cattanach, A.W., Dexter, A.G., and Oplinger, E.S. 1991. Sugar beets. *In*: W.C. Agronomy: Alternative fields crop manual. University of Winsconsin cooperative extension service, the University of Minessota extension service and the Center for Alternative plant and animal products. www.hort.purdue.edu/newcrop/afcm/sugar beet.html.

Chang, K.F., Hwang, S.F., Gossen, B.D., Turnbull, G.D., Howard, R.J., and Blade, S.F. 2004. Effects of soil temperature, seeding depth, and seeding date on rhizoctonia seedling blight and root rot of chickpea. Canadian Journal of Plant Science 84: 901-907.

Chittoor, J.M., Leach, J.E., and White, F.F. 1997. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interactions 10: 861-871.

Ciampi, M.B., Kuramae, E.E., Fenille, R.C., Meyer, M.C., Souza, N.L. and Ceresini, P.C. 2005. Intraspecific evolution of *Rhizoctonia solani* AG-1 IA associated with soybean and rice in Brazil based on polymorphisms at the ITS-5.8S rDNA operon. Eur. J. Plant Pathol. 113: 183-196.

Cohen, Y., Niderman, T., Mosinger, E., and Fluhr, R. 1994. Beta-aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon*

esculentum L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. Plant Physiology 104: 59-66.

Cohen, Y.R. 2002. Beta-aminobutyric acid-induced resistance against plant pathogens. Plant Dis. 86: 448-457.

Conrath, U., Thulke, O., Schwindling, S., and Kohler, A. 2001. Priming as a mechanism in induced systemic resistance of plants. Eur. J. Plant Pathol. 107: 113-119.

Cools, H.J., and Ishii, H. 2002. Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (*PAL1*) for enhanced expression upon attack with a pathogenic fungus. Physiol Mol. Plant Pathol.61: 273-280.

Cornelissen, B.J.C., Does, M.P., and Melchers, L.S. 1996. *In*: *Rhizoctonia* Species: Taxonomy, Molecular biology, Ecology, Pathology and Disease control. *Edited by* B. Sneh, S. Jabaji-Hare, S. Nate and G Dijst. Kluwer Academic Publishers, Netherlands, pp.529-536 .

Cramer, C.L., Edwards, K., Dron, M., Liang, X.W., Dildine, S.L., Bolwell, G.P., Dixon, R.A., Lamb, C.J., and Schuch, W. 1989. Phenylalanine ammonia-lyase gene organization and structure. Plant Mol. Biol. 12: 367-383.

Csinos, A.S., Pappu, H.R., McPherson, R.M., and Stephenson, M.G. 2001. Management of Tomato spotted wilt virus in flue-cured tobacco with acibenzolar-S-methyl and imidacloprid. Plant Dis. 85: 292-296.

Delgado, A., Franco, G.M., Paez, J.I., Vega, J.M., Carmona, E., and Aviles, M. 2005. Incidence of cotton seedling diseases caused by *Rhizoctonia solani* and *Thielaviopsis basicola* in relation to previous crop, residue management and nutrients availability in soils in SW Spain. J. Phytopathology 153: 710-714.

de Souza, A.A., Takita, M.A., Pereira, E.O., Coletta-Filho, H.D., and Machado, M.A. 2005. Expression of pathogenicity-related genes of *Xyllela fastidiosa* in vitro and in planta. Curr. Microbiol. 50: 223-228.

Durango, D., Quinones, W., Torres, F., Rosero, Y., Gil, J., and Echeverri, F. 2002. Phytoalexin accumulation in Colombian bean varieties and aminosugars as elicitors. Molecules 7: 817-832.

Durrant, W.E., and Dong, X. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42: 185-209.

El-Tarabily, K.A. 2004. Suppression of *Rhizoctonia solani* diseases of sugar beet by antagonistic and plant growth-promoting yeasts. J. Appl. Microbiol. 96: 69-75.

Epple, P., Apel, K., Bohlmann, H. 1995. An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol. 109: 813-820.

Flors, V., Miralles, M.C., Gonzalez-Bosch, C., Carda, M., and Garcia-Agustin, P. 2003. Induction of protection against the necrotrophic pathogens *Phytophthora citrophthora* and *Alternaria solani* in *Lycopersicon esculentum* Mill. by a novel synthetic glycoside combined with amines. Planta 216: 929-938.

Gachon, C., Mingam, A., and Charrier, B. 2004. Real-time PCR: what relevance to plant studies? Journal of Experimental Botany 55: 1445-1454.

Garcia-Olmedo, F., Molina, A., Segura, A., Moreno, M. 1995. The defensive role of nonspecific lipid-transfer proteins in plants. Trends in Microbiology 3: 72-74.

Geetha, H.M., and Shetty, H.S. 2002. Induction of resistance in pearl millet against downy mildew disease caused by *Sclerospora graminicola* using benzothiadiazole,

calcium chloride and hydrogen peroxide - a comparative evaluation. Crop Prot. 21: 601-610.

Glare, E.M., Divjak, M., Bailey, M.J., and Walters, E.H.. 2002. Beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax 57: 765-770.

Gorlach, J., Volrath, S., Knauff-Beiter, G., Hengy, G., Beckhove, U. K.H. Kogel, M. Oostnedorp, T. Staub, S., Ward, E., Kessman, H., and Ryals, J. 1996. Benzothiadiazole a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:62-643.

Gomez, K.A., and Gomez, A. A. 1983. Chi-square test. *In*: Statistical Procedures for Agricultural Research. *Edited by* John Wiley and Sons Inc, pp. 467-471.

Gottschalk, T.E., Mikkelsen, J.D., Nielsen, J.E., Nielsen, K.K., and Brunstedt, J. 1998. Immunolocalization and characterization of a beta-1,3-glucanase from sugar beet, deduction of its primary structure and nucleotide sequence by cDNA and genomic cloning. Plant Sci. 132: 153-167.

Gouillon, C., St-Arnaud, M., Hamel, C., and Jabaji-Hare, S. 2002. Differential and systemic alteration of defence-related gene transcript levels in mycorrhizal bean plants infected with *Rhizoctonia solani*. Canadian Journal of Botany, Revue Canadienne De Botanique 80: 305-315

Gozzo, F. 2003. Systemic acquired resistance in crop protection: From nature to a chemical approach. J. Agric. Food Chem. 51: 4487-4503.

Graham, L.S. and Sticklen, M.B. 1994. Plant Chitinases. Canadian Journal of Botany-Revue Canadienne De Botanique 72: 1057-1083.

Grosch, R., Schneider, J.H.M., and Kofoet, A. 2004. Characterisation of *Rhizoctonia solani* anastomosis groups causing bottom rot in field-grown lettuce in Germany. Eur. J. Plant Pathol. 110: 53-62.

Hamiduzzaman, M.M., Jakab, G., Barnavon, L., Neuhaus, J.M., and Mauch-Mani, B. 2005. Beta-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Mol. Plant-Microbe Interact. 8: 819-829.

Hammerschmidt, R. 1999. Induced disease resistance: how do induced plants stop pathogens? Physiol. Mol. Plant Pathol. 55: 77-84.

Hammerschmidt, R., Metraux, J.P., and van Loon, L.C. 2001. Inducing resistance: A summary of papers presented at the First International Symposium on Induced Resistance to Plant Diseases, Corfu, May 2000. Eur. J. Plant Pathol. 107: 1-6.

Hecker, R.J. and Ruppel, E.G. 1976. Inheritance of resistance to *Rhizoctonia* root rot in sugar beet. Crop Sci. 15: 487-490.

Heil, M. and Bostock R.M. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. Annals of Botany 89: 503-512.

Herr, L.J. 1996. Sugar beet diseases incited by *Rhizoctonia* spp. *In*: *Rhizoctonia* Species: Taxonomy, Molecular biology, Ecology, Pathology and Disease control. *Edited by* B. Sneh, S. Jabaji-Hare, S. Nate and G Dijst. Kluwer Academic Publishers, Netherlands, pp.341-351 .

Hofgaard, I.S., Ergon, A., Wanner, L.A., and Tronsmo, A.M. 2005. The effect of chitosan and Bion on resistance to pink snow mould in perennial ryegrass and winter wheat. J. Phytopathology 153: 108-119.

Hong, J.K., Hwang, B.K., and Kim, C.H. 1999. Induction of local and systemic resistance to *Colletotrichum coccodes* in pepper plants by DL-beta-amino-n-butyric acid. J. Phytopathology 147: 193-198.

Hwang, J. and Benson, D.M.. 2003. Expression of induced systemic resistance in poinsettia cuttings against *Rhizoctonia* stem rot by treatment of stock plants with binucleate *Rhizoctonia.* Biological control 27: 73-80.

Ishii, H., Tomita, Y., Horio, T., Narusaka, Y., Nakazawa, Y., Nishimura, K., and Iwamoto, S. 1999. Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and Japanese pear diseases. Eur. J. Plant Pathol. 105: 77-85.

Jakab, G., Cottier V., Toquin, V., Rigoli, G., Zimmerli, L., Metraux, J.P., and Mauch-Mani, B. 2001. Eur. J. Plant Pathol. 107: 29-37.

Jensen, B., Latunde-Dada, O., Hudson, D., Lucas, J.A. 1998. Protection of *Brassica* seedlings against downy mildew and damping-off by seed treatment with benzothiadiazole, an inducer of systemic acquired resistance. Pesticide Sci. 52: 63-69.

Julian, M.C., Acero, J., Salazar, O., Keijer, J., and Rubio, V. 1999. Mating type-correlated molecular markers and demonstration of heterokaryosis in the phytopathogenic fungus *Thanatephorus cucumeris* (*Rhizoctonia solani*) AG 1-IC by AFLP DNA fingerprinting analysis. Journal of Biotechnology 67: 49-56.

Kaszprzewska, A. 2003. Plant chitinases-regulation and function. Cellular & Molecular Biology Letters 8: 809-824.

Kataria, H.R., Hugelshofer, U., and Gisi, U. 1991. Sensitivity of Rhizoctonia species to different fungicides. Plant Pathol. 40: 203-211.

Kauss, H., Seehaus, K., Franke, R., Gilbert, S., Dietrich, R.A., and Kroger, N. 2003. Silica deposition by a strongly cationic proline-rich protein from systemically resistant cucumber plants. Plant J. 33: 87-95.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S., and Ryals, J. 1994. Induction of systemic acquired disease resistance in plants by chemicals. Annu. Rev. Phytopathol. 32: 439-459.

Kogel, K.H., Beckhove, U., Dreschers, J., Munch, S., and Y. Romme 1994. Acquired resistance in barley. Plant Physiol. 106: 1269-1277.

Kombrink, E. and Somssich, I.E. 1995. Defence responses of plants to pathogens. Advances in Botanical Research 21: 1-34.

Kuc, J. 1995. Phytoalexins, Stress metabolism, and disease resistance in plants. Annu. Rev. Phytopathol. 33: 275-297.

Kuc, J. 2001. Concepts and direction of induced systemic resistance in plants and its application. Eur. J. Plant Pathol. 107: 7-12.

Kunz, W., Scharter, R., and Maetzke, T. 1997. The chemistry of benzothiadiazole plant activators. Pesticide Sci. 50: 275-282.

Lagrimini, L.M., and Rothstein, S. 1987. Tissue-specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic-virus infection: Plant Physiology 84: 438-442.

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

Latunde-Dada, A.O., and Lucas, J.A. 2001. The plant defence activator acibenzolar-S-methyl primes cowpea [*Vigna unguiculata* (L.) Walp.] seedlings for rapid induction of resistance. Physiol. Mol Plant Pathol. 58: 199-208.

Lawton, K.A., Potter, S.L., Uknes, S. and Ryals, J. 1994. Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell 6: 581–588.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. 1996. Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J. 10: 71-82.

Li, Y.Z., Zheng, X.H., Tang, H.L., Zhu, J.W., Yang, J.M. 2003. Increase of β-1, 3-glucanase and chitinase activities in cotton callus cells treated by salicylic acid and toxin of *Verticillium dahliae*. Acta Botanica Sinica 45: 802-808.

Liu, W.H. and Saint, D.A. 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Analytical Biochemistry 305: 291-291.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25: 402-408.

Lopez, A.M.Q. and Lucas, J.A. 2002. Effects of plant defence activators on anthracnose disease of cashew. Eur. J. Plant Pathol. 108: 409-420.

Matheron, M.E., and Porchas, M. 2002. Suppression of Phytophthora root and crown rot on pepper plants treated with acibenzolar-S-methyl. Plant Dis. 86: 292-297.

McMaugh, S.J., and Lyon, B.R. 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. BioTechniques 34: 982-986.

Meins, F., Jr., Fritig, B., Linthorst, H. J. M., Mikkelsen, J. D., Neuhaus, J. M., and Ryals, J. 1994. Plant chitinase genes. Plant Mol. Biol. Rep.12: 22-28.

Metraux, J.P., AHL-Goy, P., Staub, T., Speich, J., Steinermann, A., Ryals, J., and Ward, J. 1991. Induced systemic resistance in cucumber in response to 2, 6-dichloro-isonicotinic acid and pathogens. *In* Advances in Molecular genetics of Plant-Microbe interactions vol. I. *Edited by* Hennecke H. and D.P.S. Kluwer Academic Publishers, Dordrecht. pp: 432-439.

Mukhopadhyay, A.N. 1987. *In*: CRC handbook on disease of sugar beet. *Edited by*: Boca Raton, Florida.190 pp.

Nagendran, S., and McGrath, J.M. 2004. Seedling resistance to *Rhizoctonia solani* in sugar beet. Phytopathology [Abstr.] 94: s74.

Neuhaus J.M. 1995. Proposal presented at the 4th International workshop on pathogenesis-related proteins in plants: biology and biotechnological potential held September 3-7, 1995, Irsee, Germany.

Nicot, N., Hausman, J.F., Hoffmann, L., and Evers, D. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany 56: 2907-2914.

Nielsen, K.K., Mikkelsen, J.D., Kragh, K.M., and Bojsen, K. 1993. An acidic class-III chitinase in sugar-beet-induction by *Cercospora-beticola*, characterization, and expression in transgenic tobacco plants. Mol. Plant-Microbe Interact. 6: 495-506.

Nielsen, K.K., Bojsen, K., Collinge, D.B., and Mikkelsen, J.D. 1994. Induced resistance in sugar-beet against *Cercospora-beticola*-induction by dichloroisonicotinic acid is independent of chitinase and beta-1,3-glucanase transcript accumulation. Physiol.Mol. Plant Pathol. 45: 89-99.

Nielsen, K.K., Nielsen, J.E., Madrid, S.M., and Mikkelsen, J.D. 1996. New antifungal proteins from sugar beet (*Beta vulgaris* L) showing homology to non-specific lipid transfer proteins. Plant Mol. Biol. 31: 539-552.

Nielsen, M.N., Sørensen, J., Fels, J., and Pedersen, H.C. 1998. Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. Appl. Environ. Microbiol. 64: 3563-3569.

Oka, Y., and Cohen, Y. 2001. Induced resistance to cyst and root-knot nematodes in cereals by DL-beta-amino-n-butyric acid. Eur. J. Plant Pathol. 107: 219-227.

Okey, E.N., Duncan, E.J., SirjuCharran, G. and Sreenivasan, T.N. 1996. Factors affecting the susceptibility of six cocoa clones to *Phytophthora palmivora* (Butl) Butler bark canker in Trinidad. Plant Pathology 45: 84-91.

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T. 2001. Induced disease resistance in plants by chemicals. Eur. J. Plant Pathol. 107: 19-28.

Panella, L. Registration of four multigerm sugar beet germplasms resistant to rhizoctonia root rot: FC716, FC717, FC718, and FC719. 1995. Crop Sci. 35: 291-292.

Panella, L. 1998. Evaluation of Beta PIs from the USDA-ARS National Plant Germplasm System for resistance to Rhizoctonia root rot. Biological and Cultural Test for Control of Plant Diseases. Am. Phytopathol. Soc. 14: 154.

Parmeter, J.R. 1970. *Rhizoctonia solani*, biology and pathology. University of California Press, Berkeley, CA. 300 pp.

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

Paulitz, T.C. and Schroeder, K.L. 2005. A new method for the quantification of *Rhizoctonia solani* and *R. oryzae* from soil. Plant Dis. 89: 767-772.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: 2002-2007.

Pffafl, M.W., Horgan, G.W., Dempfle, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acid Res. 30: e36.

Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26: 509-515.

Plant Biosafety Office 2001. The biology of *Beta vulgaris* L. (Sugar beet). http://www.inspection.gc.ca/english/plaveg/bio/dir/bio0201e.pdf.

Pradhanang, P.M., Ji, P., Momol, M.T., Olson, S.M., Mayfield, J.L., and Jones, J.B. 2005. Application of acibenzolar-S-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. Plant Dis. 89: 989-993.

Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., and Nitsche, A. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313: 856-862.

Restrepo, S., Myers, K.L., del Pozo, O., Martin, G.B., Hart, A.L., Buell, C.R., Fry, W.E., and Smart, C.D. 2005. Gene profiling of a compatible interaction between

Phytophthora infestans and *Solanum tuberosum* suggests a role for carbonic anhydrase. Mol. Plant-Microbe Interact. 18: 913-922.

Rodriguez-Kabana, R. and Curl, E.A. 1980. Nontarget effects of pesticides on soilborne pathogens and disease. Annu. Rev. Phytopathol. 18: 311-332.

Rohilla, R., Singh, U.S., and Singh, R.L. 2002. Mode of action of acibenzolar-S-methyl against sheath blight of rice, caused by *Rhizoctonia solani* Kuhn. Pest Manag. Sci. 58: 63-69.

Ross A.F. 1961. Systemic acquired resistance induced by localized virus infection in plants. Virology 14: 340-358.

Rubie, C., Kempf, K., Hans, J., Su, T.F., Tilton, B., Georg, T., Brittner, B., Ludwig, B., and Schilling, M. 2005. Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues: Mol.Cell. Probes 19: 101-109.

Ruppel, E.G., Schneider, C.L., Hecker, R.J., and Hogaboam, G.J. 1979. Creating epiphytotics of Rhizoctonia root rot and evaluating for resistance to *Rhizoctonia solani* in sugar beet field plots. Plant Dis. Reporter 63: 518-522.

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

SAS Institute. 1999. SAS 8 software. SAS Institute., Cary, N.C.

Schenk, P.M., Kazan, K., Manners, J.M., Anderson, J.P., Simpson, R.S., Wilson, I.W., Somerville, S.C., and Maclean, D.J. 2003. Systemic gene expression in *Arabidopsis* during an incompatible interaction with *Alternaria brassicicola*. Plant Physiology 132: 999-1010.

Schmid, H., Henger, A., Luckow, B., Irrgang, S., Cohen, C.D., Grone, H.J., Schlondorff, D., and Kretzler, M. 2003. A mRNA expression marker set for the differential diagnosis of renal allograft rejection. Journal of the American Society of Nephrology 14: 181a-181a.

Schmidt, K., Heberle, B., Kurrasch, J., Nehls, R., and Stahl, D.J. 2004. Suppression of phenylalanine ammonia lyase expression in sugar beet by the fungal pathogen *Cercospora beticola* is mediated at the core promoter of the gene. Plant Mol. Biol. 55: 835-852.

Schmidt, T.A.H.-H., J.S.1998. The physical and genomic organization of microsatellites in sugar beet. Proc. Natl. Acad. Sci. USA 93: 8761-8765.

Scholten, O., Panella, L.W., De Bock, T.S.M., and Lange, W. 2001. A green house for testing sugar beet (*Beta vulgaris*) for resistance to *Rhizoctonia solani*. Eur. J. Plant Pathol. 107:161-166.

Si-Ammour, A., Mauch-Mani, B., and Mauch, F. 2003. Quantification of induced resistance against Phytophtora species expressing GFP as a vital marker: β-aminobutyric acid not BTH protects potato and Arabidopsis from infection. Mol. Plant Pathol. 4: 237-248.

Siddiqui, L.A. and S.S. Shaukat. 2002. Resistance against damping off fungus *Rhizoctonia solani* systemically induced by the plant-growth-promoting rhizobacteria *Pseudomonas aeruginosa* (IE-6S^{+}) and *P. Fluorescens* (CHAO). J. Phytopathol. 150: 500-506

Singh, A., Rohilla, R., Singh, U.S., Savary, S., Willocquet, L., and Duveiller, E. 2001. Can. J. Plant Pathol. 24: 65-68.

Smidt, K., Wogensen, L., Brock, B., Schmitz, O., and Rungby, J. 2006. Real-time PCR: Housekeeping genes in the INS-1E beta-cell line. Hormone and Metabolic Research 38: 8-11.

Srivastava, A.K., Singh, T., Jana, T.K., and Arora, D.K. 2001. Induced resistance and control of charcoal rot in *Cicer arietinum* (chickpea) by *Pseudomonas fluorescens*: Canadian Journal of Botany-Revue Canadienne De Botanique 79: 787-795.

Stepniewska-Jarosz, S., Manka, M. and Asiegbu, F.O. 2006. Studies on anastomosis groups of Rhizoctonia solani isolates causing disease in two forest nurseries in Poland. Forest Pathology 36: 97-109.

Sticher, L., Mauch-Many, B., and Metraux, J. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35: 235-270.

Strausbaugh, C.A., Bradley, C.A., Koehn, A.C. and Forster, R.L. 2004. Survey of root diseases of wheat and barley in southeastern Idaho. Can. J. Plant Pathol. 26: 167-176.

Sunwoo, J.Y., Lee,Y.K, and Hwang, B.K. 1996. Induced resistance against *Phytophthora capsici* in pepper plants in response to DL-β-amino-n-butyric acid. Eur. J. Plant Pathol. 102: 663-670.

Suo, Y., and Leung, D.W.M. 2001. Elevation of extracellular β-1,3-glucanase and chitinase activities in rose in response to treatment with acibenzolar-S-methyl and infection by *D. rosae*. J. Plant Physiol. 158: 971-976.

Takenaka, S., Nishio, Z., and Nakamura, Y. 2003. Induction of defence reactions in sugar beet and wheat by treatment with cell wall protein fractions from the mycoparasite *Pythium oligandrum*. Phytopathology 93: 1228-1232.

Terras, F.R.G., Schoofs, H., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A., Broekaert, W.F. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sati.us* L.) seeds. Journal of Biological Chemistry 267: 15301-15309.

Thrane, C., Nielsen, M.N., Sorensen, J., and Olsson S. 2001. *Pseudomonas fluorescens* DR54 reduces sclerotia formation biomass development, and disease incidence of *Rhizoctonia solani* causing damping-off in sugar-beet. Microbial Ecology 42: 438-445.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. and Heinen, E. 1999. Housekeeping genes as internal standards: use and limits. Journal of Biotechnology 75: 291-295.

Tian, Y., Fan, L.J., Thurau, T., Jung, C., and Cai, D., 2004, The absence of TIR-type resistance gene analogues in the sugar beet (Beta vulgaris L.) genome. Journal of Molecular Evolution 58: 40-53.

Ton, J., and Mauch-Mani, B. 2004. Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 38: 119-130.

Ton, J., Jakab, G., Toquin, V., Flors, V., Iavicoli, A., Maeder, M.N., Metraux, J.P., and Mauch-Mani, B. 2005. Dissecting the beta-aminobutyric acid-induced priming phenomenon in arabidopsis. Plant Cell 17: 987-999.

Tosi, L., Luigetti, R., and Zazzerini, A. 1999. Benzothiadiazole induces resistance to *Plasmopara helianthi* in sunflower plants. J Phytopathol. 147: 365-370.

Trognitz, F., Manosalva, P., Gysin, R., Nino-Liu, D., Simon, R., Herrera, M.D., Trognitz, B., Ghislain, M. and Nelson, R. 2002. Plant defence genes associated with

quantitative resistance to potato late blight in *Solanum phureja* x dihaploid *S. tuberosum* hybrids. Mol. Plant-Microbe Interact.15: 587-597.

Tsror (Lahkim) L., Barak, R., and Sneh, B. 2001. Biological control of black scurf on potato under organic management. Crop Prot. 20:145-150.

Tuzun, S. 2001. The relationship between pathogen-induced systemic resistance (ISR) and multigenic (horizontal) resistance in plants. Eur. J. Plant Pathol. 107: 85-93.

Vallad, G.E., and Goodman, R.M. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 44: 1920-1934.

Van Bruggen, A.H.C., N.J. Grunwald, M. Bolda. 1996. Cultural methods and soil nutrient status in low and high input agricultural systems, as they affect *Rhizoctonia* species. In: *Rhizoctonia* Species: Taxonomy, Molecular biology, Ecology, Pathology and Disease control. B. *Edited by* Jabaji-Hare, S. Nate and G Dijst . Kluwer Academic Publishers, Netherlands, pp: 407-423.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:1-11.

Van Dijken, R. 2001. Science shop for biology.http://www.consubiotech.nl/pub/pdf/suikerb.pdf.

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

Van Loon, L.C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. Eur. J. Plant Pathol. 103: 753-765.

Van Loon, L.C. and Van Strien, E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55: 85-97.

Watanabe T., Sekizawa, Y., Shimura, M., Suzuki, Y., Matsumoto, K., Iwata, M., Mase, S. 1979. Effects of probenazole (Oryzemate) on rice plants with reference to controlling rice blast. J. Pest. Sci. 2: 395-404.

Watzinger, F. Ebner, K., and Lion, T. 2006. Detection and monitoring of virus infections by real-time PCR. Molecular Aspects of Medicine 27:254-298.

Wen, K., Seguin, P., St-Arnaud, M., and Jabaji-Hare, S. 2005. Real-time quantitative RT-PCR of defence-associated gene transcripts of *Rhizoctonia solani*-infected bean seedlings in response to inoculation with a nonpathogenic binucleate *Rhizoctonia* isolate. Phytopathology 95: 345-353.

Whitney, E.D., and Duffus, J.E. 1986. Compendium of beet diseases and insects, Amer. Phytopathological Society, St. Paul, MN. 116 pp.

Wu, G.S., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C. and Shah, D.M. 1995. Disease resistance conferred by expression of a gene encoding H2O2-generating glucose-oxidase in transgenic potato plants. Plant Cell 7: 1357-1368.

Yamada, T., Hashimoto, H., Shiraishi, T., and Oku, H. 1989. Suppression of pisatin, phenylalanine ammonia-lyase messenger-RNA, and chalcone synthase messenger-RNA accumulation by a putative pathogenicity factor from the fungus *Mycosphaerella-Pinodes*. Mol. Plant-Microbe Interact. 2: 256-261.

Yamada, T., Sriprasertsak, P., Kato, H., Hashimoto, T., Shimizu, H., and Shiraishi, T. 1994. Functional-analysis of the promoters of phenylalanine ammonia-lyase genes in pea. Plant and Cell Physiol. 35: 917-926.

Yanar, Y., Yilmaz, G., Cesmeli, I. and Coskun, S. 2005. Characterization of *Rhizoctonia solani* isolates from potatoes in Turkey and screening potato cultivars for resistance to AG-3 isolates. Phytoparasitica 33: 370-376.

Yoshioka, K., Nakashita, H., Klessig, D.F., and Yamaguchi, I. 2001. Probenazole induces systemic acquired resistance in *Arabidopsis* with a novel type of action. Plant J. 25:149-157.

Xue, L., Charest, P.M., and Jabaji-Hare, S.H. 1998. Systemic induction of peroxidaes, 1-3 beta-glucanases, chitinases, and resistance in bean plants by binucleate Rhizoctonia species. Phytopathology 88: 359-365.

Zhang, Y, N.H. Haunerland and Punja, Z.K. 1996. Chitinase profiles in mature carrot (Daucus carota) roots and purification and characterization of a novel isoform. Physiologia Plantarum 96: 130-138.

Zhang, S., Schisler, D.A., Boehm, M.J. 2005. Evaluation of chemical SAR inducers in combination with *Cryptococcus Nodaensis* Oh 182.9 for Fhb control in wheat [abstract]. American Phytopathological Society Annual Meeting. Phytopathology 95: 117.

Zhao, G., Ablett, G.R., Anderson, T.R., Rajcan, I. and Schaafsma, A.W. 2005. Anastomosis groups *of Rhizoctonia solani* associated with soybean root and hypocotyl rot in Ontario and resistance of accession PI 442031 to different anastomosis groups. Can. J. Plant Pathol. 27: 108-117.

Zimmerli, L., G. Jakab, J.P. Metraux, B. Mauch-Mani. 2000. Potentiation of pathogen-specific defence mechanisms in Arabidopsis by beta-aminobutyric acid. Proc. Natl. Acad. Sci. USA 97: 12920-12925.

Zimmerli, L., Metraux, J.P., and Mauch-Mani, B. 2001. Beta-aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*: Plant Physiology 126: 517-523.

Zou, J., Young, S., Zhu, F., Xia, L., Skeans, S., Wan, Y.T., Wang, L.Q., McClanahan, T., Gheyas, F., Wei, D., Garlisi, C., Jakway, J. and Umland, S. 2002. Identification of differentially expressed genes in a monkey model of allergic asthma by microarray technology. Chest 121: 26s-27s.