EVALUATION OF SYSTEMIC PLANT PROTECTION MECHANISM INDUCED BY HYPOVIRULENT *RHIZOCTONIA* SPP.

THESIS SUBMITTED FOR THE DEGREE

"DOCTOR OF PHILOSOPHY"

BY

Michal Sharon

Submitted to the senate of Tel Aviv University

May, 2010

EVALUATION OF SYSTEMIC PLANT PROTECTION MECHANISM INDUCED BY HYPOVIRULENT *RHIZOCTONIA* SPP

THESIS SUBMITTED FOR THE DEGREE

"DOCTOR OF PHILOSOPHY"

BY

Michal Sharon

Submitted to the senate of Tel Aviv University

May, 2010

Acknowledgements

First of all, I wish to thank my supervisors: Prof. Baruch Sneh, Dr. Stanley Freeman and Dr. Nurit Katzir for their guidance - providing me their valuable suggestions, encouragement, support and good advice, whether the results were challenging or encouraging.

My special gratitude to my laboratory colleagues: Aida Zvibel, Adi Biton, Ifat Abraham, Sigal Horowitz, Tomer Gershon and especially Marcel Maymon for helping me out in all aspects during my work, whether by good advice, technical support or a kind word during the numerous experiments.

My special thanks to my friend and colleague Youlia Denisov for her moral and technical support, and for being such a great company during the challenging years of my research.

I wish to thank my family for their constant support and encouragement in all my academic endeavors.

This work is dedicated to the memory of Efrat Atinsky, may her soul be bound up in the bond of everlasting life.

This work was carried out under the supervision of

Prof. Baruch Sneh

Department of Molecular Biology and Ecology of Plants, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel.

Dr. Stanley Freeman

Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan, Israel.

Dr. Nurit Katzir

Department of Vegetable Crops, ARO, Newe Ya'ar Research Center, Ramat Yishay, Israel.

Table of contents

1. Abstract

It has been established thus far that protective *Rhizoctonia* isolates trigger defense-related responses in the plants they protect. To our knowledge, by now, no study has examined thoroughly which plant resistance pathways are induced in plants colonized by hypovirulent *Rhizoctonia* spp. isolates. The SAR (Systemic acquired resistance) pathway is effective mostly against biotrophic pathogens and involves PR (pathogenesis related) protein induction. It is usually induced in plants by biotrophic pathogens, SA (Salicylic acid – which is a key element in this pathway) and some chemical, such as BTH and Bion®. The ISR (Induced systemic resistance) is mostly effective against necrotrophic pathogens. It is usually induced in plants by rhizobacteria, necrotrophic pathogens, insects, and meJA (methyl jasmonate), – which is a key element of this pathway, along with ethylene. Phytoalexins production is sometimes associated with the ISR pathway, although phytoalexins can be induced in a separate pathway from ISR, and thus protect the plants in an independent way.

The objective of the present work was to evaluate which of the induced systemic resistance pathways are involved in the protection of plants by hypovirulent *Rhizoctonia* spp. isolates against pathogenic *Rhizoctonia*. To establish this, it was first validated that the virulence levels of the isolates used in this study were very low on various plant species. Protection levels which the hypovirulent isolates induced on cucumber plants were evaluated when the pathogen was introduced at the same site as the hypovirulent isolate on the plant, or at a distance away, and it was revealed that although the protective hypovirulent isolates induced systemic resistance in the plants they colonize, it is most

likely not the only method of protection involved. Local protections, which were not examined in this study, are probably an important part in the defense of plants by the hypovirulent *Rhizoctonia* spp. isolates. Extracts of radish plants colonized by one of the hypovirulent *Rhizoctonia* isolates inhibited growth of pathogenic *Rhizoctonia* and indicated the involvement of phytoalexins in the resistance induced by the hypovirulent isolates. Comparing changes in protection levels of *Arabidopsis thaliana* mutants defected in defense-related genes to the protection of wt plants by the same isolates revealed that *A. thaliana* mutants defected in prime genes involved in both SAR and ISR were protected to a lower extent than the wt plants. On the other hand, the protection rates of the wt plants were similar to the protection of a mutant whose SAR deficiency was compensated by ISR expression as a result of removing the inhibition on ISR (*SALK-025198c* mutant), and the protection of mutants constantly expressing SAR (*snc1, CS6571*) was decreased compared to wt. These results indicate that the systemic induced resistance is a substantial part of the protection mechanism of plants by colonization of hypovirulent isolates against pathogenic *Rhizoctonia*, and that the ISR pathway has a major role in this protection. Monitoring prime genes in the SAR, ISR, and phytoalexin production pathways, in plants inoculated with hypovirulent *Rhizoctonia* spp., showed elevated levels of *Pr5* (SAR), *Pdf1.2, Lox2, Lox1,Cori3* (ISR), and *Pad3* (phytoalexin production) gene expression which indicated that all of those pathways are induced in hypovirulent-inoculated plants. When SAR or ISR were induced separately in plants through application of chemical inducers (Bion and meJA, respectively), only ISR protected the plants against pathogenic *Rhizoctonia* isolate, and this protection, though significant, was not high. Concluding this study: hypovirulent *Rhizoctonia* spp. isolates

induce SAR, ISR and phytoalexin production in plants they colonize. Even though the SAR pathway may protect plant against other pathogens, it is probably not a significant part in the defense of inoculated plants against pathogenic *Rhizoctonia*. Apart from inducing SAR, ISR and phytoalexin production it is likely that the protective hypovirulent isolates also defend the plants they colonize locally, and although local protection was not investigated in this work, it should be examined in future research. The high protection levels of plants by hypovirulent *Rhizoctonia* spp. isolates is probably due to the combination of various protection modes, local and systemic, physical and induced in the plant. Therefore, the comprehensive reaction of protection which is achieved by the hypovirulent isolates could not be reconstructed by application of chemical substances in conventional pest control.

Abbreviations

UNR – Uni-Nucleate *Rhizoctonia*

BNR – Bi-Nucleate *Rhizoctonia*

MNR – Multi-Nucleate *Rhizoctonia*

ISR – Induced Systemic Resistance

SAR – Systemic Acquired Resistance

SA – Salicylic Acid

JA – Jasmonic Acid

Et – Ethylene

BTH - Benzo 1,2,3-thiadiazole-7-carbothioic acid S-methyl ester

ROS – Reactive Oxygen Species

PR – Pathogenesis Related

2. Introduction

Biological control, by itself or as a component of integrated control of plant-pathogens, which includes environmentally-friendly agro-chemicals and other agricultural practices (Harris, 1995; Harris, 2000b; Sneh, 1996; LaMondia *et al.*, 2002), is an ideal substitute for the current worldwide immense use of toxic pesticides in agriculture. On the one hand, chemical pesticides cause harmful effects on humans, both in the short and long terms. On the other hand, pesticides with known harmful effects on humans, animals or the environment prohibited from use (such as methyl bromide, for example), suggested replacements are sometimes less effective, resulting in increased crop damage by pathogens (Martin and Bull, 2002). In addition, pathogens evolve resistance to continued use of the same chemical pesticides (Brent and Hollomon, 2007), while biocontrol agents are living organisms that can counter-act when a pathogen-resistance occurs and thus, resistance to them may evolve at a slower pace. Since some hypovirulent *Rhizoctonia* spp. isolates protect plants they colonize against pathogenic *Rhizoctonia* (Honeycutt and Benson, 2001; Ichielevich-Auster *et al*. 1985; Sneh and Ichielevich-Auster, 1998; Sneh, 1996), it is important to clarify the modes of action of this protection in order to make use of the knowledge for future development of more effective biological control agent preparations and application procedures.

2.1. *Rhizoctonia* spp. Pathogenic *Rhizoctonia* spp. isolates cause many diseases on a wide host range of plant and consequently inflict serious losses on a wide variety of highly important cash crops.

Rhizoctonia De Candolle anamorph (asexual stage) classification belongs to the Mitosporic Fungi (syn.: Deuteromycotina, Deuteromycetes, Fungi Imperfecti, Asexual

Fungi) (Hawksworth *et al.,* 1995). Since it does not form asexual spores it belongs to Mycelia Sterilia (Ulloa and Hanlin, 2000). As DNA sequencing analysis made it possible to clade the anamorphic fungi to their correct teleomorphic (sexual stage) groups, the present classification code uses the teleomorph classification (Kirk *et al.,* 2008). *Rhizoctonia* classified as a 'Genus form' which includes a wide variety of fungal teleomorphs. It is subdivided according to the nuclear number in the young hyphal cells to: uni-, bi- or multi-nucleate (also known as UNR, BNR and MNR, respectively) (Sneh *et al.,* 1991), and according to its teleomorph associations into; *Moniliopsis* (MNR, anamorph of *Thanatephorus*, *Ceratorhiza* (BNR, anamorphs of *Ceratobasidium*), *Chrysorhiza* (MNR, anamorph of *Waitea*), *Epulorhiza* (BNR, anamorph of *Tulasnella*) and *Opadorhiza* (BNR, anamorph of *Sebacina*). The *Ceratobasidium*, *Thanatephorus* and *Waitea* all belong to one clade, while *Botryobasidium* and *Tulasnella* to another (Mclaughlin *et al.,* 2001). *Ceratobasidium*-, *Thanatephorus*- and *Waitea*-genus belongs to the family ceratobasidiaceae, order cantharellales, subclass incertae sedis, class agaricomycetes and phylum basidiomycota, kingdom: Fungi (Kirk *et al.,* 2008). *Rhizoctonia* spp. are classified by anastomosis groups and sub-groups, which according to Sharon *et al.,* (2006; 2007; 2008) have a strong phylogenetic foundation.

A significant proportion of the *Rhizoctonia* spp. isolates are pathogenic to a wide range of host plants, belonging to many botanical families. Some of the isolates are hypovirulent or even form mycorrhizal associations with orchids and other plants (Sneh *et al.,* 1991).

2.1.1. Colonization and infection. When pathogenic *Rhizoctonia solani* isolates colonize seedlings the hyphae grow along the hypocotyl. At some stage the hyphae

branch and form hyphal clusters. Secretion of mucilage enables them to adhere to each other and to the plant surface and the hyphae continue to branch into T like structures, while creating short cells and infection cushions from which many infection pegs emerge and penetrate the epidermis. Under the infection cushions the first hypocotyl discoloration appeares, which subsequently become necrotic (Armentrout and Downer, 1987; Weinhold and Jerome, 1973).

2.1.2. Protective hypovirulent *Rhizoctonia* spp. isolates. Hypovirulent *Rhizoctonia* spp. isolates protected plants efficiently (75-95% protection) against pathogenic *Rhizoctonia* (Ichielevich-Auster, 1987; Harris *et al.,* 1997; Harris and Adkins, 1999; Harris, 2000a; Ross *et al.,* 1998; Sneh, 1996; Sneh et al., 1989a; Wen *et al.,* 2005) as well as other pathogens such as *Pythium ultimum, Pseudomonas syringae* (Sneh and Icihelevich-Auster, 1998), *Fusarium oxysporum* (Muslim *et al.,* 2003) and *Botrytis cinerea* (Cardinale *et al.,* 2006). The hypovirulent isolates colonized the surface of the epidermis densely but did not penetrate the cells (Harris *et al.,* 1997; Harris and Adkins, 1999; Harris, 2000a; Siwek *et al.,* 1997a; Siwek *et al.,* 1997b; Sneh et al., 1989b; Jabaji-Hare *et al.,* 1999). The possibilities of protection by antibiotic secretion, parasitism, or competition for root exudates containing sugars and amino acid were tested but neither was found to be involved (Ichielevich-Auster, 1987; Sneh et al., 1989a).

2.1.3. Plant responses to colonization by protective hypovirulent *Rhizoctonia* spp. isolates. Suberin, a part of the hydrophobic cell wall barriers, involved in protection plants against biotic and abiotic stresses (Pollard *et al.*,2008), accumulated in cell walls of plants colonized by hypovirulent *Rhizoctonia* spp. isolates but not in plants infected by the pathogenic isolate. Pectic substances accumulated in plants colonized by hypovirulent

isolates were restricted to the outer section of the epidermal cells; while pectic substances accumulated in all of the epidermal and cortical tissues as well as the parenchyma cells in plants infected by the pathogen (Jabaji-Hare *et al.,* 1999). Bean plants inoculated with hypovirulent isolates accumulated more salicylic acid (SA) than non-inoculated control plants or plants infected by the pathogen. Plants colonized first by the hypovirulent isolates and subsequently by the pathogen exhibited higher peroxidase activity than plants colonized solely by the pathogen. Similar effects were obtained for 1,3-β-glucanase (PR-2 family), phenylalanine ammonia lyase (PAL –lignin synthesis, phenylpropanoid and flavonoid pathway) and chitinase (PR-3 family) (Xue, 1999; Cardinale *et al.,* 2006; Walski *et al.,* 2005). On the other hand, Wen *et al.,* (2005) reported that bean plants colonized by a protective hypovirulent *Rhizoctonia* isolate had a reduced expression of 1,3-β-D-glucanase, PAL and chalcone lyase (phenylpropanoid and flavonoid pathway). Poromarto et al., (1998) reported that hypovirulent *Rhizoctonia* induced systemic resistance in colonized soybean plants, by inhibiting growth of the pathogenic *Rhizoctonia* isolate on the colonized plants without hyphal contact between the isolates, when the pathogen was placed at a distance of one centimeter away from the hypovirulent isolate. Convincing evidence for induction of systemic resistance by hypovirulent isolates on colonized plants was published by Hwang and Benson (2003) who established that poinsettia mother plants colonized by a hypovirulent *Rhizoctonia* isolate produced cuttings that were resistance to pathogenic *Rhizoctonia*. The protection observed was time dependent, and best resistance results were found in cuttings taken from plants which were colonized with the hypovirulent isolate for duration of at least 7

days. This resistance was not present in plants treated with the SAR inducing agent acibenzolar.

So far, to our knowledge, there has been no attempt to classify plant resistance reactions induced by hypovirulent *Rhizoctonia* specifically to the SAR or ISR pathways.

2.2. Plant defense responses to various pathogens. Plants, as sessile organisms, have developed an array of defense mechanisms in response to attack by pathogens. These defense mechanisms were either constitutively expressed (such as thorns, waxes, secondary metabolites) or induced in response to pathogen attack, as local induced resistance, Phytoalexins production **(**Glawischnig, 2007), induced systemic resistance (ISR), systemic acquired resistance (SAR), and Pr proteins production (Kombrink and Somssich, 1997).

2.2.1. Local induced defenses. When a host plant senses a pathogen it initiates a series of reactions to trigger its defense responses. The first of which consists of induction of the local defense response - which is a rapid reaction, mostly restricted to the infected area, transcription independent and consists initially of the oxidative burst and ion fluxes followed by cytoskeletal rearrangements, protein phosphorylation or dephosphorylation, nitric oxide synthesis and induction of a hypersensitive response (HR). These responses occur very rapidly, within a matter of minutes after exposure to the pathogen and prior to the systemic induction responses. The oxidative burst can affect the invading microorganisms directly due to its toxicity, or indirectly by oxidative cross-linking of cell wall components, increasing cell wall lignification, and inducing phytoalexin synthesis. Reactive components created by the oxidative burst could also act as a signal for other defense reactions – such as the HR (Talarczyk and Hennig, 2001).

2.2.2. Systemic acquired resistance (SAR). A systemic defense network that is induced in plants when they are attacked by some pathogens - usually a biotroph (van Loon, 1997; Pieterse *et al.,* 2001; Klessing *et al.*, 2000; Heil and Bostock, 2002; Perl-Treves *et al.,* 2004) involves salicylic acid (SA) or its derivative methyl salicylate as the key signals (Shah, 2009). SAR was also induced by applying abiotic substances such as BTH (Benzothiadiazole) or Bion® (Acibenzolar-S-methyl, also known as ACTIGARD® manufactured by Syngenta) which contains acibenzolar-*S*-methyl (Wade, 2006). This artificial induction of SAR functioned as a SA triggering factor resulting in a 4-80% reduction in disease severity (Walters and Heil, 2007). SA levels were raised after pathogen attack, both locally and systemically, and exposure of the plant to SA was sufficient to trigger PR protein expression (Beckers and Spoel, 2006; Glazebrook, 1999). A constitutive expression of SAR may reduce plant fitness (Cipollini, 2002), though without SA production and/or accumulation SAR did not occur (Beckers and Spoel, 2006; Glazebrook, 1999). Application of BTH (Benzo 1,2,3-thiadiazole-7-carbothioic acid S-methyl ester) induced SAR in the *nahG* mutant (a mutant that expresses a bacterial enzyme that destroys SA in the plant) (Friedrich *et al.,* 1996). The SAR pathway has been represented as a linear model where *Eds1*/*Pad4* and other genes increased SA production that activated NPR1, which in turn activated the transcription factors for PR gene (Delaney, 1997; Ryals *et al.* 1996). As research in this area progressed it became clear that this model was a misconception and that the actual SAR network is much more complicated (Glazebrook *et al.,* 2003; Lu, 2009), consisting of several pathways, some of which do not rely on *Npr1* (Zhang and Shapiro, 2002, Lu, 2009).

2.2.3. Induced systemic resistance (ISR). ISR is a systemic resistance response that is triggered in plants by a variety of microorganisms and insects, mostly rhizobacteria (Ton *et al.,* 2002) and necrotrophic fungi (Perl-Treves *et al.,* 2004). Contrary to SAR, ISR is not associated with PR genes (van Wees *et al.,* 1999). ISR is dependent on jasmonic acid (JA) or its derivative methyl jasmonate (meJA) and ethylene (Et) signals and is independent on SA (Pieterse *et al.,* 2001; Klessing *et al.,* 2000), although Stintzi *et al.,* (2001) have demonstrated previously-known JA-depended plant resistance to some fungal and insect pathogens in a mutant which did not generate JA or meJA. It is necessary for the plant to have a functional *Npr1* gene for ISR induction (Pieterse *et al.,* 2001, Kawamura *et al.,* 2009). *Arabidopsis thaliana* mutants that over expressed JA-biosynthetic genes constitutively expressed PDF1.2 and exhibited enhanced resistance to *Botrytis cinerea* (Kunkel and Brooks, 2002).

2.2.4. SAR and ISR interactions. There is a tradeoff between the SAR and ISR systems, SAR-induced plants were more susceptible to pathogens which plants usually defend against by inducing ISR (Bostock, 2005; Kliebenstein and Rowe, 2008; Murry and Jonathan 2009). Similarly, JA application inhibited responses which were induced by SAR (Glazebrook *et al.,* 2003; Kliebenstein and Rowe, 2008; Mur *et al.,* 2006). On the other hand, sometimes there were synergistic responses when components from either SAR or ISR pathway enhanced the responses of the ISR or SAR pathway (Durrant and Dong, 2004; Koornneef and Pieterse, 2008; Mur *et al.,* 2006; Schenk *et al.,* 2000), one pathway triggered the other (Walters *et al.,* 2006), or one pathway did not affect the other (Mur *et al.,* 2006). The various different responses triggered in plants in response to different pathogens attacks indicated that even though SA, JA and Et play an important

role in induced resistance, the ultimate response of the plant to the attack is composed of the sum of the genes triggered in those pathways, and these genes differ in their response to different kinds of pathogens (Bari and Jones, 2009; Walters and Heil, 2007; Zarate *et al.,* 2007; Smart *et al.,* 2003). Although SAR and ISR have been investigated extensively, there appear to be new unknown pathways which can partially trigger either system (van Wees *et al.,* 1999; Attaran *et al.,* 2009).

2.2.5. The main plant genes involved in the systemic defense responses

2.2.5.1. Nonexpresser of PR genes (*Npr1*). Also known as 'non-inducible immunity1' (*Nim1*) and 'Salicylic acid insensitive1' (*Sai1*). The NPR1 protein contains an ankyrin-repeat domain and a BTB/POZ (Broad-complex, tramtrack, bric-a-brac/poxvirus, zinc finger), both involved in protein-protein interaction (Durrant and Dong 2004), containing an NLS signal positioned at the carboxylic end (Dong, 1998). Mutant *npr1* plants of *A. thaliana* did not activate PR proteins after pathogen infection even though they could accumulate high levels of SA (Beckers and Spoel, 2006), but mutants that constitutively expressed NPR1 did not express PR1 constantly since the NPR1 protein must be activated in order to trigger SAR (Durrant and Dong 2004). Wild type plants were found to express a constant basal level of NPR1 that accumulated as oligomers in the cytosol. After pathogen attack, treatment with SA or BTH, SA was triggered, there was an oxidative burst and the NPR1 oligomers were reduced to monomers (Beckers and Spoel, 2006; Dong, 2004; Grant and Lamb, 2006), the transcription of the NPR1 gene was elevated and its mRNA levels rose by 2-3 fold (Durrant and Dong 2004). NPR1 monomers were reported to enter the nucleus (Dong, 2004), where they interacted with transcription factors from the TGA family (containing

basic leucine zipper) and triggered PR proteins from the SAR pathway (Koornneef and Pieterse 2008; Johnson *et al.,* 2008). The ankyrin-repeat (Durrant and Dong 2004) and BTB/POZ (Rochon *et al.,* 2006) domains in the NPR1 protein were essential for the interaction with the TGA transcription factors, while the NPR1 N-terminal increased the NPR1-TGA connection. The *Npr1* gene was also necessary for resistance responses activated by the JA/ethylene pathway (Dong, 1998), though in this case *Npr1* was not involved in PR1 transcription (Pieterse *et al.,* 2007). NPR1 was also involved in local defense responses, probably by limiting advance of the pathogen from the infection site (Durrant and Dong 2004).

2.2.5.2. Non race specific disease resistance1 gene (*Ndr1*). *Ndr1* encodes a protein with two transmembrane domains (Glazebrook, 1999). The gene's mRNA expression was increased in the plant after bacterial infection, starting 4 hours after exposure (Century *et al.,* 1997). An intact *Ndr1* gene was necessary for the function of most PR proteins in the subgroup of Leucin-zipper (LZ) in the gene group of leucin rich repeats-(LRR) NBS (Glazebrook, 2001). It was approximated that one of the *Ndr1* roles was to maintain resistance (R) proteins in proximity to the membrane (Glazebrook, 1999), and the Ndr1 protein may also act as a transducer of an elicitor signal after the R gene product interacted with a specific AVR signal or served as a transporter or a receptor for the elicitor signal (Century *et al.,* 1997). *ndr1*-1 mutants were impaired in reactive oxygen species (ROS) response, both production and sensing (Zhang and Shapiro, 2002), which could affect or be affected by the *Ndr1* role as a SA regulator, acting independently from *Eds1* (Lu, 2009).

2.2.5.3. Enhanced disease susceptibility (*Eds1*) and Phytoalexin deficient4 (*Pad4*).

eds A. thaliana mutants were identified by their enhanced susceptibility to disease after infection with a pathogenic isolate of *Pseudomonas syringae* pv. *maculicola* (Dong, 1998), *eds1* and *pad4* mutants are defected in lipase-like proteins (Durrant and Dong 2004). Both genes are upstream from the SA signal in the SAR pathway (Glazebrook, 2001) and their mRNA levels increase in response to induction of the SA-dependent systemic resistance (Falk *et al.,* 1999; Jirage *et al.,* 1999). A defected *pad4* gene partially reduced the accumulation of Eds1 transcripts, while a defected *eds1* gene prevented accumulation of *Pad4* mRNA. Eds1 dimerized and interacted with Pad4, through different domains. According to the hypothesis of Feys *et al.,* (2001), *Eds1* is necessary at two stages during the activation of SAR - upstream of *Pad4*, triggering earlier defense responses, and later – with Pad4, strengthening plant defense by accumulating SA (Feys *et al.,* 2001). An intact *Eds1* gene was necessary for the function of PR proteins in the subgroup of proteins with *Drosophila* Toll-like and animal Interleukin2-like N-terminal (TIR-NBS-LRR) in the group of NLS-LRR proteins (Glazebrook, 2001), and defective *pad4* mutants weakened the local resistance intermediated by the same PR (TIR-NBS-LRR) proteins (Durrant and Dong 2004). Expression of *Eds1* reduced accumulation of *Pdf1.2* mRNA, which is an ISR-related gene (Brodersen *et al.,* 2006).

2.2.5.4. Pathogenesis related protein5 (*Pr5*). The *Pr5* gene family was first found in tobacco and described as thaumatin-like proteins (van Loon *et al.,* 2006). These proteins are induced by viral and fungal infection and were found to have antifungal activity (Kombrink and Somssich, 1997). The transcription of the gene *Pr5* in *A. thaliana* was

induced in response to activation of the SAR pathway and therefore was used as a marker for SAR-dependent defense triggering (Kawamura *et al.,* 2009).

2.2.5.5. Lipoxygenases (LOX). Genes from the Lox family are dioxygenases, in association with iron and without heme. They were found to catalyze the hydroperoxidation of specific unsaturated fatty acids in plants, animals and microorganisms (Melan *et al.,* 1993), including initiating JA synthesis in plants (Schaller and Stintzi, 2008), where they mediate the conversion of linolenic acid (LA) to 13-hydroperoxylinolenic acid (Bell *et al.,* 1995). The lipoxygenase genes were divided into two main groups – the 9-lipoxygenases and 13-lipoxygenases, according to the position of the oxygen they incorporated into the linoleic acid and/or linolenic acid (Bannenberg *et al.,* 2009), the 13-lipoxygenases catalyzed the first step of JA biosynthetic pathway, while the 9-lipoxygenases were involved in defense responses (Lopez *et al.,* 2008).

Lox1 is a 9-lipoxygenase gene (Bannenberg *et al.,* 2009), its mRNA levels increased when plants were treated with abscisic acid (ABA) and methyl-jasmonate (meJA) and maintained elevated levels for at least 96 hours after induction. The same effect was also achieved by pathogen (*Pseudomonas syringae*) inoculation (Melan *et al.,* 1993).

Lox2 is a 13-lipoxygenase gene (Bannenberg *et al.,* 2009) and was found to be targeted to the chloroplast. *Lox2* mRNA accumulated rapidly after JA induction (Bell and Mullet, 1993) and transgenic plants defected in *Lox2* did not accumulate JA after wounding, though the basal level of JA was unaffected in the transgenic plants (Bell *et al.,* 1995).

2.2.5.6. Plant defensin1.2 (*Pdf1.2*). The *Pdf1.2* gene encodes a small protein with antifungal activity (Penninckx *et al.,* 1996). *Pdf1.2* transcription was triggered in

response to JA and ethylene (Solano *et al.,* 1998; Brodersen *et al.,* 2006) but not SA (Manners *et al,* 1998), and the increase in mRNA levels has been used as an indication for induction of the ISR pathway (Falk *et al.,* 1999; Kawamura *et al.,* 2009) and was not expressed in mutants insensitive to ethylene (Penninckx *et al.,* 1998). Also, *Pdf1.2* transcription was induced by *Atpep1* which also induces H_2O_2. Blocking H_2O_2 induction (by using an NADPH inhibitor) has also inhibited *Pdf1.2* induction (Huffaker *et al.,* 2006).

2.2.5.7. Coronatine induced3 (*Cori3*)/Jasmonic acid Responsive gene (*Jr1*). The *A. thaliana Cori3* gene encodes cystine lyase (Jones *et al.,* 2003). This gene's mRNA was induced in response to coronatine, meJA and ABA in a dosage depended manner. It was also induced by wounding, but its mRNA levels declined 6 hours after wounding, while 24 hours after meJA induction *Cori3* mRNA continued to accumulate (Castillo *et al.,* 2004; Leon *et al.,* 1998).

2.2.5.8. Phytoalexin deficient3 (*Pad3*). One of the defense mechanisms induced in plants after pathogen attack includes the phenylpropanoid pathway. This leads to accumulation of phenolic compounds such as lignins which help to fortify the cell walls, SA (the systemic resistance signal), and phytoalexins which have an antifungal effect (Talarczyk and Hennig, 2001). van Wees *et al.,* (2003) reported that the phytoalexin-related gene *Pad3* is essential for *A. thaliana* defense against *Alternaria brassicicola*. *Pad3* encodes the cytochrome P450 enzyme CYP71B15 that catalyzes the last step of camalexin (an *A. thaliana* phytoalexin) biosynthesis – the conversion of dihydrocamalexic acid to camalexin (Bottcher *et al.,* 2009; Schuhegger et al., 2006).

2.2.5.8. GH3.12/avrPphB susceptible 3 (*Pbs3*). This *A. thaliana* gene was proposed to act up-stream from SA, inducing SA biosynthesis, while SA inhibits GH3.12 activity, and hence regulates its own synthesis (Vlot *et al.,* 2009). A mutant in this gene (*pbs1-3*) carries two point mutations in a highly conserved region of the protein's C-terminal (Nobuta, et al. 2007), which results in reduced SA accumulation and SA-dependent gene expression (Okrent *et al.,* 2009).

Although previous studies confirmed that colonization of plants by protective hypovirulent *Rhizoctonia* spp. isolates triggered resistance-related responses in the protected plants (Cardinale *et al.,* 2006; Jabaji-Hare *et al.,* 1999; Walski *et al.,* 2005; Xue, 1999), the modes of action (SAR, ISR, ROS, Phytoalexins and genes) involved in the defense induced by protective hypovirulent *Rhizoctonia* spp. isolates have not yet been identified. The present study objective was to identify the modes of induced systemic resistance in plants colonized by protective hypovirulent *Rhizoctonia* spp. isolates, focusing on the plant protection systems which are effective against *R. solani*. Utilizing *A. thaliana* mutants harboring defective genes involved in induced resistance pathways accompanied with quantitative real time PCR to identify some of the prime genes and pathways involved in the mode of induced resistance in plants, triggered by the hypovirulent *Rhizoctonia* spp. isolates.

Clarifying these modes of action may facilitate and advance more efficient screening for more efficient protective hypovirulent isolates and consequently better defense of plants against pathogens (Fravel, 2005).

3. Materials and Methods

3.1. Media.

3.1.1 WAcm (Water Agar)

Table 1. WAcm preparation.

Component	Weight/Volume
Agar	17g
Chloramphenicol	0.25g
H_2O distilled	1000mL

The medium was dispensed 18mL/90mm plate.

3.1.2. YDAcm (Yeast Extract Agar)

Table 2. YDAcm preparation.

Component	Weight/Volume
Yeast Extract	5g
Peptone	5g
Sucrose	5g
Agar	17g
Chloramphenicol	0.25g
H_2O distilled	1000mL

The medium was dispensed 18mL/90mm plate.

3.1.3. MS (Murashige and Skoog agar). Prepared as described in Murashige and Skoog, 1962.

The medium was dispensed 33mL/90mm plate.

3.2. *Rhizoctonia* spp. isolates used in the present study.

Table 3. The *Rhizoctonia* spp. isolates used and their affiliations.

Isolate name	Virulence	Anastomosis group	origin[c]
Ru18-1	Hypovirulent	AG-B(o)[a]	Maryland
Ru89-1	Hypovirulent	AG-B(o)[a]	Connecticut
Ru521	Hypovirulent	AG-A[a]	Israel
Ru56-8	Hypovirulent	AG-A[a]	West Virginia
RS13	Pathogenic	AG 4[b]	Israel

[a] – According to Sharon *et al.* (2008).

[b] - According to Sharon *et al.* (2006).

c – According to Sneh and Ichielevich-Auster (1998).

3.3. Inoculum preparation of *Rhizoctonia* spp. isolates. Inoculation with *Rhizoctonia* isolates was preformed either with colonized YDAcm plugs, or with colonized wheat grains.

3.3.1. Colonized YDAcm agar. A wheat grain colonized with *Rhizoctonia* isolate was placed in the middle of a YDAcm plate, and incubated at 25°C for 3 days. Colonized 4×4 mm^2 agar pieces were taken from the fresh hyphal growth at the perimeter of the colony for plant inoculation.

3.3.2. Wheat grains. Wheat grains were prepared as described in Sneh and Ichielevich-Auster (1998), without chloramphenicol supplementation. The inoculated grains were stored at 4°C, up to a year before use.

3.4. Seeds germination. Except for *Arabidopsis thaliana*, the seeds of all the plant species were surface disinfected for 5 minutes in 1% sodium hypochlorite solution. Then the seeds where thoroughly washed with sterile water and placed on disinfected trays

lined with moist, sterilized paper towels. The trays were wrapped in plastic bags and placed in 25°C until the seeds germinated. The germinating seeds were planted in $6\times6.5\times7.5cm^3$ pots, containing 200ml of autoclaved sandy soil.

3.5. *Arabidopsis thaliana* seed disinfection. Approximately 50µL *A. thaliana* seeds were placed in a 1.5mL sterile vial and surface disinfected with 250µL of 10% sodium hypochlorite and 750µL of 100% ethanol. The content was vigorously stirred, and incubated for 15min. The liquid was discarded and the seeds were washed 4 times with 750µL 100% ethanol and dried.

3.6. Evaluation of *Rhizoctonia* spp. isolates' virulence on various plant species. The seedlings were planted: 5 seeds/pot, 6 pot/repeat, 3 repeats. With each germinating seed a *Rhizoctonia*-colonized wheat grain was added. The plants were grown for 30 days at 25°C, 12:12 light:dark conditions before survival assessment.

3.7. Cucumber plants inoculation with hypovirulent *Rhizoctonia* spp. isolates in soil and challenge inoculation with the pathogen at consecutive time intervals either in soil, or on the hypocotyl. Two-day old cucumber germinating seeds were planted 10 seeds/pot, 4 pot/repeat, 3 repeats. Ten days after planting, wheat grains (one per plant) colonized with the hypovirulent isolates were placed in soil, adjacent to each plant. One to 6 days later the pathogenic isolate RS13 was challenge inoculated, either in the soil (4 inoculated grains/pot), or on the hypocotyl (by a colonized YDAcm 4×4mm square), at 24 hours intervals. Twelve days later the survival of the plants was evaluated and the protection rate calculated according to the formula: plant protection (%) =100*(A-B)/(C-B), where A = the percentage of surviving plants after colonization with

the tested hypovirulent isolate and challenge inoculation with the pathogen; B = is the percentage of surviving plants after inoculation only with the pathogen, and C = the percentage of the surviving non-inoculated plants (Sneh and Ichielevich Auster, 1998). Non-inoculated negative controls and RS13-inoculated positive controls were used for each time interval since in each time interval tested the plants were a day older compared to the previous experiment.

3.8. Staining *Rhizoctonia* hyphae on cucumber. A thin layer of cucumber tissue inoculated with *Rhizoctonia* hyphae was dissected from the plant and stained with 0.1% (w/v) trypan blue in 10% (v/v) acetic acid for 2 minutes, followed by 2 minutes in 10% (v/v) acetic acid to remove excess dye and 5 minutes in water to remove the acetic acid. The samples were viewed under a microscope (Wild Heerbrugg M20-57287, Switzerland) at ×200 magnifications.

3.9. *In vitro* hyphal growth inhibition of the pathogenic *Rhizoctonia solani* isolate by extracts from radish plants colonized by hypovirulent *Rhizoctonia* spp. isolates. Radish germinating seeds (prepared as described in section 3.4) were placed on trays lined with moist, sterilized paper towels, 1.5cm apart. The trays were wrapped in plastic bags and placed in 25°C, 12:12 light:dark conditions. After 48 hours, wheat grains colonized with hypovirulent isolates were placed among the seedlings. The trays were re-wrapped and the plants were placed at 25°C, 12:12 light:dark conditions for another 48 hours. Then, the plastic bags were removed and the plants continued to grow for another 8 days. The paper towels were kept moist throughout the duration of the trial. The plants were then separated to: roots, lower hypocotyls, upper hypocotyls, and leaves. Two

grams pooled from each plant part were ground in liquid nitrogen, supplemented with 4 ml aliquots of water:ethanol:acetone (1:2:2 respectively) and stored for 48 hours at -20°C. The samples were centrifuged at 10,000 rpm in 4°C for 20 minutes. The supernatant was transferred to a new vial containing 2 5.5 mm diam. filter paper disks (Whatman, #5). The vials' contents were lyophilized. The impregnated paper discs were placed in the center of a WAcm plates. WAcm discs (5 mm in diameter) from a 3 days old RS13 culture were placed 30 mm to the right and the left of the paper disks. Hyphal growth was recorded after 24 hours incubation at 25°C in the dark. The difference in growth of the hyphae measured towards the paper disc from the hyphae growth at 90° angle from the paper disk was calculated. The experiment was repeated three times.

3.10. Comparison of protein maps from extracts of cucumber plants colonized with hypovirulent *Rhizoctonia* spp. isolates with that of non colonized plants.

3.10.1. Protein extraction. Germinated cucumber seeds (two-day old) were planted in $6\times6.5\times7.5$ cm^3 pots, containing 200 mL sandy soil, (5 plants/pot, 6 pots/treatment) grown at 25°C, 12:12 light:dark conditions for 10 days. Wheat grains (1/plant) colonized with hypovirulent *Rhizoctonia* isolates were placed in soil (approximately 5 mm deep) adjacent to the cucumber plants. After 48 hours the hypocotyls were harvested. Combined samples of 4 g (from 25-30 plants) were used for protein extraction. The protein extraction was conducted according to the 'phenol extraction' method in Hurkman and Tanaka (1986), with adjustments. The extraction buffer consisted of 0.1M Tris 8.0, 5% sucrose, 2% SDS, 50mM DTT, 2.5% Protease Inhibitor Cocktail (Sigma, US) and 2 mM PMSF. After centrifugation (4°C, 7000 rpm,

30 min), 5 mL of phenol-saturated H_2O was added to the supernatant, shaken and placed on ice for 10min. The extracts were centrifuged (4°C, 7000 rpm, 30 min), the phenolic (lower) fraction was transferred to a new vial, and 5 mL of extraction buffer was added, shaken, and placed on ice for 10 min. The extracts were centrifuged (4°C, 7000 rpm, 30 min), the phenolic (lower) fraction was transferred to a new vial and 15mL of 0.1M ammonium acetate added. The vials where incubated over night at -20°C. The following day, the extracts were centrifuged (4°C, 7000 rpm, 30 min) and the pellet was dissolved in 15 mL of 0.1M ammonium acetate. The extracts were centrifuged (4°C, 7000 rpm, 30 min), and the pellet was dissolved in 15 mL cold acetone. The extracts were centrifuged (4°C, 7000 rpm, 30 min), the supernatant discarded and the pellet (proteins) was air-dried and kept at -80°C until use. Before use, the samples were dissolved in 300 µL rehydration buffer containing: 9M urea, 3% CHAPS, 0.5% Tris 8.0 and 2% IPG. Protein quantification was preformed according to the Bradford method (Bradford, 1976), using BSA as standard. The experiment was repeated 3 times.

3.10.2. First dimension analysis. Using immobiline drystrip pH3-10 and pH4-7, the first dimension analysis was performed as instructed by the manufacturer (Amerrsham, US), loading 80 µg proteins/strip. The strips were run at: 300 v for 15 min, 500 v for 15 min, 1000 v for 15 min, 1500 v for 15 min, 2000 v for 15 min, 2500 v for 15 min, 3000 v for 15 min and 3500 v for 5 h. The strips were frozen (-80°C) overnight, and the second dimension analysis was carried out the following day.

3.10.3. Second dimension analysis. Equilibration was preformed as instructed by the manufacturer (Amerrsham- GE Healthcare, US). The 12% SDS-PAGE gel was

prepared and loaded with the strip as described in Gharahdaghi *et al.* (1999) and run at 100 v for 8 hours. The gels were stained by equilibration with G-250 Coomassie blue, according to Anderson *et al*. (1991), for at least 4 days. The gels were compared using the Z3 program ver. 2.0 (Compugen, Israel).

3.10.4. Protein identification. Differential protein spots were cut from the gels and sent to the Weizmann institute for analysis using the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) system and mass spectrometry (MS). The results were compared to the protein database in NCBI (National Center for Biotechnology Information).

3.11. Development of virulence and protection assays for hypovirulent *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* plants in MS plates. *A. thaliana* wild-type seeds (provided courtesy of Liora Meiri, Tel-Aviv University) were disinfected as described in section 3.5, and sown in excess at the perimeter of MS plates. The plates were sealed with parafilm and kept overnight at 4°C. The parafilm was then perforated in four places around the plates and the plants were grown at 25°C, 12:12 hour dark:light in the greenhouse. Seven days later the plants were thinned out to leave 20 plants/plate and sealed with perforated parafilm. Ten days later, 4 squares ($4\times4mm^2$) of *Rhizoctonia* isolate culture on YDAcm (as described in section 3.3.1) were placed at a distance of 1 cm from the plantlets to the center of the plate, for the virulence assay. For the protection assay, the hypovirulent isolates were first placed at the center of the plates, as described in the virulence test, and after 48 hours the pathogen culture was challenge inoculated at the center of each plate with one YDAcm square ($4\times4mm^2$). Plant survival was

monitored during 14 days from inoculation with the hypovirulent isolates. Day 8 after hypovirulent-inoculation was found to be the optimal time to evaluate plant survival of the subsequent virulence and protection experiments. Results of the protection test were calculated according to the protection rate formula described in section (3.7). The virulence and protection experiments were repeated 3 times, 6 plates/repeat.

3.12. The defense-related *Arabidopsis thaliana* mutants.

Table 4. The *Arabidopsis thaliana* mutants used in the present study.

TAIR affiliation	**Mutant name**	**Defected gene/locus**	**Origin**
CS3726	*npr1-1*	*Npr1*/AT1G64280.1	Cao *et al.* 1997.
CS3801	*npr1-2*		
NK[a]	*ndr1-1*	*Ndr1*/AT3G20600.1	Century *et al.* 1997.
CS6355	*npr1-2* + *ndr1-1*	Npr1/AT1G64280.1 *Ndr1*/AT3G20600.1	Zhang and Shapiro, 2002.
CS6571	*cim6*	*Cim6*/1005840138	Maleck *et al.* 2002
SALK_025198c		*WRKY70*/ AT3G56400	Bari and Jones, 2009
NK	*snc1*	*RPP5*/AT4G16890.1	Li *et al*. 2001.
NK	*pbs3*	*Pbs3*/AT5G13320	Warren *et al.* 1999.
NK	*pbs1-2*	*Pbs1*/AT5G13160.1	

[a] - Not Known. Mutants who were received courtesy of researchers listed in the correlated manuscript versus the rest of the mutants which were from TAIR (the *Arabidopsis* information resource).

3.13. Exogenic induction of SAR or ISR. *A. thaliana* plants were grown, as described in section 3.5, for seventeen days before inoculation.

3.13.1. SAR induction: Control – two 5 µL drops of sterile water were placed on the leaves of each plant, Bion – two drops of 5 µL, 1% (v/w) Bion® (Acibenzolar-S-methyl) (Syngenta, Switzerland) were added on the leaves. RS13 – Plants treated as the control and 48 hours later infected with the pathogen RS13 as described in the *A. thaliana* protection assay. Bion+RS13 – Plants treated with Bion and 48 hours later infected with the pathogen RS13 as described in the *A. thaliana* protection assay. The plates were sealed and the plants incubated at 25°C, 12:12 hour dark:light in the greenhouse. After six days plant survival was evaluated and the protection rate was calculated. The experiment was repeated 3 times, 6 plates/repeat.

3.13.2. ISR induction: a propylene disk (8mm in diameter) was placed at the center of the plate. Five mm diameter paper disks were placed on top of the propylene disk and was saturated with: Control - 20µL of ethanol 20%, meJA - 20µL of 2% meJA (Duchefa Biochemies, The Netherlands) in 20% ethanol. The RS13 treatment - prepared as the control and 48 hours after adding the paper disks, the plants were challenge inoculated with the pathogen RS13 by placing an inoculated YDAcm square at the center of the plate. MeJA+RS13 treatment - prepared as the meJA treatment and 48 hours after adding the paper disks, the plants were infected with the pathogen RS13 by placing an

inoculated YDAcm square at the center of the plate. The plates were sealed and the plants grown at 25°C, 12:12 hour dark:light in the greenhouse. After six days plant survival was evaluated and plant protection rate was calculated. The experiment was repeated 3 times, 6 plates/repeat.

3.14. Evaluation of defense-related gene expression in *Arabidopsis thaliana*.

3.14.1. Plant growth and inoculation. *A. thaliana* plants were grown as described in the *A. thaliana* virulence assay (10 plants/plate, 12 plates/treatment, each experiment carried out twice). YDAcm plugs of the hypovirulent isolate cultures were placed adjacent to each plant (for control plants non-inoculated YDAcm plugs). The plants were incubated at 25°C, 12:12 hour dark:light for 48 hours, harvested and immediately frozen in liquid nitrogen. The plants were kept frozen at -80°C until RNA extraction.

3.14.2. RNA extraction and cDNA synthesis. The plants were finely ground with a mortar and pestle, while liquid nitrogen was added to keep the tissue frozen throughout the process. RNA extraction was carried out using Tri Reagent ™ (Sigma, USA), including the modifications for RT-PCR, as specified in the manufacturer's instructions. The resulting RNA was inspected on 1.5% agarose gel to confirm that the RNA had not been degraded. If the RNA was of a high-quality, the TURBO DNA-free™ kit (Applied Biosystems, USA) was used to clean the RNA from DNA traces. cDNA was prepared from 1 µg of clean RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) with a primer of oligo-$dT_{(17)}$. The cDNA was stored at -80°C.

3.14.3. Quantitative real-time PCR (qRT-PCR). The cDNA was diluted to calculate calibration curves for each primer. The reaction volumes were 15 µL,

containing: 4 μL cDNA, 2 μL 0.03 mM sense and antisence primers and ×1 SYBR® Premix Ex Taq™ II Mix, (Takara, Japan). Real-time PCR amplification was performed in a Rotor-Gene 3000 machine (Corbett Research, Australia).

3.15.4. qRT-PCR results analysis. Relative quantification of the examined genes' expression was analyzed using REST-2005© program, which is based on the mean CP deviation of control and sample group, normalized by a reference gene (β-Tubulin) (Pfaffl *et al.*, 2002). This method is also based on efficiency corrected calculation of qRT-PCR amplification. The Pair Wise Fixed Reallocation Randomization Test© (Pfaffl *et al.*, 2002) was used to analyze the mean of at least five technical and two independent biological repeats, detecting the relative quantification, and the level of significance. The qRT-PCR experiments for each treatment consisted of triplicates/5 technical repeats/2 biological repeats.

3.15.5. Reference gene and conditions. The reference gene for all experiments was *A. thaliana* β-tubulin. For evaluation of difference in genes' expression due to colonization by the hypovirulent *Rhizoctonia* isolates, the reference condition was non-inoculated control plants. For evaluation of difference in genes' expression due to mutation in defense-related genes, the reference condition was wt plants.

3.15.4. The genes and primers used in the qRT-PCR analysis.

Table 5. The primers used in the qRT-PCR analysis and the genes they represent.

Primer[a]			Gene	
Name	**Sequence**	**Tm**	**Name**	**NCBI affiliation of mRNA**
Tub9-R[b]	TTTCGGTCTTCCCATCTC	60.1	tubulin 9	NM_118207.2
Tub9-L[c]	ATACATTCATCAGCATTCTCAAC	61.1		
Pad4-3R	CTTATCCTCCGATGAACCTCTAC	63.6	phytoalexin-deficient 4 protein	AF188329
Pad4-3L	ACCTAACAATTCCAATTCCAATCC	63.5		
Pr5-2R	GTAACGGCGGCGGAGTTC	67.6	Pathogenesis-related gene 5	NM_106161
Pr5-2L	TTGTAACCATCTACGAGGCTCAC	66.2		
Cori3-R	ACTGGTTGGCTCACGCTAC	66.5	Coronatine induced 1	NM_001036628
Cori3-L	TCGGAGGGTTATTGTTTATCTGGAG	66.1		
Lox1-R	GACTATGCTTACTACAATGATTTAG	59.4	Lipoxygenase 1	NM_104376.2
Lox1-L	CGGTTCTTCCTCTTCTTG	58.8		
Lox2-R	AGTGAAGTGCGGAACATAGG	63.9	Lipoxygenase 2	NC_003074
Lox2-L	CAATCGTAGTTACCACACCAATC	63.4		
Pdf1.2-R	CACATACATCTATACATTGAAAAC	57.9	Plant defensin 1.2A	NM_123809.3
Pdf1.2-L	CAGCAAAGAGAACAAGAG	57		
Pad3-R	CGTGGTCAAGGAGACATTAAGG	64.8	Phytoalexin deficient 3	NM_113595
Pad3-L	CGCAGGAACATCGTAGCC	64.7		
Ndr1-3R	CTATCAAGGACACAAGAAGAAG	60.3	non-race specific disease resistance protein1	BT002004.1
Ndr1-3L	AACAGCCGATCCATTAGG	60.6		

[a] – All amplicons were 100 bases long.

[b] – Primer name ended with an R is a sense primer.

[c] - Primer name ended with an L is an anti-sense primer.

3.16. Statistical analyzes. Apart from the quantitative real-time PCR results all of the statistical analyzes were performed using the JMP IN program, version 5.0.1a (SAS Institute, US). The results were transformed, if necessary, according to statistical standards.

4. Results

4.1. Evaluation of virulence of *Rhizoctonia* spp. isolates on various plant species. Hypovirulent isolates may be mildly virulent on some plant species. Results summarized in Fig. 1 indicate that all of the hypovirulent isolates examined were considerably less virulent (67-100% survival of all of the tested plant species) than the pathogenic isolate RS13 (only 0-3% survival of most of the tested plant species, with 53-73% survival among the Poaceae family members, indicating that this pathogenic isolate was less virulent on these plants). There were some differences in virulence of the hypovirulent isolates on the same plants: Ru56-8 was mildly virulent on some of the plant species (survival rates of lettuce, carrot, tomato, and radish were 89, 72, 89 and 67%, respectively) similar to the hypovirulent strain Ru18-1 (survival rates of carrot, tomato, and cotton were 84, 87, and 84%, respectively). Apart from these cases, survival rate of plants colonized by the different hypovirulent isolates was not significantly different (α=0.05) from that of the non-inoculated control plants. The survival rates of wheat, corn and rice (Poaceae) inoculated with the hypovirulent isolates were high and not significantly different from the respective non-inoculated controls and, as mentioned, the pathogenic isolate (RS13) used was less virulent on these plants than on the other tested plant species (Fig. 1). In cucumber plants, the survival rate of plants inoculated with the hypovirulent isolates was high and not significantly different from the non-inoculated control plants, and the mortality of the plants inoculated with the pathogenic isolate (RS13) was very high. Considering these results and the convenience of growing cucumber plants (rapid growing, rigid and erect hypocotyl), cucumber plants, pre-

inoculated in soil with the hypovirulent isolates were used in the subsequent protection experiments, where either the roots or the hypocotyls were challenge inoculated with the pathogenic isolate.

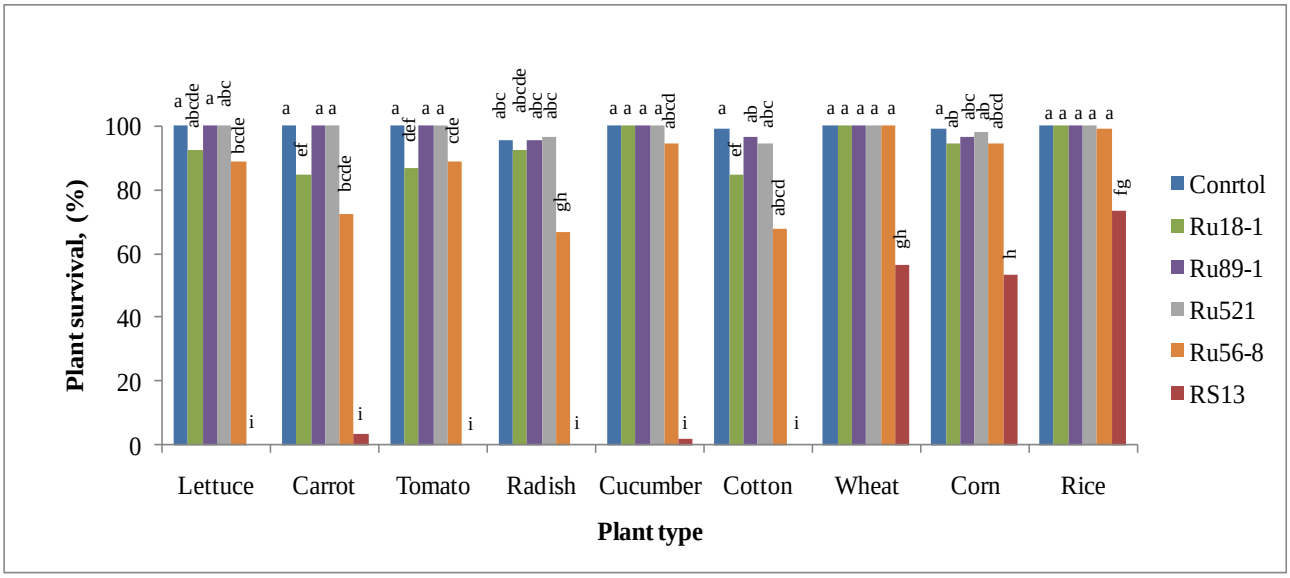

Fig. 1. Virulence of *Rhizoctonia* spp. isolates on various plant species. Survival rate of plants inoculated with hypovirulent isolates (Ru18-1, Ru89-1, Ru521 and Ru56-8), pathogenic isolate RS13 or non-inoculated control. The plants were inoculated with the isolates at planting time of the germinating seeds and the survival rate was recorded one month later.

- Columns sharing common letters are not significantly different (Two way ANOVA followed by Tukey-HSD, α=0.05).

4.2. Protection of cucumber plants by hypovirulent *Rhizoctonia* spp. isolates against pathogenic *R. solani* – effect of pre-inoculation time with the hypovirulent isolates and challenge inoculation site. Cucumber plants were inoculated with hypovirulent *Rhizoctonia* spp. isolates in soil and challenge inoculated with the pathogen

at consecutive time intervals either in soil, or on the hypocotyl. In order to evaluate the possibility that a longer pre-colonization with the hypovirulent isolates may provide better protection against the pathogen, cucumber plants were inoculated with hypovirulent isolates in the soil, and thereafter were challenge inoculated one to six days later with the pathogenic *Rhizoctonia* isolate. Results summarized in Fig. 2I, indicate that when both the hypovirulent isolates and the challenging pathogen were inoculated in soil, all of the hypovirulent isolates protected the plants from the first day and there was rarely a statistically significant difference in plant protection when the hypovirulent isolates had a longer pre-colonization period before challenge inoculation with the pathogen. Plants colonized with Ru18-1 were better protected when challenge inoculated after 3-5 days than on the first 1-2 days. This may indicate that longer pre-colonization with this isolate enabled enhanced induction of resistance in the plants. Even though there was a decrease in protection of plants inoculated for 2 days with Ru89-1 and plants inoculated for 3 days with Ru56-8, this did not represent a trend.

When the plants were inoculated only with the pathogen at increasing time intervals (from 11 to 17 days old plants) there was no significant difference in plant mortality, indicating that plants at these different ages were not more susceptible to the pathogen. When the hypovirulent isolates were inoculated in soil and the pathogen was challenge inoculated on the hypocotyl (Fig. 2II, Fig. 3), the hypovirulent isolates did not protect the plants at all from the first day following inoculation, and a high percentage of plants infected solely by RS13 survived (55%). On the second day, only Ru18-1 and Ru56-8 protected the plants, on the third day – Ru56-8, Ru521 and Ru18-1, on the fourth and fifth days only Ru89-1 and on the sixth day – Ru56-8 and Ru18-1. When a healthy-

looking 48 hour-old infection site of RS13 on a cucumber hypocotyl was stained and inspected microscopically (Fig. 4A) the hyphae appeared long and narrow, branching out sporadically and there was no visible hyphal penetration of the isolate into the plant tissue. When a diseased infection site (exhibiting water stains and tanning) was inspected (Fig. 4B) the RS13 hyphae were short and swollen, some of which penetrated the plant's tissue. A similar phenomenon was observed in a diseased infection site of RS13 on plants which were colonized 48 hours earlier with the hypovirulent isolate Ru18-1(Fig. 4C).

Overall, there was no clear trend of significant increased protection in cucumber neither for the consecutive time periods of colonization by the hypovirulent isolates nor by the different hypovirulent isolates.

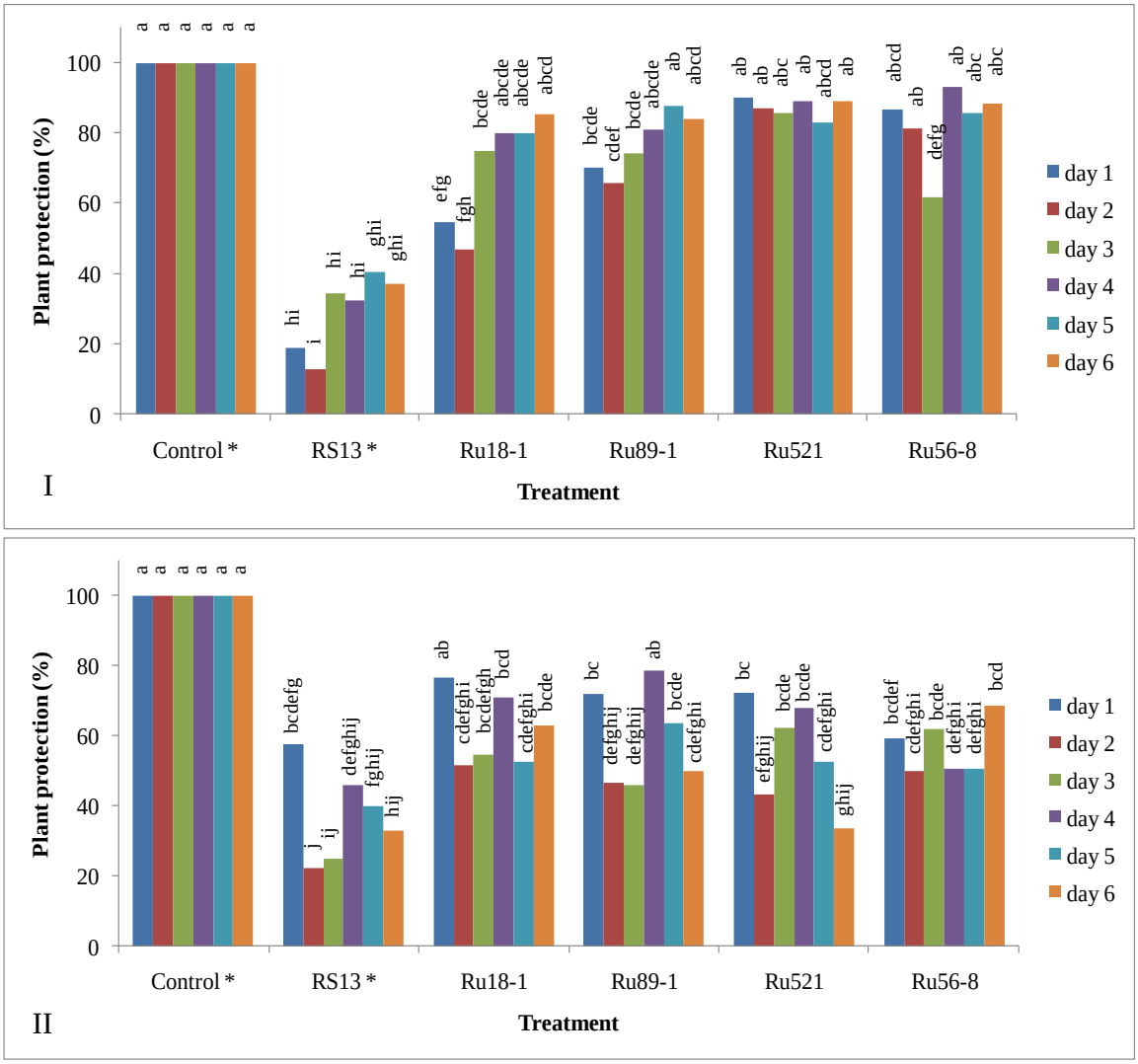

Fig. 2. Protection of cucumber plants by hypovirulent *Rhizoctonia* spp. isolates (Ru18-1, Ru89-1, Ru521, and Ru56-8) against challenge inoculation with the pathogenic isolate RS13. The hypovirulent isolates were applied in soil at planting time of the germinating seeds, while the pathogen was applied at increasing time intervals after the hypovirulent isolate: (I) In soil, (II) On the hypocotyl. Plant protection was calculated according to the following formula: plant protection (%) =100*(A-B)/(C-B), where A is the percentage of surviving plants after colonization with the tested hypovirulent isolate and challenge inoculation with the pathogen; B is the percentage of surviving plants after

inoculation only with the pathogen, and C is the percentage of the surviving non-inoculated plants.

* Results for control plants and Rs13-infected plants are of the plants survival rates.

- There was no mortality in the non-inoculated control at all of the tested time periods (There is a control for each time period since the plants grew a day older as the time interval between hypovirulent colonization and challenge inoculation were a day longer).
- P values for the tests: (I) between treatments = 0.00, between times = 0.00, combined = 0.00. (II) Between treatments =0.00, between times =0.00, combined =0.00.
- Columns sharing common letters are not significantly different (Two way ANOVA followed by Tukey-HSD, α=0.05).

Fig. 3. Protection of plants inoculated by hypovirulent *Rhizoctonia* spp. isolates in soil and challenge inoculated with the pathogen on the plant hypocotyl. (A) non-inoculated control, (B) Plants infected with the pathogen RS13 on the hypocotyl, (C) Plants inoculated with the hypovirulent isolate Ru18-1 in soil and 7 days later challenge inoculated with the pathogen RS13 on the hypocotyl (protected).

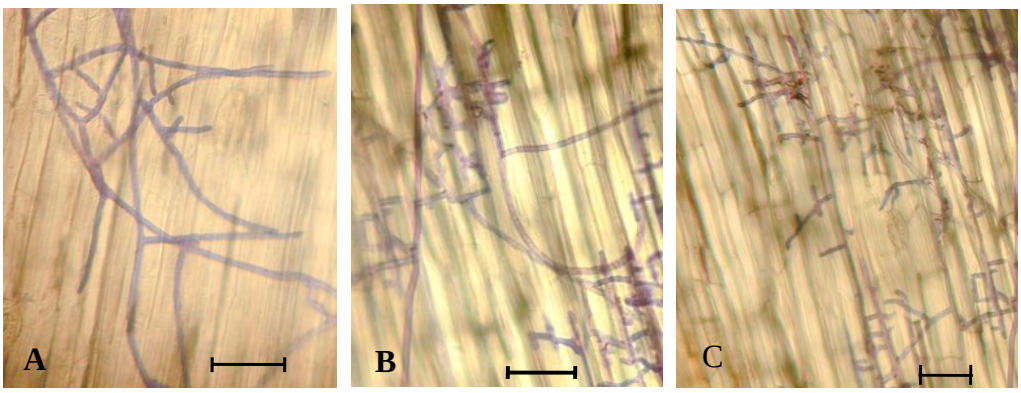

Fig. 4. Hyphae of the pathogenic *Rhizoctonia solani* isolate RS13, 48 hours after challenge inoculation on cucumber hypocotyls: (A) healthy-looking infection site of RS13, protected by prior (48 hours) root colonization with the hypovirulent isolate Ru18-1, (B) diseased infection site of RS13 (C) diseased infection site of RS13, protected by prior (48 hours) root colonization with the hypovirulent isolate Ru18-1. Bars represent 400µm.

- The samples were stained with 0.1% (w/v) trypan blue in 10% (v/v) acetic acid. (A) and (B) were viewed at ×200, (C) at ×100 magnification.

The data summarized in Fig. 2I represents an experimental system where the hypovirulent and the pathogenic isolates were inoculated at the same site (soil). Therefore, the protection provided by the different hypovirulent isolates on these plants may be a consequence of one or more of the following modes of action: local competition for space and/or infection sites, or local and systemic resistances. The data summarized in Fig. 2II represents an experimental system where the hypovirulent isolates were applied in soil and the pathogen was applied on the hypocotyl. Therefore, the protection provided by the different hypovirulent isolates on these plants may be a consequence of only induced systemic resistance. Since the plants were grown in a greenhouse, exposed to

different microorganisms through air, water and soil results of some treatments (plants inoculated with Ru89-1, for example) were not always consistent, and therefore it was not possible to determine a general trend of protection over time for the hypovirulent isolates. Results summarized in Table 6 compare the protection provided by the hypovirulent isolates when the pathogen was challenge inoculated in soil with the hypovirulent isolate when the pathogen was applied on the hypocotyl, for each day. Except from day 1 after the pathogen application, there was no significant difference in survival rates of plants inoculated in soil or on the hypocotyl – only with the pathogen. When the pathogen was applied one or two days after the hypovirulent isolate, Ru56-8 protected the soil-challenge inoculated plants better than the plants challenge inoculated on the hypocotyl at both times (59 and 50% plant survival on day 1 and 2, respectively). When the incubation period of the hypovirulent isolates before pathogen challenge was 3 days, isolates Ru89-1 and Ru521 protected the plants better via soil-challenge inoculation compared with challenge inoculation on the hypocotyl (Table 6, Fig. 2). When the incubation period of the hypovirulent isolates before pathogen challenge was 4 days, isolates Ru521 and Ru56-8 protected the plants better via soil-challenge inoculation compared with challenge inoculation on the hypocotyl (Table 6, Fig. 2). Incubation periods of 5-6 days with the hypovirulent isolates before pathogen challenge resulted in high protection of plants challenged in soil (80-88 and 84-89% plant protection, respectively) than challenge-inoculation on the hypocotyl (51-64 and 34-69% plant protection, respectively).

Table 6. Differences in protection between soil- and hypocotyl-challenge of cucumber seedling by different hypovirulent *Rhizoctonia* isolates.

Isolates	Time period of inoculation with hypovirulent isolates before challenge inoculation with RS13, (days)											
	1		2		3		4		5		6	
	S[b]	H[c]	S	H	S	H	S	H	S	H	S	H
RS13	f	de	f	ef	ef	f	e	e	d	d	d	d
RU18-1[a]	e	bcd	cd	cd	bc	cde	bc	c	ab	cd	ab	c
RU89-1[a]	cde	bcde	bc	cd	bc	def	bc	bc	a	bc	ab	cd
Rs521[a]	ab	bcde	de	ab	ab	cd	ab	cd	a	cd	a	d
RU56-8[a]	abc	de	ab	cd	cd	bcd	ab	de	a	cd	a	bc
Control[d]	a	a	a	a	a	a	a	a	a	a	a	a

- Statistical significance (Two-way ANOVA followed by Tukey-HSD, α=0.05) of the protection data obtained from cucumber seedling inoculated in soil by hypovirulent *Rhizoctonia* spp. isolates and challenge inoculated with the pathogen RS13 either in soil or on the hypocotyl 1 to 6 days after the hypovirulent isolates. Each day was analyzed separately.

[a] The hypovirulent isolates (Ru18-1, Ru89-1, Ru521 and Ru56-8) were applied in soil after the cucumber plantlets emerged.

[b] S – The pathogenic isolate RS13 was challenge inoculated in soil.

[c] H - The pathogenic isolate RS13 was challenge inoculated on the hypocotyl.

[d] Control – non-inoculated plants

4.3. Induction of proteins in cucumber plants colonized with protective hypovirulent *Rhizoctonia* spp. isolates, which may be involved in induced resistance.

In addition to protection against the pathogen of plant organs colonized by the protective hypovirulent *Rhizoctonia* spp., isolates were shown to also protect plant organs at a distance from the colonized sites, indicating that these isolates induce systemic resistance

by triggering protein synthesis involved in the mechanism(s) of induced resistance cascades. In order to identify such differentially expressed proteins, which may be involved in the protection processes, proteins were extracted from cucumber plants colonized with hypovirulent or pathogenic isolates. Since all of the hypovirulent *Rhizoctonia* isolates tested were found to be protective, a non-protecting hypovirulent control isolate could not been used. Therefore, non-treated plants were used as controls. The proteins were separated on two dimensions protein gels at a 3-10 pH range and compared. Since there was a mass of proteins in the central areas of these 3-10 pH gels they could not be distinguished individually, therefore, gels with pH range of 4-7 pH were used (Fig. 5) to achieve a better separation of proteins (inevitably at the expense of the external margins). Results summarized in Table 7 show 17 differentially expressed proteins (designated MC and a consecutive number) in plants colonized with the protective hypovirulent isolates Ru18-1, Ru56-8, and the pathogen RS13. Three of the proteins were up-regulated and seven were down-regulated in the hypovirulent-inoculated plants compared to non-colonized ones. The remaining seven differentially expressed proteins had no distinguishable trend compared to the non-inoculated control. Out of the 17 proteins sampled, 12 were identified.

The identified proteins were found to be involved in either general metabolic processes and/or in stress and defense responses. The former group included the proteins MC1, MC3, MC4(2), MC8, MC9, MC10, MC13(1) and MC16 and the latter group: MC8, MC10 (which were also included in the former group), MC5-7 and MS13(2).

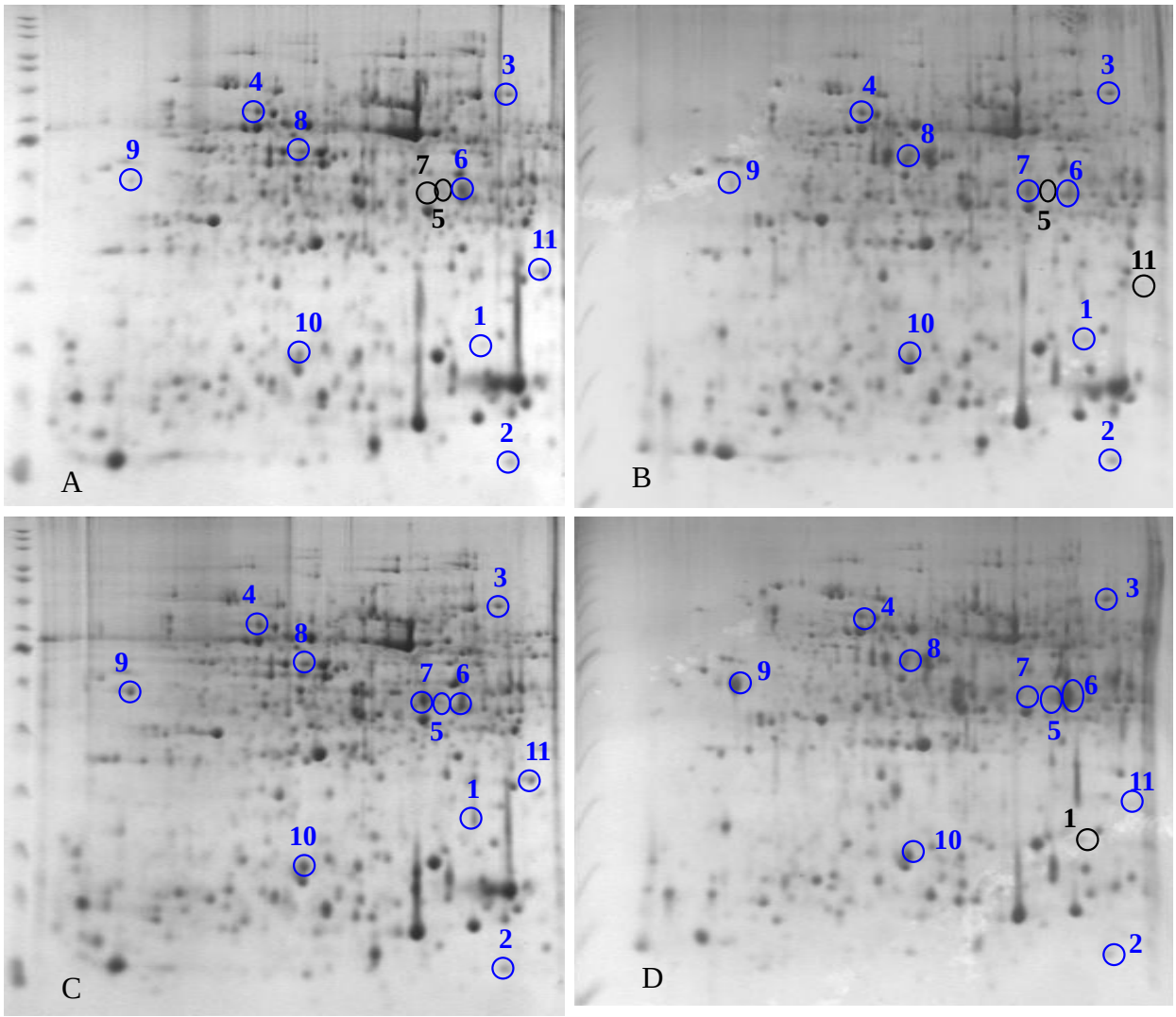

Fig. 5. Two dimension (4-7 pH range) protein gels of proteins extracted from plants inoculated with the hypovirulent *Rhizoctonia* isolates (A) Ru18-1, (B) Ru56-8 ,(C) the pathogen RS13, and (D) non-treated plants. The gels possessed 224, 240, 258 and 217 spots, respectively. Up- and down-regulated proteins [according to the Z3 program from Compugen, Tel-Aviv, Israel (Smilansky, 2001)] were circled in blue, absent spots in black.

Table 7. Differentially expressed proteins isolated from two dimension gels and identified according to the MS/MS procedure.

Spot No.	PI[c]	Treatment				Match (%)	Gi no.[d]	Known protein name[e]
		Ru18-1	Ru56-8	Control	RS13			
MC1[a]	6.5	+	+	0	+	20	77416955	GTP-binding protein SAR1
MC2	6.9	+	++	+	++	-		No known protein
MC3	6.8	+	++	+	++	36	7528270	Poly-A binding protein
MC4	5.2	++	++	+	++	35	806808	Chaperonin precursor
						26	18831	Mitochondrial ATP synthase β-subunit
MC5	6.2	0	0	+	+	25	38373428	Netting associated peroxidase
MC6	6.5	++	++	+++	++	25	38373428	Netting associated peroxidase
MC7	6.3	0	++	+	++	35	38373428	Netting associated peroxidase
MC8	5.5	+	+	++	++	25	13540316	S-adenosyl-L-methinine synthetase
MC9	4.5	++	+	+++	+++	15	18072799	Glyceraldehyde 3-phosphate dehydrogenase
MC10	5.5	++	++	+++	+++	31	19702	Eukaryotic initiation factor 5A
MC11	6.9	+	0	+	++	-		No known protein
MC12	4.4	+	ND[b]	+	++	-		No known protein
MC13	9.0	++	ND	++	+	37	15077030	Tasselseed2-like protein
						20	6469139	Malate dehydrogenase
MC14	7.4	++	ND	+	++	-		No known protein
MC15	7.1	+	ND	++	+	30	7528270	Poly-A binding protein
MC16	8	+	ND	++	+	15	50878379	Putative Fructose bisphosphate aldolase
MC17	6.3	+	ND	-	-	21	22137284	At1g09630/F21M12-2

[a] Spots MC1-11 were sampled from the 4-6 pH range gels, spots MC12-17 from 3-7 pH range gels.

[b] ND – not determined - no 3-7 pH gels were available for plants inoculated with Ru56-8.

[c] PI- isoelectric point of the protein (the pH value where the protein sum of charges is 0).

[d] Gi- the protein sequence version number in the ncbi protein data base.

[e] Protein name as identified by comparing fragments of the protein sequence to the NCBI protein data base.

- The proteins were extracted from cucumber plants colonized with the hypovirulent *Rhizoctonia* isolates Ru18-1, Ru56-8, the pathogen RS13 and non-treated plants, and separated on two-dimension gels presented in Fig 7.

Despite many attempts to repeat the two-dimension gel analyses of the protein extracts from the plants, the clear results of Fig. 5 and Table 7 could not be repeated. Therefore, the proteomics approach of the present research work could not be pursued and a molecular genetic approach was adopted in order to continue the study on induced resistance mechanisms of plants protected by hypovirulent *Rhizoctonia* isolates and challenged with the pathogenic *Rhizoctonia* RS13.

4.4. *In vitro* hyphal growth inhibition of the pathogenic isolate with extracts from radish plants colonized by hypovirulent *Rhizoctonia* spp. isolates. Since root-colonization by the hypovirulent isolates protected against challenge inoculation on the hypocotyl, the possibility of induced production of inhibitory secondary compounds (such as phytoalexins) was evaluated in radish seedling extracts. Results summarized in Fig. 6 indicate that the various parts of radish plants colonized by isolate Ru56-8

significantly inhibited hyphal growth of the pathogen. There was no difference in inhibition when extracts from different plant parts were used. Inhibition of the RS13 isolate by extracts from plants colonized with the hypovirulent isolates Ru18-1, Ru89-1 or Ru521 were not significantly reduced compared to inhibition by the extracts from the non-colonized plants.

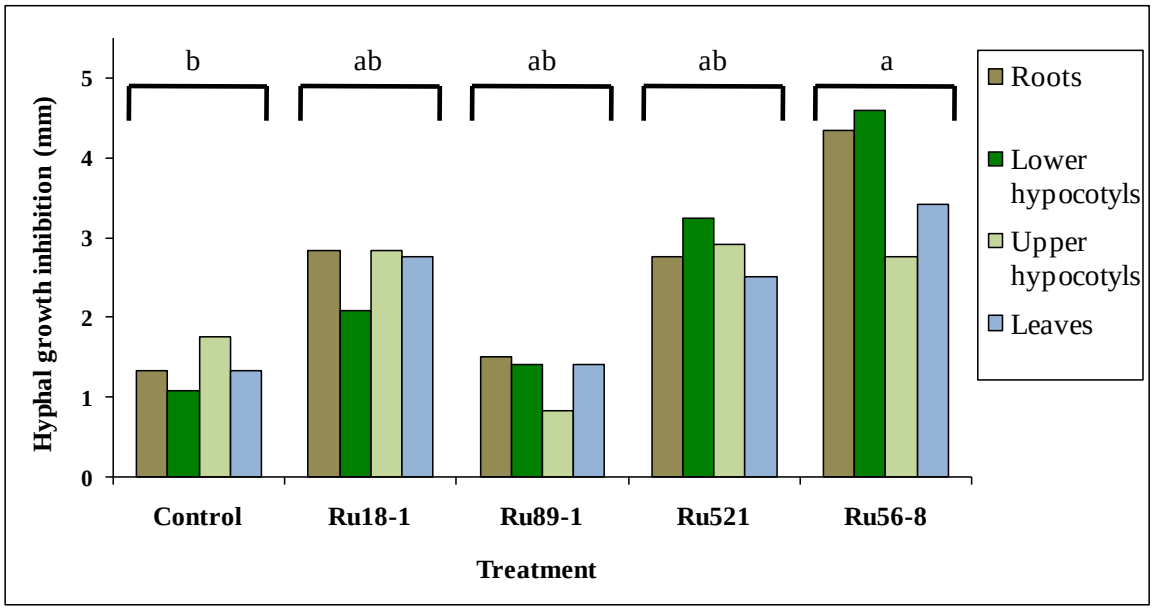

Fig. 6. Hyphal growth inhibition of the pathogenic *Rhizoctonia solani* (RS13) by extracts (water:ethanol:acetone 1:2:2) of sections from radish plants colonized with the hypovirulent isolates (Ru18-1, Ru89-1, Ru521 and Ru56-8) on paper disks.

- Differences in hyphal growth were calculated as the growth of the RS13 isolate opposite the saturated paper disk, subtracted from the growth of the same RS13 colony at a 45° angle from the first measurement.

- Control represents extracts from non-inoculated plants.

- There were no extracts from RS13-inoculated plants since this pathogenic isolate killed most of the plants.

- Groups sharing common letters are not significantly different (Two way ANOVA followed by Tukey-HSD, α=0.05).

4.5. Development of reliable virulence and protection tests for hypovirulent *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* plants in MS plates. *A. thaliana* plants are widely investigated as a research model plant. The plant genome had been mapped, and thus, a significant number of mutants are available for investigators worldwide. Therefore, *A. thaliana* was chosen as the most appropriate candidate for the following part of the research. In order to evaluate the involvement of different plant defense genes in the protection process it was necessary to develop reliable virulence and protection tests for the hypovirulent isolates on *Arabidopsis thaliana* plants. The requirements for these tests were based on: reliability, quantitative generation of data, a possibility to examine a number of different plant genotypes and *Rhizoctonia* spp. isolates, limited time and space, and minimal effect of external factors. Therefore, the *A. thaliana* plants were grown in MS (Murashige and Skoog, 1962) plates. For the virulence tests, 5 mm plugs of *Rhizoctonia* isolates culture on YDAcm were placed on the outer circle of the plates, on the 17th day of plantlet development (Fig 7A). For protection tests (Fig. 7B), 48 hours after the hypovirulent isolates were applied, a RS13-colonized plug of YDAcm was placed at the center of the plate.

4.5.1. Virulence assay. Results summarized in Fig. 7A indicate that mortality of *A. thaliana* plants infected by the pathogenic isolate began on the second day after

inoculation, increased rapidly by the 4th day, and reached more than 97% by day 8. On the contrary, all of the plants colonized by the hypovirulent isolates survived, even after 10 days. The greatest difference in survival rates between the plants colonized by the pathogen and those colonized by the hypovirulent *Rhizoctonia* spp. isolates was from day 8 to 10, even though there were no significant differences in survival between the different hypovirulent isolate-inoculated plants and the pathogen-inoculated plants from day four (Fig. 7B).

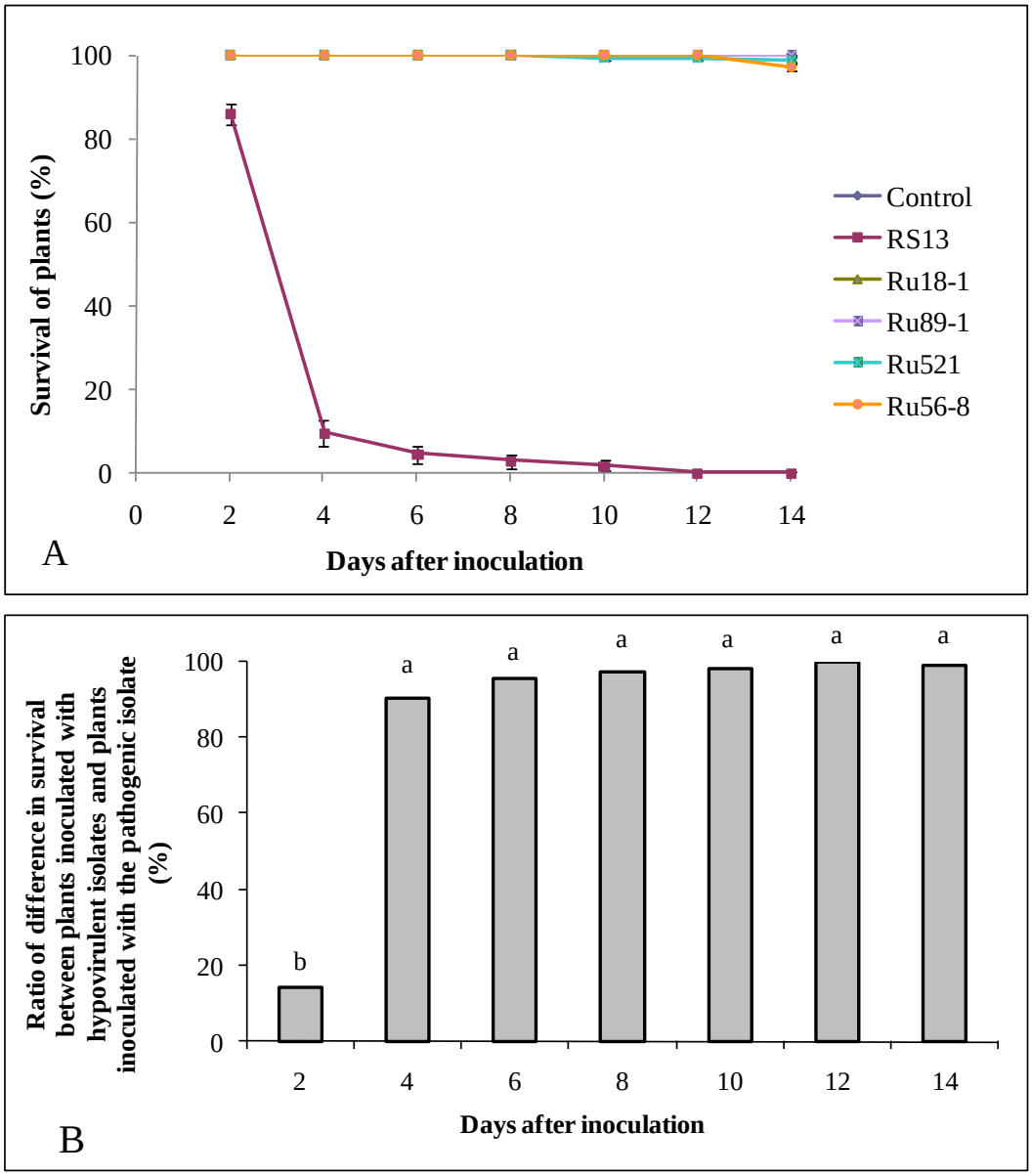

Fig. 7. Development of a virulence test for *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* plants. (A) Survival rate of plants inoculated with *Rhizoctonia* spp. isolates. Control – non-inoculated plants, RS13 – pathogenic isolate, Ru18-1, Ru89-1, Ru521 and Ru56-8 – hypovirulent isolates. The bars represent standard errors of the mean. (B) The differences between survival rates of plants inoculated with the hypovirulent *Rhizoctonia* isolates to plants inoculated with the pathogen RS13, over time.

- Columns sharing common letters are not significantly different (Tukey-HSD test, α=0.05).

4.5.2. The protection assay. Mortality of *A. thaliana* plants colonized by the pathogen alone was observed 2 days following inoculation (Fig. 8A). Some of the plants which were colonized with the hypovirulent isolates prior to challenge inoculation with the pathogen survived for 4-10 days. The highest difference in protection rates between the protected plants and the pathogen-infected control was on day 6 (Tukey-HSD, α=0.05; Fig. 8B and Fig. 9). Therefore, subsequent protection experiments were monitored 6 days after challenge-inoculation with the pathogen.

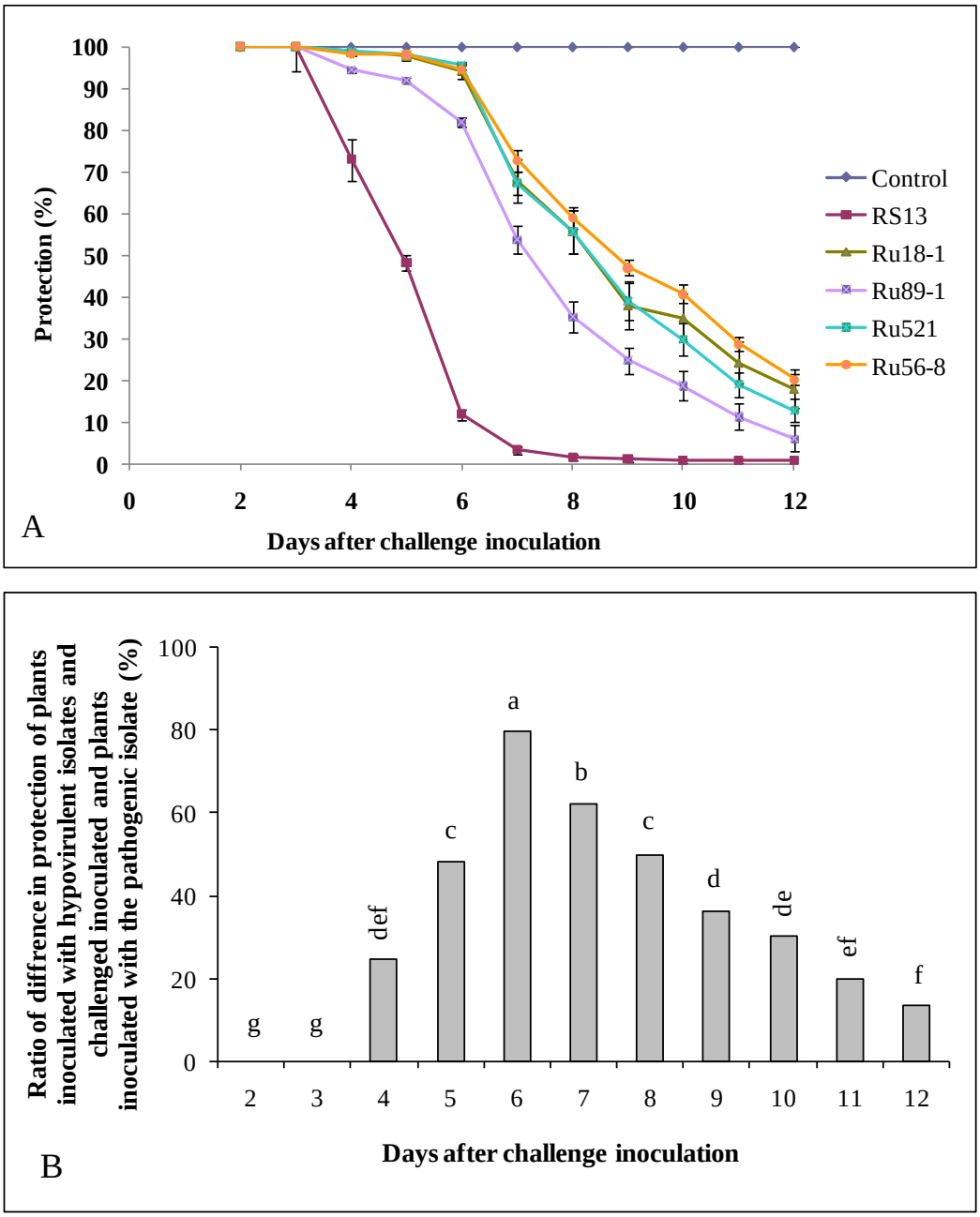

Fig. 8. Development of a protection assay for *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* plants. (A) Protection rate of plants inoculated with *Rhizoctonia* isolates. Control – non-inoculated plants, RS13 – pathogenic isolate alone, Ru18-1, Ru89-1, Ru521 and Ru56-8 – hypovirulent isolates. The plants were challenge-inoculated with the pathogenic isolate 48 hours after inoculation with the

hypovirulent isolates. The bars represent standard errors of the mean. (B) The differences between protection rates of plants inoculated with the pathogenic isolate RS13 to plants inoculated with the hypovirulent *Rhizoctonia* isolates and 48 hours later with RS13, over time.

Ratio of diffrence in protection of plants inoculated with hypovirulent isolates and challenged inoculated and plants inoculated with the pathogenic isolate

- Columns sharing common letters are not significantly different (Tukey-HSD test, α=0.05).

- Protection (%) was calculated as explained previously in Fig. 3.

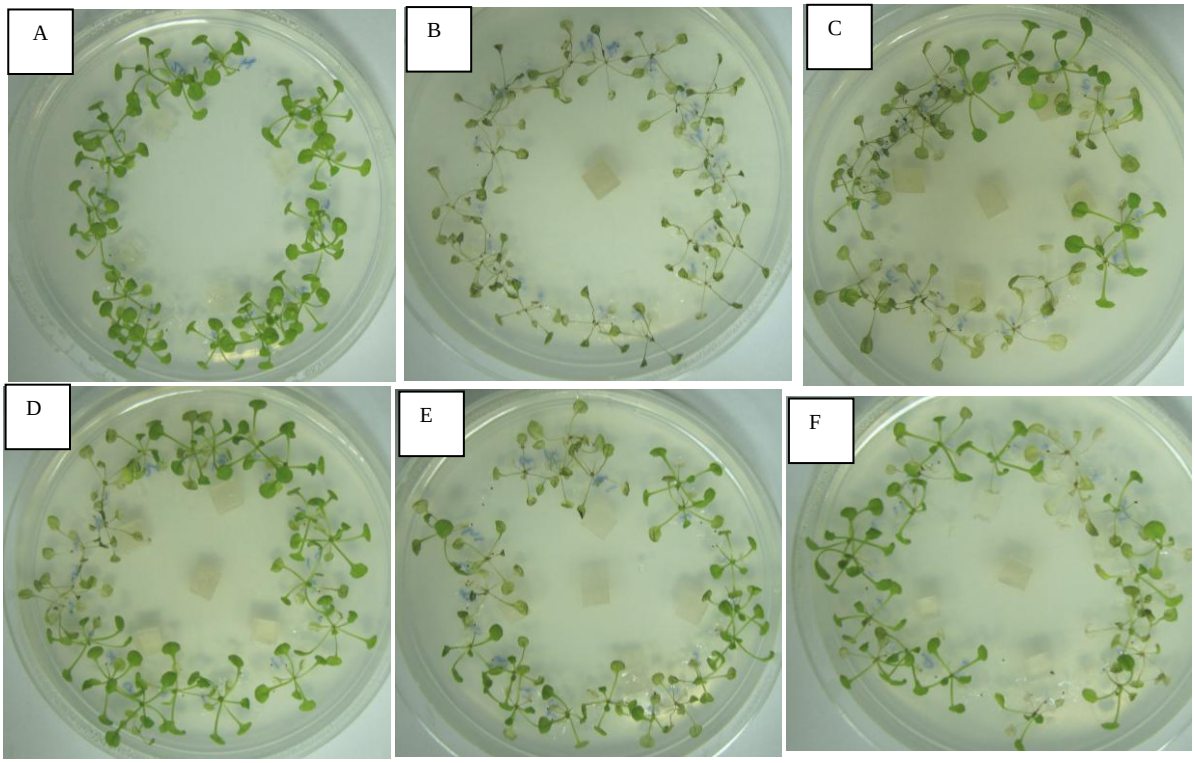

Fig. 9. Protection of *Arabidopsis thaliana* plants inoculated with hypovirulent *Rhizoctonia* spp. isolates against pathogenic *Rhizoctonia* as depicted in Petri plates. (A) Non-inoculated control plants, (B) plants inoculated with the pathogenic

isolate RS13 alone, (C)-(F) plants inoculated with Ru18-1, Ru89-1, Ru521 and Ru56-8, respectively, and challenge-inoculated 48 hours later with the pathogenic isolate (RS13). Six days after challenge inoculation with the pathogenic isolate, plant mortality was evaluated.

4.6. Changes in virulence or protection rates by *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* mutants defected in genes involved in defense pathways compared with virulence and protection of wt plants by the same isolates. In order to identify genes involved in the induced resistance pathways, which were triggered in plants colonized by hypovirulent *Rhizoctonia* spp. isolates, changes in virulence of the isolates and their protection ability of *A. thaliana* mutants, harboring defected defense-related genes, were examined, and compared with the reactions obtained on wt plants.

4.6.1. *npr1* mutants. No difference was observed in virulence between the *Rhizoctonia* isolates on the *npr1-1* mutant compared to that on wt plants (Fig. 10A). While isolate Ru89-1 protected the mutant plants similarly to those of the wt, most of the hypovirulent isolates did not protect the *npr1-1* mutant to the same extent that they protected the wt plants. For example: the decrease in protection by isolates Ru18-1 and Ru56-8 was reduced by 28% (Fig. 10B). For further evaluation of reduced protection of this mutant, RNA was extracted from *npr1-1* plants after inoculation with isolates Ru18-1 or Ru56-8 and used in a quantitative real-time PCR assay to evaluate the changes in expression of selected genes involved in the induced systemic resistance pathways, as summarized in Fig. 13, 14. No increase in virulence of the hypovirulent *Rhizoctonia* isolates on the other *npr1* mutant – *npr1-2* was observed, and while isolates Ru18-1,

Ru521 and Ru56-8 did not protect this mutant compared to the wt plants, there was no significant change in the protection rate by Ru89-1 on *npr1-2*.

4.6.2. *ndr1-1* mutant. There was no change in the virulence of the hypovirulent *Rhizoctonia* spp. isolates on these mutant plants compared to that on the wt plants (Fig. 10A), but there was a significant decrease in the protection (over 60% for Ru18-1 and over 30% for the rest of the tested hypovirulent isolates) (Fig. 10B). For further evaluation of the decreased protection of this mutant, RNA was extracted from *ndr1-1* plants after inoculation with isolates Ru18-1 or Ru56-8 and used in quantitative real-time PCR to evaluate the changes in expression of selected genes involved in the induced systemic resistance pathways. The results are summarized in Fig. 13, 15.

4.6.3. CS6355 mutant. This is a double mutant containing *npr1-2* and *ndr1-1* mutations. The hypovirulent isolate Ru56-8 was slightly virulent on this mutant (causing 8% mortality). Protection of *ndr1-1* plants by all of the hypovirulent isolates was impaired compared to the wt plants. When the protection rates of the CS6355 and *npr1-2* mutant plants were compared, significantly improved protection (α=0.05) was observed for *npr1-2* plants compared to those of CS6355, while Ru56-8 colonization protected both CS6355 and *npr1-2* plants to the same extent. No significant difference was observed between plant survival rates of the CS6355 and *ndr1-1* mutant plants inoculated with the tested hypovirulent isolates, apart from slight increase in protection (8%) by isolate Ru521 of the *ndr1-1* plants compared to protection of CS6355 plants.

4.6.4. CS6571 (*cim6*) mutant. This mutant constitutively expresses genes involved in the SAR pathway, including accumulation of SA, PR-1,5 and Npr1. Isolates Ru521 and

Ru56-8 were slightly virulent on this mutant (6 and 3% mortality, respectively - Fig. 10B), which may be the consequence of impaired fitness due to constitutive SAR expression. The protection of these mutants by the hypovirulent isolates was impaired compared with the wt *A. thaliana* plants (Fig. 10B).

4.6.5. SALK-025198c (*wrky70.1*) mutant. This mutant was not over-susceptible to inoculation by the different *Rhizoctonia* spp. isolates (Fig. 10A) and there was no difference in protection of the wt and mutant plants by any of the tested hypovirulent isolates (Fig. 10B).

4.6.6. *snc1* mutant. This mutant, similar to CS6571, also constitutively expressed genes involved in the SAR pathway and its virulence and protection results resembled those for CS6571: two of the hypovirulent isolates (Ru521 and Ru56-8) were slightly more virulent on the *snc1* mutant than on wt plants (92 and 86% survival, respectively) (Fig. 10A), and all of the *Rhizoctonia* spp. isolates protected these mutant plants at a reduced level than that for the wt (Fig. 10B).

4.6.7. *pbs3-1* mutant. There was no increase in virulence of the isolates on these mutant plants (Fig. 10A), but all of the hypovirulent isolates protected this mutant at a reduced level than that for the wt plants (Fig. 10B).

4.6.8. *pbs1-2* mutant. None of the *Rhizoctonia* spp. isolates were more virulent on these mutant plants than those of the wt (Fig. 10A). The *pbs1-2* plants were protected by the hypovirulent isolates at the same levels of the wt plants, and even better by isolate Ru89-1 (Fig. 10B).

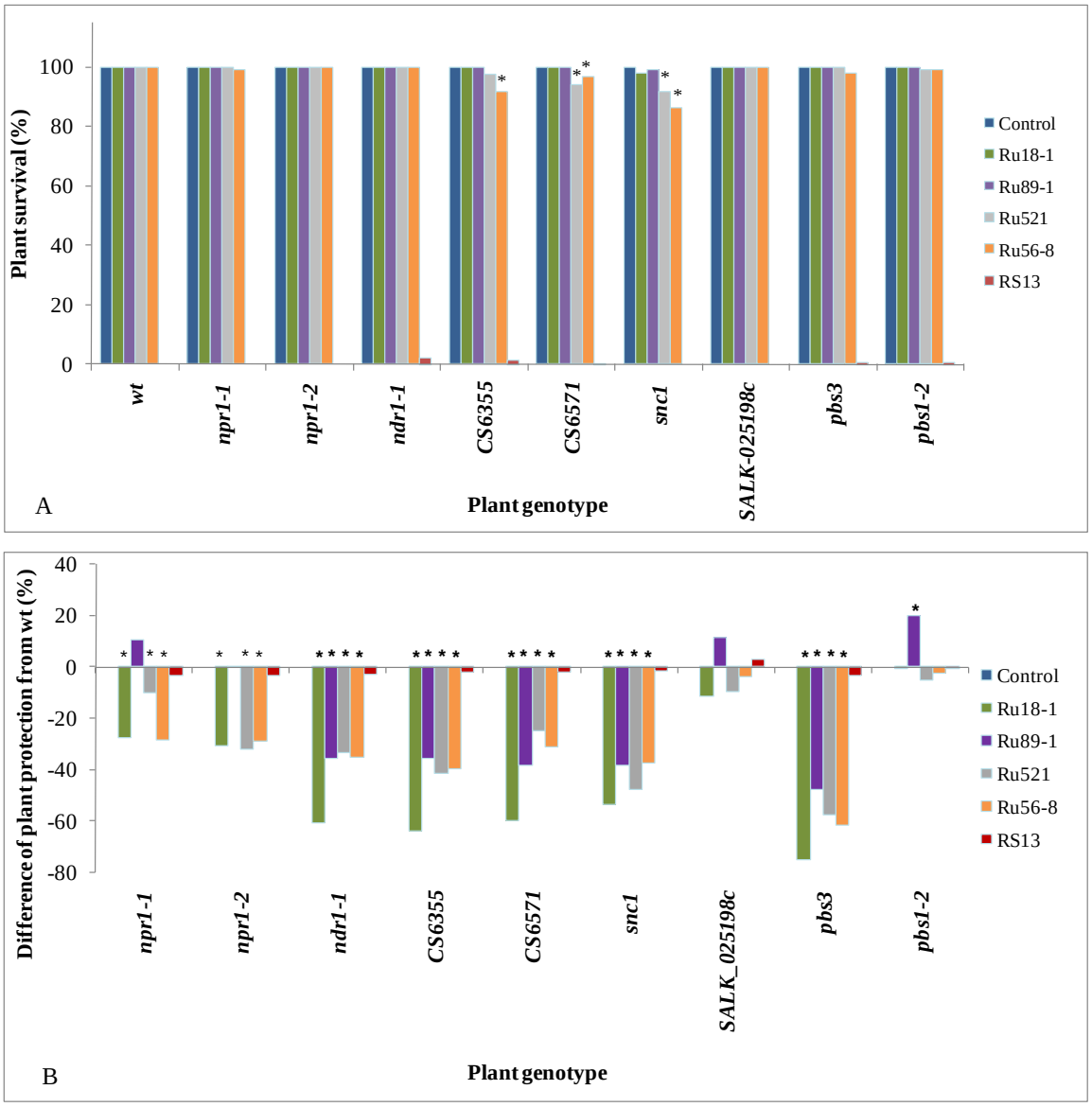

Fig. 10. Virulence (A) and protection (B) assays of *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* mutant plants.

- Control – non-inoculated plants, RS13 – pathogenic *Rhizoctonia* isolate, Ru18-1, Ru89-1, Ru521 and Ru56-8 – hypovirulent *Rhizoctonia* isolates. Columns marked with an asterisk are significantly different from the values of the same treatment on wt plants (Two way ANOVA followed by Tukey-HSD, α=0.05).

- Percent protection was calculated as previously described in Fig. 2.

4.7. Evaluation of the protection levels induced in wt *Arabidopsis thaliana* plants by the exogenic SAR or ISR inducers - Bion and meJA, respectively. Changes in protection of the tested *A. thaliana* mutants indicated the involvement of induced resistance pathways in protection of the plants colonized with the tested hypovirulent isolates. To evaluate the extent that each of the induced resistance pathways contributed to the protection of the wt *A. thaliana* plants against the pathogenic *Rhizoctonia* isolate, Bion was used to trigger the SAR pathway and meJA to trigger the ISR pathway. Results summarized in Fig. 11 (A) and (B) indicate that Bion did not protect the plants against the pathogenic *Rhizoctonia* isolate (Fig. 11A) and meJA provided a very low protection, significantly weaker than the protective hypovirulent isolates (Fig. 11B). It is likely that the high protection levels the hypovirulent isolates induced in plants they colonized are due to a combined series of responses – local resistance - by mechanisms of competition with the pathogenic isolate, induced local resistance in the plants, ROS (reactive oxygen species) production, phytoalexin production, and induced systemic resistance – by both the SAR and ISR pathways.

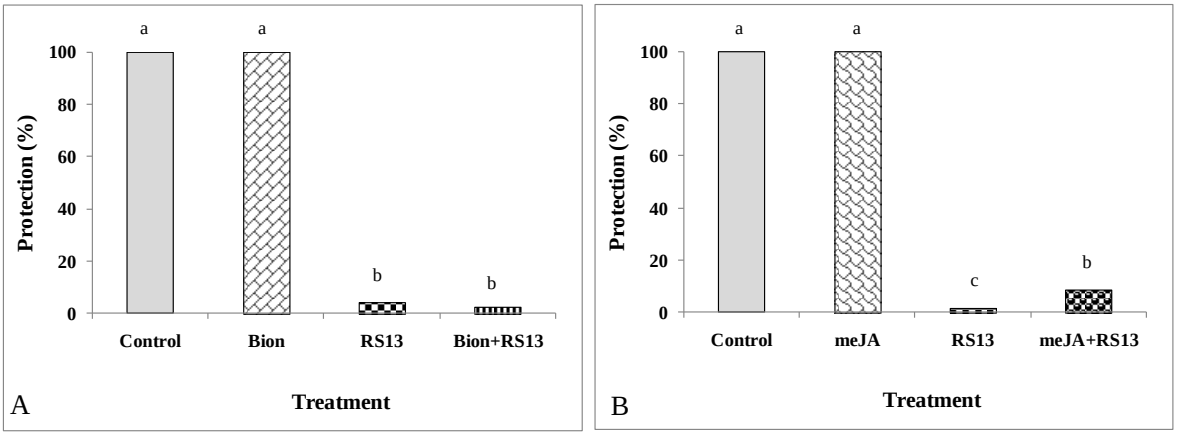

Fig. 11. Protection of *Arabidopsis thaliana* plants by exogenic induction of resistance pathways. (A) Bion treated plants to trigger SAR. Control – sterile water, Bion – 0.5µM Bion. RS13 – Challenge inoculation with the pathogen RS13. **(B) MeJA treated plants to trigger ISR.** Control - ethanol 20%. meJA – 2% meJA in 20% ethanol. RS13 – challenge inoculation of control plants with the pathogen RS13.

The percent protection was calculated as described in Fig. 2.

4.8. Evaluation of defense related gene expression in *Arabidopsis thaliana* induced by colonization with hypovirulent *Rhizoctonia* spp. isolates, using quantitative real-time PCR. To further clarify which mechanisms are involved in the induction of resistance in plants colonized by the protective hypovirulent isolates, a selected number of genes involved in different modes of systemic induced resistance pathways were monitored by using the quantitative real-time PCR technique. The genes tested were induced either via the SAR (*Pad4*, *Pr5*), or the ISR (*Lox1*, *Lox2*, *Cori3*, *Pdf1.2*) pathways, the phytoalexin camalexin production pathway (*Pad3*) or involved in ROS triggering and function of some PR and R proteins (*Ndr1*). The hypovirulent *Rhizoctonia* isolates Ru18-1 and Ru56-8 induced *Pr5* (SAR related), *Lox2* and *Pdf1.2* (ISR) genes in *A. thaliana* plants (Fig. 12), and also induced the *Pad3* gene (phytoalexin pathway). The *Lox1* gene which

is involved in jasmonic acid production and *Cori3* were induced only in plants colonized with hypovirulent isolate Ru56-8. *Pad4* expression increased by a statistically insignificant level in the colonized plants, which indicates that the increased expression of *Pad4* may not be the essential component in the induction of SAR by the hypovirulent isolates. Also, expression levels of the *Ndr1* gene were unchanged in plants colonized with the hypovirulent isolates. In addition, Bion and meJA (methyl jasmonate) were used to induce either SAR or ISR, respectively, in *A. thaliana* plants to provide an additional view on how the tested genes would react when either defense system was induced and for comparison of this expression pattern to the one induced by the colonization of the plants with the hypovirulent *Rhizoctonia* spp. isolates. MeJA-treated plants responded with higher levels of mRNA of all the tested genes involved in the ISR pathway, except for *Lox1*. The SAR related genes (*Pad4* and *Pr5*) were not induced in meJA-treated plants, nor was the *Ndr1* gene (related to R-Avr gene recognition and to the induction of ROS). Expression levels of the phytoalexin-related gene *Pad3* increased slightly in meJA-treated plants, but their increase was statistically significant only in one of the two biological repeats. Therefore, it is not possible to draw any conclusion for expression levels of this gene when the plants were treated with meJA. Bion-treated plants had significantly higher levels of mRNA of both SAR related genes (*Pad4* and *Pr5*), the *Pad3* and *Ndr1* genes. None of the ISR related genes (*Lox1, Lox2, Cori3* and *Pdf1.2*) were induced in Bion-treated plants. To summarize this section: while Bion and meJA induced either SAR or ISR, respectively, the hypovirulent *Rhizoctonia* isolates induced both SAR- and ISR-related genes, as well as phytoalexin production-related genes in the *A. thaliana* plants. The locations of the defense-related genes whose expression was monitored and the defected genes of the *A. thaliana* mutants used in the present study, are illustrated in Fig. 13.

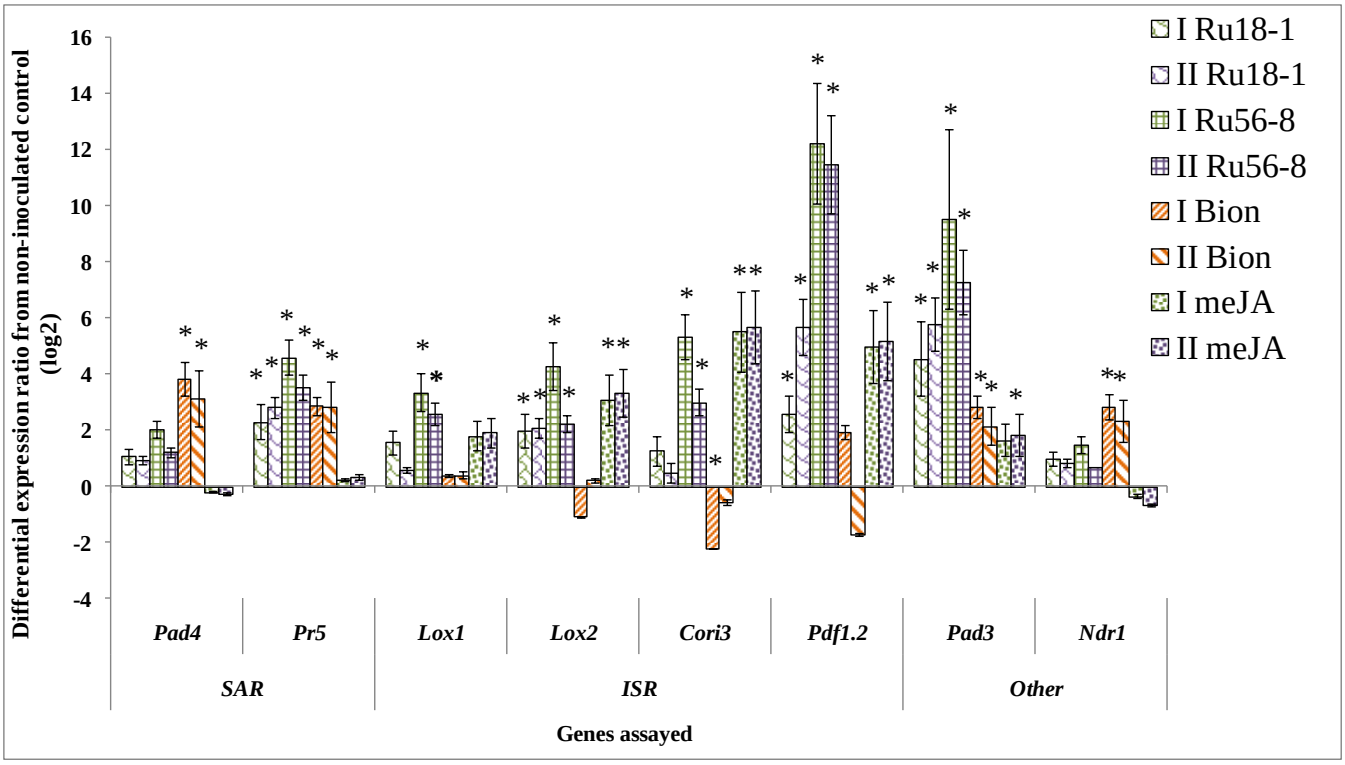

Fig. 12. Expression ratio of genes induced by hypovirulent *Rhizoctonia* spp. isolates, meJA or Bion, compared with non-inoculated control in wt *Arabidopsis thaliana* plants.

- The controls used were plants which received the same treatment as the tested condition, described in Fig. 10, 11.

- Ru18-1and Ru56-8 – hypovirulent isolates. Bion and meJA – as described in Fig. 11.

- The results are from two biological repeats (I and II).

The tested genes: both *Pad4* and *Pr5* are a part of the SAR pathway, *Pad4* is upstream from SA (Glazebrook, 2001) while *Pr5* is induced in response to SAR activation (Kawamura *et al.*, 2009). *Lox1*, *Lox2*, *Cori3* and *Pdf1.2* genes are induced in the ISR pathway. *Lox1* is involved in defense responses but its precise

function is yet unclear, *Lox2* catalyzes the first step of JA biosynthesis (Bannenberg *et al*. 2009), *Cori3* mRNA is induced in response to meJA, and *Pdf1.2* encodes a protein with antifungal activity (Penninckx *et al.*, 1996). Other genes: *Pad3* encodes an enzyme which catalyzes the last step in the phytoalexin camalexin production (Bottcher *et al.* 2009) and *Ndr1* is involved in ROS production and accumulation (Century *et al.* 1997). For more elaborate clarification, see Fig. 13.

- The examined treatments were: inoculation with hypovirulent isolates Ru18-1 or Ru56-8, addition of an artificial SAR inducing reagent (Bion) or meJA – the ISR inducing derivative of jasmonic acid.

- Data was analyzed using the REST program (Pffalf *et al*. 2002).

- Results with an absolute value higher than 2 which were significantly different (Relocation randomization test, $p<0.05$) from the expression of these genes in non-inoculated control plants, are marked with an asterisk.

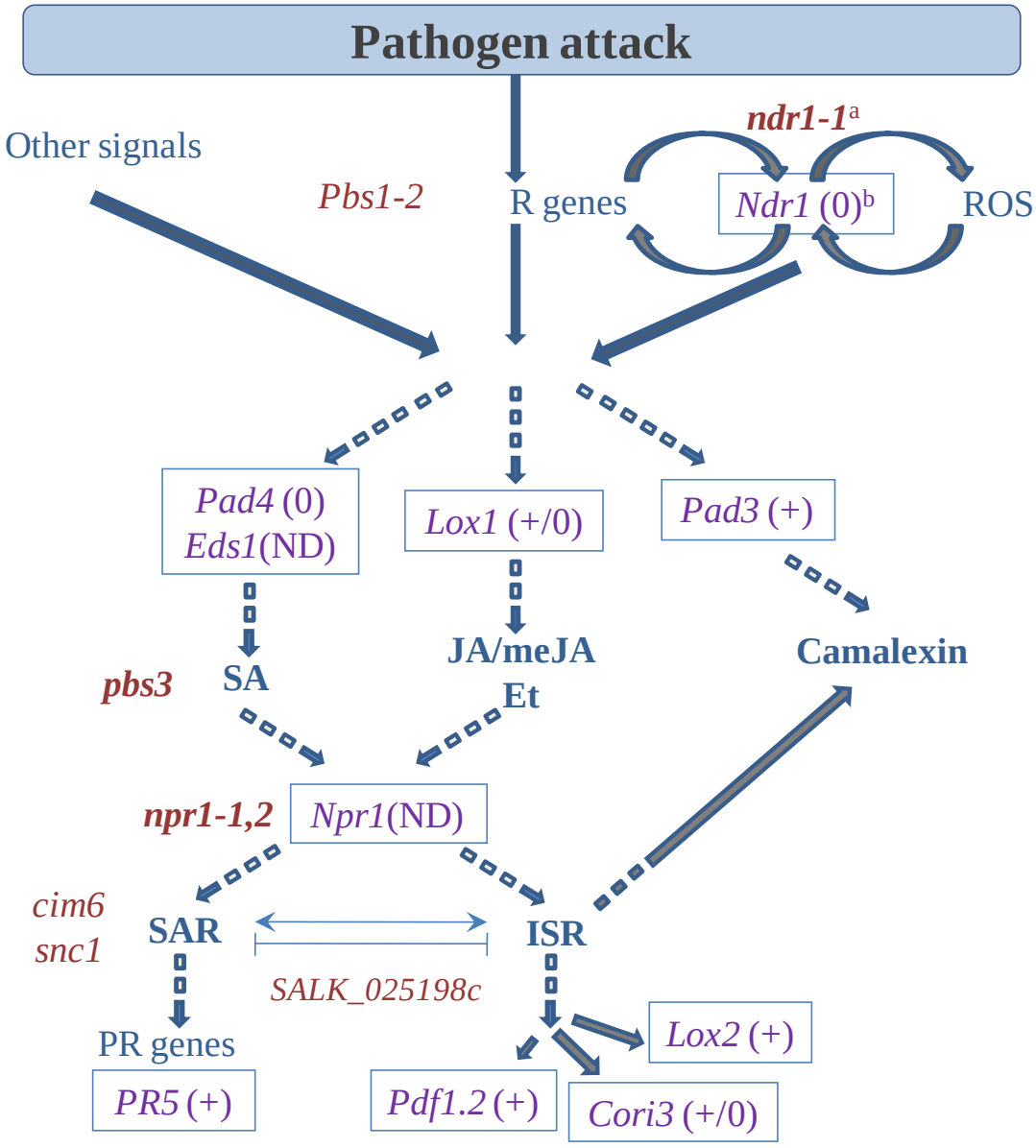

Fig. 13. A simplistic diagram illustrating the locations of the defected genes of the *A. thaliana* mutants, the defense-related genes monitored and their reactions in the present study. The genes (purple) involved are in a frame, the *A. thaliana* mutants used in the present study are in brown.

[a] *A. thaliana* mutants with reduced protection compared to the wt plants (marked in bold).

[b] Central genes involved in plant protection, the expression of these genes was examined in the present study to indicate whether they were induced by the hypovirulent *Rhizoctonia* inoculation. (+) induced expression, (0) unchanged expression, (+/0) induced expression only when one of the hypovirulent isolates colonized the plant, but not the other, (ND) the gene's expression was not determined.

4.9. Evaluation of expression levels of defense related genes in defense impaired *Arabidopsis thaliana* mutants, *npr1-1* and *ndr1-1*. Plant protection assays revealed that the *npr1-1* and *ndr1-1* mutant plants were protected to a lower extent than the wt plants. Analyzing differences in the expression of genes involved in different protection mechanisms in these mutants compared with that in the wt plants may better clarify the mechanisms involved in resistance, induced in the plants colonized by the hypovirulent *Rhizoctonia* spp. isolates. The differences in expression levels of the tested gene in *npr1-1* and *ndr1-1* mutant plants compared with that in the wt plants indicated that the mutations reduced the basic levels of some of the tested genes (Fig. 14). The SAR-related genes, *Pad4* and *PR5* mRNA expression levels, were lower in both *ndr1-1* and *npr1-1* mutants compared to mRNA expression of those genes in wt plants. In addition, the ISR-related genes, *Lox1* and *Cori3* mRNA expression levels, were lower in both tested mutants, but not those of *Lox2* gene expression. The mutant *npr1-1* also possessed lower levels of *Pdf1.2* and *Ndr1* mRNA than those in the wt plants.

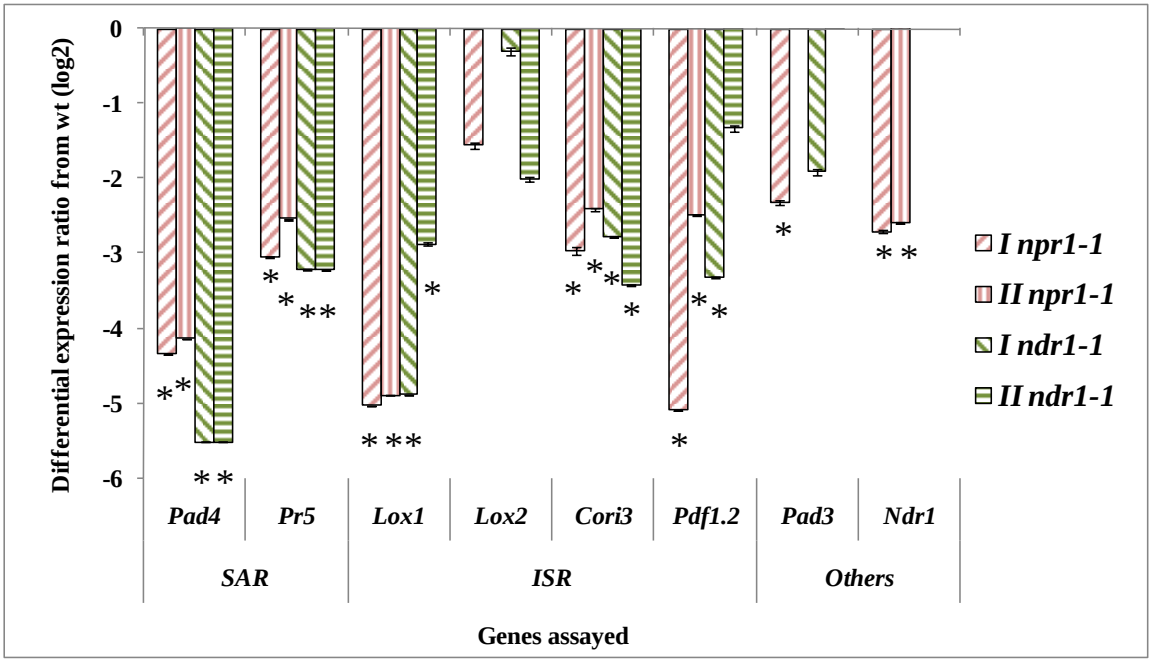

Fig. 14. Expression ratio of genes in *npr1-1* and *ndr1-1* mutants compared with that of the wt *Arabidopsis thaliana* plants

- I and II are two biological repeats.

- Expression levels of the tested genes were examined in *npr1-1* and *ndr1-1* mutants and compared to those in the wt plants.

- *Ndr1* expression in mutant *ndr1-1* was not tested as it is a null mutant for this gene.

- The tested genes were described in Fig. 12.

- Results with absolute value higher than 2, that are significantly different (Relocation randomization test, $p<0.05$) from the expression of these genes in wt plants, are marked with an asterisk.

4.10. Induction of defense related genes in the *npr1-1* mutant by colonization with hypovirulent *Rhizoctonia* spp. isolates. The Ru18-1 and Ru56-8 isolates induced *Pdf1.2* and *Pad3* gene expression in the *npr1-1* mutant plants (Fig. 15), but unlike the wt plants, there was no induction in the expression of *Pr5*, *Lox2* or *Cori3*, when the plants were colonized with Ru56-8. Although the expression of *Pdf1.2* was induced in *npr1-1* mutants by both Ru56-8 and Ru18-1 inoculation, the basic expression level of this gene in *npr1-1* mutants was lower than that in the wt plants as determined in results summarized in Fig. 14.

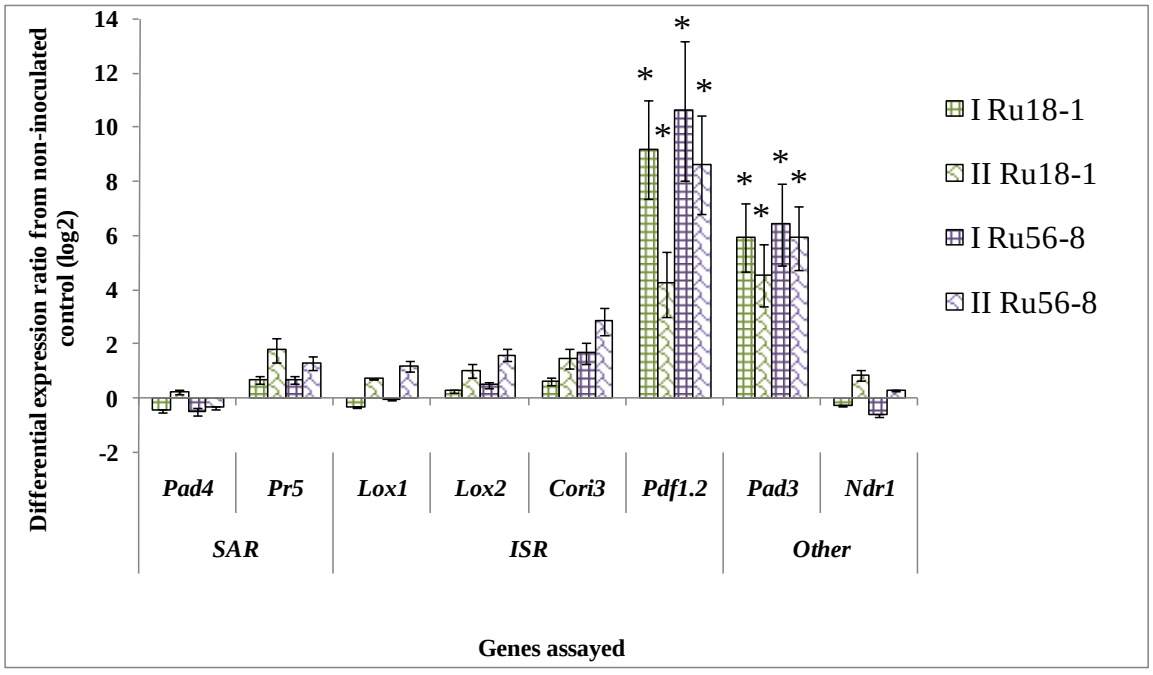

Fig. 15. Expression ratio of genes induced by hypovirulent *Rhizoctonia* spp. isolates in *npr1-1* plants compared with non-inoculated control *Arabidopsis thaliana*.

- I and II are two biological repeats.

- *npr1-1* plants were colonized with hypovirulent isolates Ru18-1 or Ru56-8 and the expression values of the tested genes were compared to the expression of the same genes in *npr1-1* non-inoculated control plants. The tested genes were described in Fig. 12.

- Results with absolute value higher than 2, that are significantly different (Relocation randomization test, $p<0.05$) from the expression of these genes in wt plants, are marked with an asterisk

4.11. Induction of defense related genes in the *ndr1-1* mutant by colonization with hypovirulent *Rhizoctonia* spp. isolates. The *ndr1-1* mutant is a null mutant of the *Ndr1* gene. This gene is necessary for the function of some PR proteins and plays a significant role in the gene for gene recognition system. *Ndr1* is also involved in the oxidative burst reaction and was found to aid the activation and sustenance of ROS. When *ndr1-1* plants were inoculated, the hypovirulent isolates Ru18-1 and Ru56-8 induced *Pr5* but not *Pad4* expression levels (Fig. 16). This may indicate that the SAR pathway was induced, but not necessarily through the *Pad4* gene pathway. Since both the basic expression of the genes *Pad4* and *Pr5* was lower in the *ndr1-1* mutants than in the wt plants (Fig. 14), the SAR reaction induced in the *ndr1-1* mutant is probably lower than that induced in the wt plants. The *Pdf1.2* gene was induced by both of the hypovirulent isolates and the mRNA levels of the *Cori3* gene increased only after colonization of plants by the Ru56-8 isolate, similar to inoculated wt plants. *Lox1* and *Lox2* expression levels did not change when the *ndr1-1* plants were inoculated by either of the hypovirulent isolates (expression of the *Lox2* gene was induced in the wt plants by both of the hypovirulent isolates, Ru18-1 and

Ru56-8, and the expression of the *Lox1* gene increased in wt plants, only when the plants were inoculated with Ru56-8).

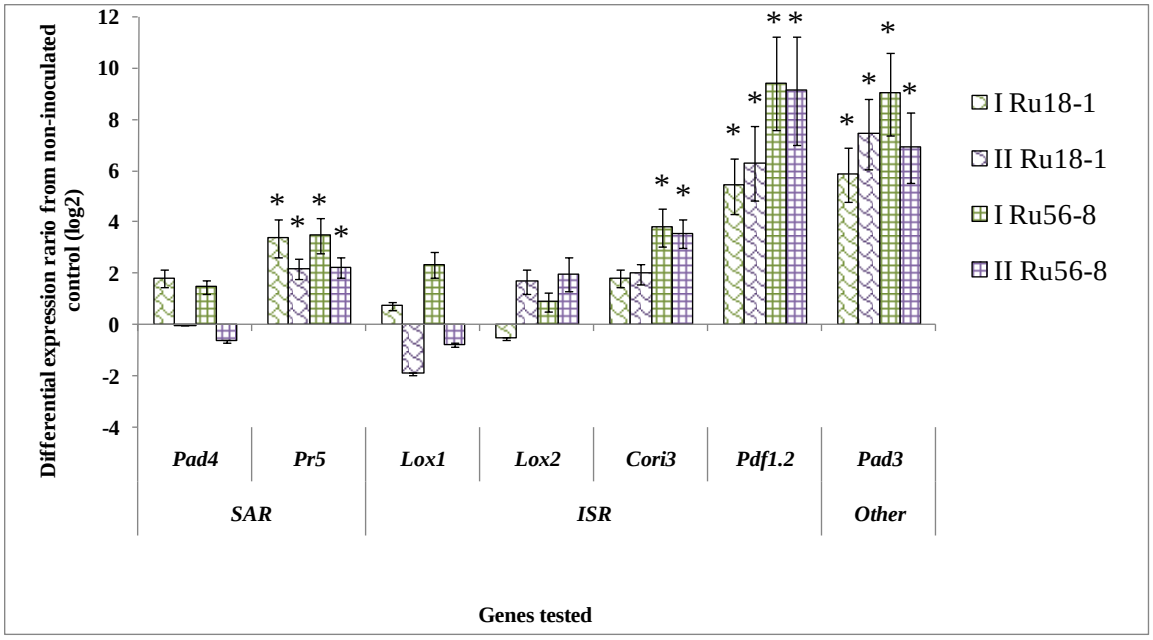

Fig. 16. Expression ratio of genes induced by hypovirulent *Rhizoctonia* spp. isolates in *ndr1-1* plants compared with non-inoculated control *Arabidopsis thaliana* mutants.

- I and II are two biological repeats.

- *ndr1-1* plants were colonized with hypovirulent isolates Ru18-1 or Ru56-8 and the expression values of the tested genes were compared to the expression of the same genes in *ndr1-1* non-inoculated control plants.

- The tested genes were described in Fig. 12.

- Results with absolute value higher than 2, that are significantly different (Relocation randomization test, $p<0.05$) from the expression of these genes in wt plants, are marked with an asterisk

5. Discussion

Hypovirulent *Rhizoctonia* spp. isolates protected a wide variety of plant species against *R. solani* with high efficacy (75-95% protection), when both hypovirulent and the challenging pathogenic isolate were applied in soil (Sneh and Icihelevich-Auster, 1998). Reports on similar protection obtained in different countries were summarized in various review articles (Herr, 1995; Sneh, 1996; 1999; Tsror *et al*. 2001). When the hypovirulent isolates were applied in soil and the challenging pathogen was applied on the hypocotyl or foliage, the plants were protected also against *Pythium ultimum and Pseudomonas syringae* (Sneh and Ichielevich-Auster, 1998), which indicates the involvement of induced resistance modes of action. In addition, when the challenging pathogen was *Rhizoctonia*, the level of protection obtained by induced resistance was lower when both the hypovirulent and the challenging pathogen were applied in soil (35-85%). A few hypovirulent isolates which protected well when both isolates were applied in soil did not protect the plants when the challenging pathogen was applied in the upper part of the plants (Sneh and Ichielevich-Auster, 1998). This could be an indication that several modes of action are involved in the phenomenon of protection by hypovirulent isolates and that different hypovirulent isolates may act via different modes of action or in different levels of the same reactions - with varying efficacies, as the results of the present study support. Unfortunately, the results of protection by the different hypovirulent isolates were inconsistent, possibly due to varying conditions in the greenhouse, influenced by the weather (humidity, sun-light est), and microbial activities in the soil and water. All those conditions might have affected the activity of the pathogen applied on the plant hypocotyl. Therefore, the requirements expected from the

assay developed for testing virulence and protection on *A. thaliana* plants included minimizing influence of the varying external conditions.

The possibilities that colonization with hypovirulent isolates may protect plants against pathogen(s) by antibiotic production, parasitism, or competition for carbon, or nitrogen sources provided by the root exudates were not found to be involved (Ichielevich-Auster, 1987; Sneh et al., 1989a). However, one study reported that some parasitism was observed on *Pythium* hyphae by a certain isolate of hypovirulent binucleate *Rhizoctonia* (Siwek et al., 1997a), and another report described antibiotic activity of a hypovirulent isolate on *Pythium* (Siwek et al., 1997b).

In histochemical studies, suberin, pectic substances accumulation in cell walls, salicylic acid, increased activity of peroxidase, 1,3-β-glucanase, chitinase and phenylalanine ammonia lyase (PAL) were found to be involved in plants protected by hypovirulent *Rhizoctonia* isolates (Jabaji-Hare *et al.*, 1999; Xue, 1999). Although these findings may indicate that the hypovirulent isolates do trigger responses in the plants they protect, they are not sufficiently specific to verify which modes of action and pathways are involved in the induced protection triggered in plants colonized by the hypovirulent *Rhizoctonia* spp. isolates.

The present work therefore, focused on studying the pathways of induced resistance involved in the protection of plants provided by colonization with several protective hypovirulent *Rhizoctonia* spp. isolates, targeting the salicylic acid pathway (SAR-systemic acquired resistance), the Jasmonic acid pathway (ISR- induced systemic resistance) and the inhibitory secondary metabolites (phytoalexins). Major genes coding

for proteins involved in these pathways were monitored using *A. thaliana* mutants harboring relevant defected genes, these mutants were tested for virulence and protection obtained by the hypovirulent isolates against the challenging *R. solani* pathogen. An additional approach was to evaluate differences in expression of genes involved in the induced protection pathways by the hypovirulent colonization.

The main results obtained from the present study clearly demonstrate that although the hypovirulent isolates have slight differences in their modes of action; ISR, SAR, and phytoalexins are all involved in the induced resistance protection response of *A. thaliana* plants by hypovirulent *Rhizoctonia* isolates.

5.1. Virulence of hypovirulent *Rhizoctonia* spp. isolates on various plant species. All of the hypovirulent isolates were of low virulence on the tested plants (9 plant species representing 7 different botanical families) than the pathogenic isolate. Most of them were a-virulent, while some of the isolates caused slight symptoms on some of the plant species. This is in accordance with previous reports (Sharon *et al.*, 2007; Sneh et al., 2004), though some plant species may be more susceptible than others (Cardinale *et al.*, 2006).

5.2. Protection of cucumber plants by hypovirulent *Rhizoctonia* spp. isolates. Based on the results of the virulence test on the different plant types and the convenience of growing cucumber plants (rapid growing, the suitability of the rigid and erect hypocotyl for the challenge inoculation), the virulence of *Rhizoctonia* spp. isolates and their ability to induce plant protection were examined on cucumber plants. All of the hypovirulent tested isolates protected the cucumber plants against the pathogen when

both the hypovirulent isolate and the challenging pathogen were applied in soil at all of the examined application time intervals of the pathogen. When the challenging pathogen was introduced at a different site (on the hypocotyl) than the hypovirulent isolate, the protection levels were not as high and in some treatments the protection was not statistically significant. This clearly indicates that in addition to the systemic resistance, the hypovirulent isolates protect the plant also by local resistance mechanisms. These mechanisms may involve competition for space, infection sites, and/or induction of local resistance responses such as strengthening of the plant cell walls components or local production of antifungal compounds.

Past attempts to nullify the presence of the hypovirulent isolate from the protected plants were made by physically removing the hyphae from the colonized areas (Icihielevich-Auster, 1987), which could eliminate competition for infection sites. However, such removal of the hyphae may also have caused some rupture to the epidermal cells and induce various responses from enhanced resistance induced in the injured plant to increased susceptibility to penetration of the pathogen. In the present work attempts were made to nullify competition with the pathogen by killing the hypovirulent hyphae colonizing surface of the plants (cucumber) with a low concentration of sodium hypochlorite solution. However, these attempts failed because mortality resulted in plants caused by the treatment while the hyphae survived. In a previous study, induced plant protection by hypovirulent isolates was obtained on cucumber seedlings challenged with the pathogen on the hypocotyl, when the hypovirulent isolates were applied in soil at planting time of the germinated seeds (Sneh and Ichielevich-Auster, 1987). In the present work, before choosing the application

method of the hypovirulent isolates, plants were colonized with the hypovirulent isolates at planting time and their hypocotyls were examined under the microscope. Small dead fragments of hyphae could be observed on the surface of the hypocotyls, sometimes wrapped around the plant hair. There is a possibility that such hyphae may trigger some local resistance mechanisms. To avoid such a possibility, the hypovirulent isolates were applied in soil after the hypocotyls emerged (10 days after sowing). Thus, no residual hyphae could be found on the site where the challenging pathogen was subsequently applied. Jabaji-Hare *et al.*, (1999) found the accumulation of suberin, phenolic compounds, and pectin substances in the cell walls of plants colonized with their hypovirulent *Rhizoctonia* isolate, using a histochemical approach. Although their findings confirmed that the hypovirulent isolates triggered certain responses in the protected plants, specific pathways and modes of action that were involved in the protection induced by the *Rhizoctonia* isolate in the plants were not specified. In the present work attempts were made to detect the differential proteins induced in plants by hypovirulent *Rhizoctonia* isolates.

5.3. Protein maps of cucumber plants colonized with hypovirulent *Rhizoctonia* spp. isolates compared with that of non-colonized plants. The differential proteins induced in plants by hypovirulent *Rhizoctonia* isolates in the present work were found to be involved in either general processes of the cell and/or in stress and defense responses. The former group assembled the proteins MC1, MC3, MC4(2), MC8, MC9, MC10, MC13(1) and MC16. MC1 is a GTP-binding protein SAR1 which is involved in protein transfer by initiating the vesicular sheath from the ER (Memon, 2004). MC3 is a poly-A binding protein which is involved in translation, translation control, RNA stabling, and

transferring (Bravo *et al.,* 2005). MC4(2) is a mitochondrial ATP synthase β-subunit which is a catalyst of the final step in oxidative phosphorylation (Ichikawa and Mizuno, 2004). MC8 is a S-adenosyl-L-methionine synthetase which is a catalyst in the biosynthesis of S-adenosyl-L-methinine, and a universal methyl donor. Its levels rose during salinity stress in tomato (Sanchez-Aguayo *et al.,* 2004). S-adenosyl-L-methinine is known to be the methyl donor in the process of converting SA to meSA – the active form of the prime substance in SAR (Loake and Grant, 2007). MC9 is a glyceraldehyde 3-phosphate dehydrogenase which is a glycolytic enzyme, involved in apoptosis induction (Hara *et al.,* 2005). MC10 is a eukaryotic initiation factor 5A which is activated post translation by HSS (homospermidine synthase) – the first specific enzyme in the path of pyrrolizidine alkaloids (Reimann *et al.,* 2004). MC13(1) is a tasselseed2-like protein which controls cell death (Calderon-Urrea and Dellaporta, 1999). MC16 is a putative fructose bisphosphate aldolase which is a glycolytic enzyme that its activity is increased in gibberellin-treated roots (Hirosato *et al.,* 2004). In addition to MC8 and MC10, which are known to participate in stress and defense reactions in plants, MC5-7 and MS13(2) are also known to be associated with stress and defense: MC5-7 is a netting associated peroxidase which is an anionic peroxidase involved in polarization of suberin aromatic area (Keren-Keiserman *et al.,* 2004) and MC13(2) is a malate dehydrogenase which its activity increases during water stress (Nath *et al.,* 2005).

Campo *et al.* (2004) found an increase in the eukaryotic initiation factor 5A and a decrease in glyceraldehyde 3-phosphate dehydrogenase, when they examined proteins extracted from pathogen-infected plants and compared them with that of control plants (among other proteins). Although this protein was identified as differentially expressed

among the different treatments in the present study, they were not necessarily higher in colonized plants compared to the non-inoculated control ones. Since in the present study we isolated proteins in their denatured form, it is possible that the identified proteins were in their inactive form. In addition, the method of protein purification and the pH spectrum which was used during protein extraction and first dimension separation, may favor certain proteins over others. Therefore, it is possible that the use of different parameters may have yielded different results. Examining other results in the present study (plant protection by induced resistance, defense-related genes expression), it was evident that colonization with the protective hypovirulent *Rhizoctonia* isolates did trigger the plant defense responses in those plants, which probably resulted in differential protein expression.

5.4. Growth inhibition of *Rhizoctonia solani* hyphae with extracts of radish seedlings colonized with hypovirulent *Rhizoctonia* spp. Radish plants were chosen for this experiment because of their rich antifungal substances contents (Soledade and Pedras, 2010; Terras *et al.* 1992). Ethanol:acetone:water extracts of radish plants colonized with isolate Ru56-8 significantly inhibited hyphal growth of the pathogen (the extracts from the separated parts of the plant – roots, lower hypocotyl, higher hypocotyl, leaves caused the same inhibition), while inhibition by extracts from plants colonized with other hypovirulent isolates was not significant. Antifungal substances such as 1,3-β-glucanase and chitinase were induced in plants in response to *Rhizoctonia* colonization (Xue, 1999; Cardinale *et al.,* 2006; Walski *et al.,* 2005). Therefore it is likely that some hypovirulent *Rhizoctonia* spp. isolates may induce production of antifungal substances in plants they colonize, though it is not clear to what extent the impact of those substances

have on the defense of the colonized plants against the pathogenic *Rhizoctonia*. Growth inhibition of the pathogen hyphae by extracts from plants colonized with isolate Ru56-8 could be incited by the induction of production and/or modification of existing inhibitory substances in the plants, by the isolate. Since extract of different sections of the plant did not differentially inhibit hyphal growth of the pathogen, it is likely that the induction of the inhibitory substances was not dependent on a specific plant part but on the hypovirulent isolate which induced production of the inhibitory substances in the plant. Since the hypovirulent isolates inhibited all of the plant organs which their extracts were tested, it is not possible to know if the induction of the fungal inhibitory substances was local or systemic.

A more precise identification of the inhibitory substances induced by the hypovirulent isolates in the plants may be obtained by separation of the colonized plant's extracts using HPLC for assaying the inhibition of the separated fractions on hyphal growth of the pathogen. By using more replicates and repeating experiments with the hypovirulent isolates Ru18-1 and Ru521, significant inhibition may be achieved.

5.5. Development of reliable virulence and protection tests for hypovirulent *Rhizoctonia* spp. isolates on *Arabidopsis thaliana* plants in MS plates. *A. thaliana* is a well studied plant and as such, researchers have developed many mutants of this plant over the years. To get a clearer view of the genes and pathways involved in the protection induced in plants by the hypovirulent *Rhizoctonia* isolates *A. thaliana* mutants that contained mutations in relevant resistance genes were chosen. In order to obtain the optimal data for virulence and protection of *A. thaliana* mutants and wt plants by the

hypovirulent *Rhizoctonia* spp. isolates a novel method had to be developed. In a previous reported study (Perl-Treves *et al.*, 2004), *Rhizoctonia* isolates were used to monitor changes in susceptibility of an *A. thaliana* mutant. The method was based on very young plants, small number of plants per plate, and the medium contained sucrose. This procedure was inappropriate for our needs. The requirements for our assays were: accuracy, reliability, quantitative data, time and space efficacy, minimal effect of external factors and the ability to examine a large number of plants, genotypes, and *Rhizoctonia* isolates. The optimal method was calibrated, including: procedure and timing of application of the hypovirulent isolates and challenging pathogen, recording incidence of virulence and protection. All of the required conditions were met in the newly developed assay, although the plants in the assay were more sensitive than plants grown under normal conditions (in soil, exposed to the greenhouse environment). This method was used to evaluate virulence and protection by the different hypovirulent isolates and the pathogenic isolate on various *A. thaliana* mutants defected in genes involved in plant induced resistance pathways.

5.6. Changes in virulence or protection rates by hypovirulent *Rhizoctonia* spp. isolates against a pathogenic *Rhizoctonia* solani isolate, on *Arabidopsis thaliana* mutants defected in genes involved in defense pathways compared with wt plants.

Both mutants of the gene *Npr1* (*npr1*-1 and *npr1*-2) resulted in decreased protection of the plants by most the hypovirulent *Rhizoctonia* isolates. On the other hand, isolate Ru89-1 protected these mutant plants at the same levels as the wt plants. It could be that this isolate induced plant protection in a different way than the other tested hypovirulent

isolates, or that protection of the plants by this isolate depended more on local resistance and competition with the pathogen on infection sites, and less on induced systemic resistance pathways, which require an intact *Npr1* gene for a complete, complicated reaction (Dong, 2004).The mutant containing *npr1-1* is defected in the ankyrin-repeat domain of the *Npr1* gene, a point mutation substitute histidine with tyrosine at the third ankyrin-repeat (Cao *et al.,* 1997). Consequently, the protein transcribed is miss-folded and loses its function in both SAR and ISR pathways (Glazebrook *et al.,* 2003). This may impair the mutant's capability to elicit both of the induced resistance pathways. The *npr1-2* has a point mutation in *Npr1* that changes the amino acid cysteine to tyrosine (Cao *et al.,* 1997) in the BTB/POZ domain. This site participates in the co-induction of PR1 by *Npr1* and *TGA2* (Rochon *et al.,* 2006). The *npr1-2* mutation is not complete and allows some *Npr1* activity, which is found by a low expression of *PR1* protein in those mutant plants (Cao *et al.,* 1997). When the difference in protection of the mutants *npr1-1* and *npr1-2*, by the different hypovirulent *Rhizoctonia* isolates was compared to that of the wt, there was no statistically significant difference between these two mutants. Hence, the decrease in the *Npr1* activity was sufficient to reduce the protection of the plants by the hypovirulent isolates to the minimum caused by the damaged *Npr1* protein. Thus, a complete loss of function of the *Npr1* protein due to a miss-folding did not reduce the protection further than that provided by the hypovirulent isolate. The decreased protection of the *npr1-1* and *npr1-2* mutants could be a consequence of faulty growth of the hypovirulent isolate on the mutant, increased virulence of the pathogen (even though the hypovirulent isolates were no more virulent on this mutant than on the wt plants), defected induced resistance pathways, which are involved in the protection of the plants

colonized by the hypovirulent isolates, or a default in the induction processes of the induced systemic resistance pathways by the hypovirulent isolates.

ndr1-1 is a null allele of the *Ndr1* gene, and is therefore defected in an array of reactions depended on ROS accumulation and activation (Century *et al.,* 1997). It has been demonstrated that Ndr1 generates plant resistance via an *Est1-Pad4* independent pathway (Aarts *et al.,* 1998). The protection rates of the *ndr1* plants by all of the hypovirulent isolates were reduced. This could be directly due to the lack of gene function as a transducer of an elicitor signal from an AVR-R gene interaction (Glazebrook, 2001; Century *et al.,* 1997), damage to the oxidative burst itself (Century *et al.,* 1997) or to reactions involved in resistance which require ROS along their pathway, such as the *Pdf1.2* gene (Huffaker *et al.,* 2006).

The CS6355 mutant is a double mutant containing both the *npr1-2* and the *ndr1-1* mutations. These two proteins have been found to act additively rather than synergistically in a previous study inducing SAR (Zhang and Shapiro, 2002). One of the hypovirulent isolates (Ru56-8) was slightly virulent on these mutant plants and the protection of this mutant by the hypovirulent isolates was lower than the protection of the same isolates on the wt *A. thaliana* plants. When protection levels of the *ndr1-1*, *npr1-2* and CS6355 by the hypovirulent isolates were compared, the protection levels of the *npr1-2* mutant plants were higher than the protection levels of the *ndr1-1* and CS6355 mutant, but there was no difference between the protection levels of the *ndr1-1* and CS6355 mutant plants by the hypovirulent isolates. These results confirm those of Zhang and Shapiro (2002), indicating that *Ndr1* and *Npr1* genes do not act synergistically.

The CS6571 and *snc1* mutants constitutively express the SAR pathway, including SA, PR-1,5 and Npr1 accumulation (Maleck *et al.,* 2002; Li *et al.,* 2001). The *snc1* mutant carries a mutation in Toll interleukin1 receptor nucleotide-binding Leu-rich repeat-type resistance gene, resulting in a constitutive activation of the R-gene and consequently - SAR. This phenotype is dependent on *Pad4* and *Eds1* and partially dependent on SA (Zhang *et al.,* 2003a). CS6571 was found to have an increased resistance to fungal pathogens (Maleck *et al.,* 2002), and its constitutive SAR expression is dependent on SA accumulation (Maleck *et al.,* 2001). Since there is a tradeoff between the SAR and the ISR pathways, it is possible that a constitutive expression of the SAR pathway led to a reduced effect of the ISR pathway (Bostock, 2005). In a previous study it was found that induction of SAR by application of acibenzolar (SAR inducer) did not inhibit disease development caused by pathogenic *Rhizoctonia* (Hwang and Benson, 2003). It is possible that another resistance mechanism is the primary one for inhibition of disease development caused by pathogenic *Rhizoctonia* and that these responses were impaired when the SAR pathway was constitutively expressed. Another possibility to this response is an impaired fitness of the mutants, which constitutively express the SAR resistance pathway, compared to that of the wt plants, as reported by Cipollini (2002). Heidel and Baldwin (2004) also demonstrated that when the SA pathway was induced there was a decrease in photosynthetic gene expression, which can contribute to a reduced fitness of the constitutively-expressing SAR mutants.

The SALK-025198c mutant is defected in the *Wrky70* - a transcription factor balancing the JA- and SA-dependent responses, by inhibiting the ISR pathway (Li *et al.,* 2006), working downstream from the ROS defense reaction and SA induction (Knoth *et*

al., 2007), as demonstrated in Fig. 1. Protection of SALK-025198c was not significantly different from protection of the wt plants by the hypovirulent *Rhizoctonia* isolates. It could be that the hypovirulent isolates induce the JA-dependent resistance pathway, but not the SA-dependent resistance pathway. This hypothesis was not supported by the quantitative real-time PCR – as it is apparent that the SAR pathway was induced by the hypovirulent isolates as well as the ISR pathway. Another possibility to the unchanged protection levels of the SALK-025198c mutant plants compared to the wt plants is that the ISR defense responses are the prime responses against the pathogenic *Rhizoctonia* isolate. This theory is supported by reduced protection of the constitutively expressing-SAR mutants CS6571 and *snc1* and well established information that necrotrophic pathogens usually induce the ISR pathway in plants (Pozo *et al.,* 2005; Thomma *et al.,* 2001; Wiemer *et al.,* 2005).

The *pbs3-1* mutant is defected in the *Gh3.12* gene, which leads to reduced SA accumulation and SA-dependent gene expression (Okrent *et al.,* 2009). SA, in addition to playing a key role in the SAR system, is also essential for the establishment of certain pathways of the ISR system (Heil and Bostock, 2002). Therefore, it is likely that in the *pbs3-1* mutant plants both the SAR and ISR systems are reduced, or even completely inhibited. The decreased protection of these mutant plants by the hypovirulent isolates is likely due to the defected induction of SAR, ISR or both.

The *pbs1-2* mutant is defected in the gene *Pbs1* which is involved in *A. thaliana* resistance against *Pseudomonas syringae* strains expressing the Avr gene -AvrPphB. The AvrPphB proteolytically cleavage PBS1 is a process required for PBS5-mediated

resistance (Shao *et al.*, 2003). The *pbs1-2* mutant has a missense mutation which changes the activation segment of PBS1 (Swiderski and Innes, 2001). Although the *Pbs1* gene mutation is an important part of the AvrPphB- PBS5 resistance system, it does not affect other resistance factors. In the present work, the *pbs1-2* mutant was used as a negative control, to demonstrate that the reduced protection of other mutants in the genes involved in plant resistance is due to the specific damage of the defected system. Protection of the *pbs1-2* mutant plants by the hypovirulent *Rhizoctonia* isolates was unaffected compared to the protection of the wt *A. thaliana* plants, confirming the results obtained with the other *A. thaliana* mutants tested in this work.

The SAR and ISR pathways are intertwined complexes with shared components. These components might act as either inducers in one pathway and inhibitors in the other, similar to the *Est1* and *Pad4* genes (Wiemer *et al.*, 2005). Other gene products may be required by both SAR and ISR pathways to assemble a complete reaction, such as Npr1 (Dong, 2004). On the other hand, there is redundancy in both of these pathways (Schenk *et al.*, 2000; Zhang *et al.*, 2003b), which may mask a defected component in a resistance pathway by the activity of the other.

5.7. Hypovirulent *Rhizoctonia* spp. isolates trigger resistance pathways in protected *Arabidopsis thaliana* wt plants. Colonization of *A. thaliana* plants by hypovirulent *Rhizoctonia* spp. isolates induced both SAR-, ISR- and Phytoalexin-related genes. While Bion induced both of the tested SAR-related genes, *Pad4* and *Pr5* in the plants, the hypovirulent isolates induced only one of these genes (*Pr5* which is expressed at the end of the SAR pathway). Bion did not protect *A. thaliana* plants against the

pathogenic *Rhizoctonia* isolate at all. Therefore it is possible that the SAR pathway, which Bion triggered in the plants, is not suitable for protection against the pathogenic *Rhizoctonia* isolate (Bion induced *Pad4* gene expression, colonization by the hypovirulent isolates did not), or that the SAR system, though induced by the hypovirulent isolates and the Bion treatment, does not protect the plants against this pathogen. Likewise, it is possible that the constant SAR activation by Bion imposed a heavy toll on the plant, which resulted in decreased protection of these plants against the pathogen. According to previous studies, necrotrophic pathogens induce ISR in plants, and this system is the one responsible for protection of plants against those pathogens (Beckers and Spoel, 2006). The hypovirulent isolates also induced ISR-related genes, as did the treatment with meJA. Induction of both ISR and SAR resistance systems was also demonstrated in *A. thaliana* by van Wees *et al.*, (2000), but to induce both systems simultaneously two different microorganisms needed to be applied - *Pseudomonas fluorescens* (to induce ISR) and *Pseudomonas syringae* carrying the a-virulence gene *avrRpt2* (to induce SAR). Similarly, when both SAR and ISR were simultaneously activated, there was an additive effect on the level of induced resistance against *P. syringae* (Durrant and Dong, 2004). Therefore, it is reasonable to assume that a biocontrol agent which triggers both of the ISR and SAR pathways could provide a more effective protection, than an agent that activates only one of the two systems.

MeJa induced only a mild protection in *A. thaliana* plants. It is possible that the relatively high protection levels provided by colonization with the hypovirulent *Rhizoctonia* isolates resulted from the combined methods of defense- both provided by the hypovirulent isolates (competition for space and infection sites) and triggered by them

in the plants they protect (local resistance, systemic resistance – both SAR and ISR). Therefore, induction of a certain resistance pathway in plants by application of a specific inducer, may not achieve the same effective protection on plants as colonization of the plants with protecting hypovirulent *Rhizoctonia* isolates. Also, similar to the earlier explanation for Bion-SAR triggering – constant ISR activation imposed a heavy toll on the plants, which resulted in decreased protection of these plants against the pathogen. The hypovirulent isolates also induced genes involved in phytoalexins production, supporting results previously obtained with extracts from radish plants colonized with Ru56-8 and previous reports demonstrated that phytoalexin play a major role in plant protection against *Alternaria* - a necrotrophic fungal pathogen (Bart *et al.,* 1999; Nafisi *et al.,* 2007; van Wees *et al.,* 2003). Additionally, the assumption that ISR and camalexin production has a shared degree of cross-talk (Feys and Parker, 2000) supports the result of combined ISR and phytoalexin protection.

5.8. The impact of *npr1-1* and *ndr1-1* mutations on resistance induced by hypovirulent *Rhizoctonia* spp. isolates.

The *npr1-1* mutation resulted in a miss-folded *Npr1* protein and henceforth – loss of function (Glazebrook *et al.,* 2003). The protection of wt *A. thaliana* plants by the hypovirulent isolates was reduced compared to the protection of colonized *npr1-1* mutant plants. The quantitative real-time PCR assay indicated that mRNA levels of the SAR-related genes *Pad4* and *Pr5* were lower in *npr1-1* plants than those in wt *A. thaliana* plants. *Pad4* is situated at the initial steps of the SAR pathway, and a reduction in expression of this gene may lead to deficiency in the later steps of this system. *Pr5* is one

of the genes induced at the end of the SAR pathway and has antifungal activity (Kombrink and Somssich, 1997). The low expression levels of the *Pad4* and *Pr5* genes demonstrated the defected SAR system in the *npr1-1*mutant, even if the hypovirulent could induce some expression of the SAR pathway in this mutant, it still would be at a considerable lower level than that induced in the wt *A. thaliana* plants, due to the lower expression levels of the SAR-related genes in the non-colonized plants. Some of the ISR-related genes had lower expression levels in the *npr1-1* plants than the wt plants. *Lox1* and *Cori3* mRNA levels were lower. Both of these genes are involved in the ISR-dependent defense responses (*Lox1* - Bannenberg *et al.,* 2009; Melan *et al.,* 1993; *Cori3* - Castillo *et al.,* 2004; Leon *et al.,* 1998). Also the expression levels of the gene *Pdf1.2* were lower in the *npr1-1* mutant than in the wt *A. thaliana* plants. This gene is triggered at the final steps of the ISR pathway (Solano *et al.,* 1998; Brodersen *et al.,* 2006) and is involved in antifungal activity (Penninckx *et al.,* 1996). The expression level of the gene *Lox2* was unchanged in the *npr1-1* mutant compared to wt *A. thaliana* plants. This gene catalyzes the first step of the JA biosynthetic pathway (Lopez *et al.,* 2008). Therefore, it is possible that the actual production of JA was not affected by the *npr1-1* mutation, but more work is required to confirm this assumption. The *Ndr1* gene's mRNA levels were lower in the *npr1-1* mutant than in the wt *A. thaliana* plants, which may cause a default in recognition of the fungi by the plant (Glazebrook, 1999), or with the oxidative burst response (Zhang and Shapiro, 2002). A defect in recognition of the hypovirulent isolates might have reduced the protection levels they induced in the colonized plants, and a faulty oxidative burst response could have reduced the ability of the *npr1-1* plants to

protect them selves against the pathogen, even if the recognition of the hypovirulent isolate was not affected by this mutation.

The quantitative real-time procedure used to evaluate the changes in expression of induced resistance-related genes in the mutant *npr1-1* by the hypovirulent isolates Ru18-1 and Ru56-8 revealed that the SAR-related genes were not induced, while in the wt *A. thaliana* plants the SAR-related end-pathway gene *Pr5,* was induced by both isolates. When taking into consideration that the expression levels of both genes were lower in the non-colonized *npr1-1* plants than in the wt plants it is plausible to assume that the SAR pathway is not functioning in this mutant. This assumption is supported by previous reports (Beckers and Spoel, 2006; Cao *et al.*, 1997). Among the ISR related tested genes, only *Pdf1.2* was induced in the *npr1-1* mutant by colonization of the plants with the hypovirulent isolates Ru18-1 and Ru56-8. While in the wt plants the expression of the *Lox2* gene was induced by both of hypovirulent isolates (Ru18-1 and Ru56-8) and the *Lox1* and *Cori3* genes were induced only by isolate Ru56-8, expression of none of these genes was induced in the *npr1-1* mutant by any of the hypovirulent isolates. *Pdf1.2* is an end-pathway gene of the ISR system. Since the ISR reaction (as well as the SAR) is a complicated network rather than a direct pathway, it might be that the hypovirulent isolate induce the ISR reaction through a number of steps, one or some of which, do not require a functional *npr1* gene and/or does not induce *Lox2*, even though the expression of the *Pdf1.2* gene was induced in *npr1-1* mutant plants by colonization with the hypovirulent isolates. Taking into consideration the low expression levels of the ISR-related genes *Lox1*, *Cori3* and *Pdf1.2* in the non-colonized *npr1-1* plants compared to the

expression levels of those genes in the wt plants, it is likely that the induction of the ISR system in the *npr1-1* mutant by the hypovirulent isolates is incomplete.

ndr1-1 plant protection by the hypovirulent isolates was reduced compared to the protection of the wt *A. thaliana* plants by the same hypovirulent isolate. The quantitative real-time PCR assay indicated that the mRNA levels of the ISR-related two tested genes (*Lox1* and *Cori3*) had lower expression levels in non-colonized *ndr1-1* mutant plants than in non-colonized wt *A. thaliana* plants. The expression levels of the *Pdf1.2* gene were significantly lower in the non-colonized *ndr1-1* plants than in the non-colonized wt *A. thaliana* plants only in one biological repeat. Therefore, clear conclusion cannot be drawn on this subject. Since the *ndr1-1* mutant is a null mutant of the gene *Ndr1*, expression of this gene was not monitored in the *ndr1-1* mutant plants. It may be concluded that some of the SAR and ISR related genes' expression is reduced in non-inoculated *ndr1-1* plants compared to that in wt plants. This could lead to a faulty expression of the resistance pathways. When the induction of the SAR- and ISR-related genes in response to hypovirulent colonization in *ndr1-1* mutant plants was examined the SAR-related gene *Pr5* and the ISR-related gene *Pdf1.2* were induced. In addition, the gene *Cori3* was induced when the plants were colonized with the hypovirulent isolate Ru56-8, but not when the plants where colonized with isolate Ru18-1. When the wt plant gene expression was evaluated, there was also an induction of the gene *Lox1* when the isolate Ru56-8 colonized the plants (but not when the isolate Ru18-1), and an induction of the gene *Lox2* by both of the hypovirulent isolates. These results may indicate that some aspects of the SAR and ISR pathways have been negatively affected by the *ndr1-1* mutation. In summary, there is still an induction of the SAR and ISR pathways, but the expression

levels of genes related to both of the pathways are reduced in *ndr1-1* mutant plants. In addition, some of the ISR-related genes which were induced in the wt plants by colonization with the hypovirulent isolates, were not induced in colonized *ndr1-1* plants.

5.9. General conclusions. The hypovirulent isolates had a significant impact on the plants they inhibited. This was confirmed by the changes in the protein profiles of plants colonized with those isolates. Several of the experiments conducted in the present study (i.e. analysis of mutant *A. thaliana* plants, monitoring resistance genes induced by the hypovirulent isolates, and fungal-growth inhibition by extracts of plants colonized with hypovirulent isolates) revealed that some of the effects were due to SAR, ISR and phytoalexin production pathways induced by the hypovirulent *Rhizoctonia* isolates examined in the present study. The protection levels of cucumber and *A. thaliana* plants by the hypovirulent *Rhizoctonia* demonstrated enhanced potential of these isolates to protect plants, although the protection levels were not as high when the protecting isolates were applied at a different site from that of the pathogen. This phenomenon indicates that the protection of the plants is a consequence of a combined effect of both local and systemic resistance, induced by the hypovirulent isolates. When different *A. thaliana* mutants defected in prime genes of the systemic resistance pathways were examined, there was a decrease in protection levels of some of the mutants, provided by the hypovirulent isolates. When combined with the results obtained from monitoring key genes in SAR, ISR and the phytoalexin production systems, It became clear that the hypovirulent isolates induced all of the 3 systems. However, SAR probably does not play

a major role in the protection of the colonized plants against the challenge inoculation with the pathogenic *Rhizoctonia* isolate. As *Rhizoctonia* pathogens are considered necrotrophic pathogens, it is an expected result. Although it is possible that the hypovirulent isolates may protect the plants against biotrophic pathogens as well, although this was not examined in the present study.

Concluding remarks and future directions to follow: the present study confirmed that the hypovirulent *Rhizoctonia* isolates induces genes involved in both known pathways of systemic resistance (SAR and ISR), and the production of phytoalexins in the colonized plants, and that this was correlated with the protection levels of the plants against pathogenic *Rhizoctonia*. Competition of the hypovirulent isolates with the pathogen for infection sites, space and local induced resistance was not evaluated in the present study. However, previous studies have indicated that this may play an important role in the modes of action of the protection of plants colonized with the hypovirulent *Rhizoctonia* isolates (Sneh *et al.* 1989a; 1989b). Additional research on mechanisms of control involved in plant protection by hypovirulent *Rhizoctonia* isolates should be conducted to better understand this subject. Some of the research directions that could be addressed:

- What is the duration of SAR and ISR induction and for how long do SAR and ISR effects prevail (if at all)? Do the plants stay primed?

- Which local mechanisms are used by the hypovirulent *Rhizoctonia* isolates to protect the inoculated plants?. The present study has focused on systemic induced resistance, but

although it is an important component of the protection of the colonized plants, it is not necessarily the major resistance mechanism.

- Which antifungal substances are induced in plant inoculated with the hypovirulent isolates, and do these substances have common characteristics in different plant species?

- Which plant species could be protected by the hypovirulent isolates?. The results presented in this work demonstrated that there could be a slight virulence effect of the hypovirulent isolates on the plants they infect, the loss of crop due to this virulence might be diminished when compared to the gain achieved by the high protection levels. This should be considered after conducting sufficient field experiments on a variety of plants.

- Which pathogens do the hypovirulent isolates protect plants against?. It was established that the hypovirulent isolates induce SAR, ISR and phytoalexin production. These three major protection systems are involved either together or separately in the resistance of plants against a variety of pathogens.

Reduced application of toxic chemical pesticides and use of other, more environmentally friendly, possibilities are the major goals of agricultural research. Biological control is one of these options. Hypovirulent *Rhizoctonia* isolates are fast growing, some of them shown also to promote plant growth (Sneh *et al.* 1986),an environmentally friendly preparation of the hypovirulent isolates can be easily produced, applied and achieve high protection levels. Therefore, such a commercial preparation represents a real possibility to utilized hypovirulent *Rhizoctonia* isolates in biological control on different crops.

6. References

Aarts, N., Metz, M., Holub, E., StasKawicz, B.J., Daniels, M.J., and Parker, J.E. 1998. Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 95:10306-10311.

Alvarez, S., Zhu, M. and Chen, S. 2009. Proteomics of *Arabidopsis* redox proteins in response to methyl jasmonate. *J. Proteomics* 73:30-40.

Anderson, N.L., Esquer-Blasco, R., Hofmann, J.P., Anderson, N.G. 1991. A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effects studies. *Electrophoresis* 12:907-930.

Armentrout, V.N. and Downer, A.J. 1987. Infection cushion development by *Rhizoctonia solani* on cotton. *Phytopathology* 77:619-623.

Attaran, E., Zeier, T.E., Griebel, T. and Zeier, J. 2009. Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. *Plant Cell* 21:954-971.

Bannenberg, G., Martinez, M., Hamberg, M. and Castresana, C. 2009. Diversity of the enzymatic activity in the lipoxygenase gene family of *Arabidopsis thaliana*. *Lipids* 44:85-95.

Bari, R. and Jones, J.D.G. 2009. Role of plant hormones in plant defense responses. *Plant Mol. Biol.* 69:473-488.

Bart, P.H.J., Thomma, I.N., Eggermont, K. and Broekaert, W.F. 1999. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J.* 19:163-171.

Beckers, G.J.M. and Spoel, S.H. 2006. Fine-tuning plant defence signalling: salicylate versus jasmonate. *Plant Biol.* 8:1-10.

Bell, E., Creelman, R.A. and Mullet, J.E. 1995. A chloroplast lipoxygenase is required for wound-induced Jasmonic acid accumulation in *Arabidopsis*. *Plant Biol*. 92:8675-8679.

Bell, E., Mullet, J.E. 1993. Characterization of an *Arabidopsis* Lipoxygenase gene responsive to methyl jasmonate and wounding. *Plant Physiol.* 103:1133-1137.

Bestel-Corre, G., Dumas-Gaudot, E. and Gianinazzi, S. 2004. Proteomics as a tool to monitor plant-microb endosymbioses in the rhizosphere. *Mycorrhiza* 14:1-10.

Bostock, R.M. 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* 43:543-580.

Bottcher, C., Westphal, L., Schmotz, C., Prade, E., Scheel, D. and Glawischnig, E. 2009. The nultifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. *Plant Cell* 1830-1845.

Bradford, M.B. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Bravo, J., Aguilar-Heronin, L., Olmedo, G. and Guzman, P. 2005. Four distinct classes of proteins as interaction partners of the PABC domain of *Arabidopsis thaliana* poly(A) binding proteins. *MGG Molacular Genetics and Gemonics* 272:651-665.

Brent, K.J. and Hollomon, D. 2007. Fungicide resistance in crop pathogens: how can it be managed? Fungicide Resistance Action committee, Brussels, Belgium, 2ed. 56 p.

Brodersen, P., Petersen, M., Nielsen, H.B., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J. and Mundy, J. 2006. *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant J.* 47:532-546.

Calderon-Urrea, A. and Dellaporta, S. 1999. Cell death and cell protection genes determine the fate of pistils in maize. *Development* 126:435-441.

Campo, S., Carrascal, M., Coca, M., Abian, J. and San Segundo, B. 2004. The defense response of germinating maize embryos against fungal infection: a proteomics approach. *Proteomics* 4:383-396.

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

Cardinale, F., Ferraris, L., Valentino, D. and Tamietti, G. 2006. Induction of systemic resistance by a hypovirulent *Rhizoctonia solani* isolate in tomato. *PMPP* 69:160-171.

Castillo, M.C., Martinez, C., Buchala, A., Metraux, J.P. and Leon, J. 2004. Gene-specific involvement of *β*-Oxidation in wound-activated responses in *Arabidopsis*. *Plant Physiol.* 135:85-94.

Century, K.S., Shapiro, A.D., Repetti, P.R., Dahlbeck, D., Holub, E. and Staskawicz, B.J. 1997. *NDR1* a pathogen-induced component required for *Arabidopsis* disease resistance. *Science* 278:1963-1965.

Cipollini, D.F. 2002. Does competition magnify the fitness costs of induced responses in *Arabidopsis thaliana*? A manipulative approach. *Oecologia* 131:514-520.

Colditz, F., Nyamsuren, O., Niehaus, K., Eubel, H., Braun, H.P. and Krajinski, F. 2004. Proteomic approach: identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*. *Plant Mol. Biol.* 55:109-120.

Delaney, T.P. 1997. Genetic dissection of acquired resistance to disease. *Plant Physiol.* 113:5-12.

Dong, X. 2004. NPR1, all things considered. *Curr. Opin. Plant Biol.* 7:547-552.

Dong, X. 1998. SA, JA, ethylene and disease resistance in plants. *Curr. Opin. Plant Biol.* 1:316-323.

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

Falk, A., Feys, B.J., Frost, N.L., Jones, J.D.G., Daniels, M.J. and Parker, J.E. 1999. EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. USA* 96:3292-3297.

Feys, B.J., Moisan, L.J., Newman, M.A. and Parker, J.E. 2001. Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* 20:5400-5411.

Feys, B.J. and Parker, J.E. 2000. Interplay of signaling pathways in plant disease resistance. *Trends Genet.* 16:449-455.

Fravel, D.R. 2005. Commercialization and implementation of biocontrol. *Annu. Rev. Phytopathol.* 43:337-359.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J.P., Kessmann, H. and Ryals, J.

1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J.* 10:61-70.

Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S., Mische, S.M. 1999. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* 20:601-605.

Glawischnig, E. 2007. Camalexin. *Phytochemistry* 68:401-406.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metrraux, J.P., Zhu, T. and Katagiri, F. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J.* 34:217-228.

Glazebrook, J. 2001. Genes controlling expression of defense responses in *Arabidopsis* — 2001 status. *Curr. Opin. Plant Biol.* 4:301-308.

Glazebrook, J. 1999. Genes controlling expression of defense responses in *Arabidopsis. Curr. Opin. Plant Biol.* 2:280-286.

Grant, M. and Lamb, C. 2006. Systemic immunity. *Curr. Opin. Plant Biol.* 9:414-420.

Hara, MR., Agrawal, N., Kim, SF., Sangwon, F., Cascio, MB., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, JH., Tankou, SK., Hester, LD., Ferris, CD., Hayward, SD., Snyder, SH. And Sawa, A. 2005. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nature Cell Biol.* 7:665-679.

Harris, A.R. 1995. Damping-off survey supports research. *Australian Nursery* 5:12-13.

Harris, A.R. 2000a. Solid formulations of binucleate *Rhizoctonia* isolates suppress *Rhizoctonia solani* and *Pythium ultimum* in potting medium. *Microbiol. Res.* 154:333-337.

Harris, A.R. 2000b. The future of biological control for plant diseases. *Agri. Sci.* 13:33-37.

Harris, A.R. and Adkins, P.G. 1999. Versatility of fungal and bacterial isolates for biological control of damping off disease caused by *Rhizoctonia solani* and *Pythium* spp. *Biol. Control* 15:10-18.

Harris, A.R., Siwek, K. and Wiseman, B.M. 1997. Interactions between damping-off fungi, antagonists and *Capsicum* seedlings. *Appl. Soil Ecol*. 6:251-263.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. 1995. Ainsworth & Bisby's Dictionary of the Fungi. 8ed. CAB International, Wallingford, UK. p. 395.

Heidel, A.J. and Baldwin, I.T. 2004. Microarray analysis of salicylic acid and jasmonic acid signalling in responses of *Nicotiana attenuata* to attack by insects from multiple feeding guilds. *Plant Cell Environ.* 27:1362-1373

Heil, M. and Bostock, R.M. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses. *Ann. Bot.* 89:503-512.

Hirosato, K., Hisakazu, Y., Masayoshi, M. and Setsuko, K. 2004. Characterization of fructose-bisphosphate aldolase regulated by gibberellin in roots of rice seedling. *Molecular Biology*. 56:839-848.

Honeycutt, E.W. and Benson, D.M. 2001. Formulation of binucleate *Rhizoctonia* spp. and biocontrol of *Rhizoctonia solani* on impatiens. Plant Dis. 85:1241-1248.

Huffaker, A., Pearce, G. and Ryan, C.A. 2006. An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc. Natl. Acad. Sci. USA* 103:10098-10103.

Hurkman, W.J. and Tanaka, C.K. 1986. Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis. *Plant Physiol.* 81:802-806.

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*. *Biol. Control* 27:73-80.

Ichielevich-Auster, M. 1987. Researches on the hypovirulent phenomenon in the fungus *Rhizoctonia solani kuhn*. Ph.D. thesis, Tel Aviv University.

Ichielevich-Auster, M., Sneh, B., Koltin, Y. and Barash, I. 1985. Suppression of damping-off caused by *Rhizoctonia* species by a nonpathogenic isolate of *R. solani*. *Phytopathology* 75:1080-1084.

Ichikawa, N. and Mizuno, M. 2004. Functional expression of hexahistidine-tagged β-subunit of yeast F1-ATPase and isolation of the enzyme by immobilized metal affinity chromatography. *Protein Expr. Purif.* 37:97-101.

Jabaji-Hare, S., Chamberland, H. and Charest, P.M. 1999. Cell wall alterations in hypocotyls of bean seedlings protected from *Rhizoctonia* stem canker by a binucleate *Rhizoctonia* isolate. *Mycol. Res.* 103:1035-1043.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker J.E., Ausubel, F.M. and Glazebrook, J. 1999. *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. USA* 96:13583-15388.

Johnson, C., Mhatre, A. and Arias, J. 2008. NPR1 preferentially binds to the DNA-inactive form of Arabidopsis TGA2. *BBA* 1779:583–589.

Jones, P.R., Manabe, T., Awazuhara, M. and Saito, K. 2003. A new member of plant CS-lyases. *J, Biol. Chem.* 278:10291-10296.

Jungblut, P. and Thiede, B. 1997. Protein identification from 2-DE gels by MALDI mass spectrometry. *Mass Spectrom. Rev.* 16:145-162.

Kavar, T., Maras, M., Kidric, M., Sustar-Vozlic, J., Meglic, V. 2008. Identification of genes involved in the response of leaves of *Phaseolus vulgaris* to drought stress. *Mol. Breeding* 21:159-172.

Kawamura, Y., Takenaka, S., Hase, S., Kubota, M., Ichinose, Y., Kanayama, Y., Nakaho, K, Klessing, D.F. and Takahashi, H. 2009. Enhanced defense responses in *Arabidopsis* induced by the cell wall protein fractions from *Pythium oligandrum* require *SGT1* , *RAR1*, *NPR1* and *JAR1*. *Plant cell Physiol.* 50:924-934.

Keren-Keiserman, A., Tanami, Z., Shoseyov, O. and Ginzberg, I. 2004. Peroxidase activity associated with subarization processes of the muskmelon (*Cucumis melo*) rind. *Physiol. Plant* 121:141-148.

Kirk, P.M., Cannon, P.F., Minter, D.W. and Stalpers, J.A. 2008. Ainsworth and Bisby's dictionary of the fungi. CAB international Wallingford, UK. 10th Edition, 784 p.

Kim, M.G., Geng, X., Lee, S.Y. and Mackey, D. 2009. The *Pseudomonas syringae* type III effector AvrRpm1 induces significant defenses by activating the *Arabidopsis* nucleotide-binding leucine-rich repeat protein RPS2. *Plant J.* 57:645-653.

Klessing, D.F., Durner, J., Noad, R., Navarre, D.A., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E, and Silva, H. 2000. Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci. USA*. 97:8849-8855.

Kliebenstein, D.J., Dietrrich, R.A., Martin, A.C., Last, R.L. and Dangl, J.L. 1999. LSD1 Regulates salicylic acid induction of copper zinc superoxide dismutase in *Arabidopsis thaliana*. *MPMI* 12:1022-1026.

Kliebenstein, D.J. and Rowe, H.C. 2008. Ecological costs of biotrophic versus necrotrophic pathogen resistance, the hypersensitive response and signal transduction. *Plant Sci.* 551-556.

Kombrink, E. and Somssich, I.E. 1997. Pathogenesis-Related Proteins and Plant Defense. From Plant Relationships part B, pages 107-128. In: K. Essesr, and P.A. Lemke, (Eds.) The Mycota. Springer-Verlag, Berlin.

Koornneef, A. and Pieterse, C.M.J. 2008. Cross talk in defense signaling. *Plant Physiol.* 146:839-844.

Knoth, C., Ringler, J., Dangl, J. and Eulgem, T. 2007. *Arabidopsis* WRKY70 is required for full *RPP4*-mediated disease resistance and basal defense against *Hyaloperonospora parasitica*. *MPMI* 20:120-128.

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

LaMondia, J.A., Elmer, W.H., Mevosh, T.L. and Cowles, R.S. 2002. Integrated management of strawberry pests by rotation and intercropping. *Crop Prot.* 21:837-846.

Lazniewska, J., Macioszek, V.K., Lawrence, C.B. and Kononowicz, A.K. 2010. Fight to the death: *Arabidopsis thaliana* defense response to fungal necrotrophic pathogens. *Acta Phisiol. Plant*. 32:1-10.

Leon, J., Rojo, E., Titarenko, E. and Sanchez-Serano, J.J. 1998. Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca^{2+}/calmodulin in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 258:412-419.

Li, J., Brader, G., Kariola, T. and Palva, T. 2006. WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* 46:477-491.

Li, X., Clarke J.D., Zhang, Y. and Dong, X. 2001. Activation of an EDS1 mediated R gene pathway in the snc1 mutant lead to constitutive, NPR1-independent pathogen resistance. *MPMI* 14:1131-1139.

Loake, G. and Grant, M. 2007. Salicylic acid in plant defense-the players and protagonists. *Curr. Opin. Plant Biol.* 10:466-472.

Lopez, M.A., Bannenberg, G. and Castresana, C. 2008. Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Curr. Opin. Plant Biol.* 11:420-427.

Lu, H. 2009. Dissection of salicylic acid-mediated defense signaling networks. *Plant Signal Behav.* 4:713-717.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L. and Dietrich, R.A. 2001. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26:403-410.

Maleck, K., Neuenschwander, U., Cade, R.M., Dietrich, R.A., Dangl, J.L. and Ryals, J.A. 2002. Isolation and characterization of broad-spectrum disease-resistant *Arabidopsis* mutants. *Genetics* 160:1661-1671.

Manners, J.M., Penninckx I.A.M.A., Vermaere, K., Kazan, K., Brown, R.L., Morgan, A., Maclean, D.J., Curtis, M.D., Cammue, B.P.A. and Broekaert, W.F. 1998. The promoter of the plant defensin gene *Pdf1.2* from *Arabidopsis* is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. *Plant. Mol. Biol.* 38:1071-1080.

Martin, F.N. and Bull, C.T. 2002. Biological approaches for control of root pathogens of strawberry. *Phytopathology* 92:1356-1362.

Mclaughlin, D.J., Mclaughlin, E.G. and Lemke, P.A. 2001. Systematics and Evolution-part B. pages. 146-147. In: K. Essesr and P.A. Lemke, (Eds.) The Mycota. Springer-Verlag, Berlin.

Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M. and Peterman, T.K. 1993. An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid and methyl jasmonate. *Plant Physiol.* 101:441-450.

Memon, A.R. 2004. The role of ADP ribosylation factor and SAR1 in vesicular trafficking in plants. *Biochim. Biophys. Acta.* 1664:9-30.

Mita, S., Nagai, Y., Asai, T. 2006. Isolation of cDNA clones corresponding to genes differentially expressed in pericarp of mume (*Prunus mume*) in response to ripening, ethylene and wounding signals. *Physiol. Plant* 128:531-545.

Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate

signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Phisiol.* 140:249-262.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.

Murray, R.G. and Jonathan, D.G.J. 2009. Hormone (dis)harmony moulds plant health and disease. *Science* 324:750-752.

Muslim, A., Horinouchi, H. and Hyakumachi, M. 2003. Control of Fusarium crown and root rot of tomato with hypovirulent binucleate *Rhizoctonia* in soil and rock wool systems. *Plant Dis.* 87:739-747.

Nath, A.K., Kumari, S. and Sharma, D.R. 2005. In vitro selection and characterization of water stress tolerant cultures of bell pepper. *Indian Journal of Plant Physiol.* 10:14-19.

Nafisi, M., Goregaoker, S., Botanga, C.J., Glawischnig, E., Olsen, C.E., Halkier, B.A. and Glazebrook, J. 2007. *Arabidopsis* cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant cell* 19:2039-2052.

Nobuta, K., Okrent, R.A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M.C. and Innes, R.W. 2007. The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in *Arabidopsis*. *Plant Phisiol.* 144:1144-1156.

Okrent, R.A., Brooks, M.D. and Wildermuth, M.C. 2009. *Arabidopsis* GH3.12 (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. *J. Biol. Chem.* 284:9742-9754.

Parker, J.E., Holub, E.B., Frost, L.N. Falk, A., Gunn, N.D. and Daniels, M.J. 1996. Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. *Plant Cell* 8:2033-2046.

Penninckx, I.A.M.A, Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M. and Broekaert, W.F. 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* 8:2309-2323.

Penninckx, I.A.M.A, Thomma, B.P.H.J., Buchala, A., Metraux, J.P. and Broekaert, W.F. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell* 10:2103-2113.

Perl-Treves, R., Foley, R.C., Chen, W. and Singh, KB. 2004. Early induction of the *Arabidopsis* GSTF8 promoter of the fungal pathogen *Rhizoctonia solani*. *MPMI* 17:70-80.

Pffalf, 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. *NAR* 30:e36.

Pieterse, C.M.J., van der Ent, S., van Pelt, J.A., van Loon, L.C. 2007. The role of ethylene in rhizobacteria-induced systemic resistance (ISR). *Advances in Plant Ethylene Research: Proc.7th Int. Symp. Plant Hormone Ethylene* 325–331.

Pieterse, C.M.J., van Pelt, J.A., van Wees, S.C.M., Ton, J., Leon-kloosterziel, K.M., Kevrentjes, J.J.B., Verhagen, B.W.M., Knoester, M., van der Sluis, I., Bakker,

P.A.H.M. and van Loon, L.C. 2001. Rhizobacteria-mediated induced systemic resistance: triggering, signaling and expression. *Eur. J. Plant Pathol.* 107:51-61.

Pollard, M., Beisson, F., Li, Y. and Ohlrogge, J.B. 2008. Building lipid barriers: biosynthesis of cutin and suberin *Trends Plant Sci.* 13:236-246.

Poromarto, S.H., Nelson, B.D., Freeman, T.P. 1998. Association of binucleate *Rhizoctonia* with soybean and mechanism of biocontrol of *Rhizoctonia solani*. *Phytopathology* 88:1056–1067.

Pozo, M.J., van Loon, L.C. and Pieterse, C.M.J. 2005. Jasmonates – signals in plant microbe interactions. *J. Plant Growth Regul.* 23:211-222.

Reiman, A., Nurhayati, N., Backenkoehler, A. and Ober, D.2004. Repeated evolution of the pyrrolizidine alkaloid-mediated defense system in separate angiosperm lineages. *Plant Cell* 16:2772-2784.

Rochon, A., Boyle, P., wignes, P., Fobert, P.R. and Despres, C. 2006. The Co activator Function of Arabidopsis NPR1 Requires the Core of Its BTB/POZ Domain and the Oxidation of C-Terminal Cysteines. *Plant Cell* 18:3670-3685.

Ross, R.E., Keinath, A.P. and Cubeta, M.A. 1998. Biological control of wirestem on cabbage using binucleate *Rhizoctonia* spp. *Crop Prot.* 2:99-104.

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.

Saito, N., Nakamura, Y., Mori, I.C. and Murata, Y. 2009. Nitric oxide functions in both methyl jasmonate signaling and abscisic acid signaling in Arabidopsis guard cells. *Plant Signal Behav.* 4:119-120.

Sanchez-Aguayo, I., Rodriguez-Galan, JM., Garcia, R., Torreblanca, J. And Pardo, JM. 2004. Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthetase in lignifying tissues of tomato plants. *Planta* 220:278-285.

Schaller, A. and Stintzi, A. 2008. Jasmonate biosynthesis and signaling for induced plant defense against herbivory. Chapter 17 In: *Induced Plant Resistance to Herbivoty*. Springer Science and Business.

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

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatos, A., Halkier, B.A. and Glawischnig, E. 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol*. 141: 1248–1254.

Shah, J. 2009. Plants under attack: systemic signals in defense. *Curr. Opin. Plant Biol.* 12:459-464.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., Innes, R.W. 2003. Cleavage of *Arabidopsis* PBS1 by a Bacterial Type III Effector. *Science* 29:1230-1233.

Sharon, M., Freeman, S., Kuninaga, S. and Sneh, B. 2007. Genetic diversity, anastomosis groups and virulence of *Rhizoctonia* spp. from strawberry. *Eur. J. Plant Pathol.* 117:247-265.

Sharon, M., Kuninaga, S., Hyakumachi, M., Naito, S. and Sneh, B. 2008. Classification of *Rhizoctonia* spp. using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping. *Mycoscience* 49:93-114.

Sharon, M., Kuninaga, S., Hyakumachi, M. and Sneh, B. 2006. The advancing identification and classification of *Rhizoctonia* spp. using molecular and biotechnological methods compared with the classical anastomosis grouping. *Mycoscience* 47:299-316.

Siwek, K., Harris, A.R. and Scott, E.S. 1997a. Mycoparasitism of *Pythium ultimum* by binucleate *Rhizoctonia* isolates on agar media and on capsicum seeds. *J. Phytopathol*. 145:417-423.

Siwek, K., Scott, E.S. and Harris, A.R. 1997b. Role of antibiosis in biological control of *Pythium ultimum* by binucleate *Rhizoctonia*. *Aust. Plant Pathol. Soc 11th Biennial Conference* p. 252.

Smart, C.D., Myers, K.L., Restrepo, S., Martin, G.B. and Fry, W.E. 2003. Partial resistance of tomato to *Phytophthora infestans* is not dependent upon ethylene, jasmonic acid, or salicylic acid signaling pathways. *MPMI* 16:141-148.

Smilansky, Z. 2001. Automatic registration for images of two-dimensional protein gels. *Electrophoresis 22:*1616–1626.

Sneh, B. 1999. Biological control of Rhizoctonia diseases: 2. Use of non-pathogenic isolates of Rhizoctonia in biological control. *Summa. Phytopathol.* 25:102-106.

Sneh, B. 1996. Non Pathogenic Isolates of *Rhizoctonia* spp. (np-R) and Their Role in Biological Control. p. 473-483 in: Sneh, B., Jabaji-Hare, S., Neate, S. and Dijst, G. (Eds.) *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Kluwer Academic Publishers Dordrecht. 578 pp.

Sneh, B., Burpee, L. and Ogoshi, A. 1991. Identification of *Rhizoctonia* Species. The American Phytopathological society Press, Minnesota USA. 135 pp.

Sneh, B. and Ichielevich-Auster, M. 1998. Induced resistance of cucumber seedlings caused by some non-pathogenic *Rhizoctonia* (np-R) isolates. *Phytoparasitica* 26:27-38.

Sneh, B., Ichielevich-Auster, M. and Plaut, Z. 1989a. Mechanism of seedling protection induced by a hypovirulent isolate of *Rhizoctonia solani*. Can. J. Bot. 67:2135-2141.

Sneh, B., Ichielevich-Auster, M. and Shomer, I. 1989b. Comparative anatomy of colonization of cotton hypocotyls and roots by virulent and hypovirulent isolates of *Rhizoctonia solani*. Can. J. Bot. 67:2142-2149.

Sneh, B., Yamoah, E. and Stewart, A. 2004. Hypovirulent *Rhizoctonia* spp. isolates from New Zealand soils protect radish seedlings against damping-off caused by *R. solani*. *New Zealand Plant Prot.* 57:54-58.

Solano, R., Stepanova, A., Chao, Q.M. and Ecker, J.R. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ethylene-insensitive3 and ethylene response-factor1. *Genes. Dev*. 12: 3703-3714.

Soledade, M. and Pedras, C. 2010. Phytoalexins from Brassicaceae: news from the front. *Phytochemistry* 71:1191-1197.

Stintzi, A., Weber, H., Reymond, P., Browse, J. and Farmer, E.E. 2001. Plant defense in the absence of jasmonic acid: The role of cyclopentenones. *Proc. Natl. Acad. Sci. USA* 98:12837-12842.

Swiderski, M.R. and Innes, R.W. 2001. The *Arabidopsis* PBS1 resistance gene encodes a member of a novel protein kinase subfamily. *Plant J.* 26:101-112.

Talarczyk, A. and Hennig, J. 2001. Early defense responses in plants infected with pathogenic organisms. *Cell. Biol. Mol. Lett.* 6:955-970.

Terras, F.R.G., Schoofs, H.M.E., 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 sativus L.*) seeds. *J. Biol. Chem.* 267:15301-15309.

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

Ton, J., van Pelt, J.A., van Loon, L.C. and Pieterse, M.J. 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *MPMI* 15:27-34.

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

Ulloa, M. and Hanlin, R. 2000. Illustrated Dictionary of Mycology. The American Phytopathological society Press, Minnesota USA. 448 pp.

van Loon, L.C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant. Pathol.* 103:753-767.

van Loon, L.C., Rep, M. and Pieterse, C.M.J. 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44:135-162.

van Wees, S.C.M., Chang, H.S., Zhu, T. and Glazebrook, J. 2003. Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiol.* 132:606-617.

van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., van Loon, L.C. and Pieterse, C.M.J. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis*is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Mol. Biol.* 41:537-549.

van Wees, S.C.M., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M.J. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 97:8711-8716.

Vlot, A.C., Dempsey, D.A. and Klessig, D.F. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.*47:177-206.

Wade, H.E. 2006. Effects of acibenzolar-*S*-methyl on the suppression of Fusarium wilt of cyclamen. *Crop Prot.* 25:671-676.

Walters, D.R., Cowley, T., Weber, H. 2006. Rapid accumulation of trihydroxyoxylipins and resistance to the bean rust pathogen *Uromyces fabae* following wounding in *Vicia faba*. *Ann. Bot.* 97:779–84.

Walters, D. and Heil, M. 2007. Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol.* 71: 3–17.

Walski, E.A., Lima, C., Agusti, R, Daleo, G.R., Andreu, A.B. and de Lederkremer, R.M. 2005. An α-glucan elicitor from the cell wall of a biocontrol binucleate *Rhizoctonia* isolate. *Carbohydr. Res.* 340:619-627.

Wan, J., Torres, M., Ganapany, A.,Thelen, J., DaGue, B.B., Mooney, B., Xu, D. and Stacey, G. 2005. Proteomic analysis of Soybean root hairs after infection by *Bradyrhizobium japonicum*. *MPMI* 18:458-467.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085-1094.

Warren, R.F., Merritt, P.M., Holub, E. and Innes, R.W. 1999. Identification of three putative signal transduction genes involved in R gene-specified disease resistance in *Arabidopsis*. *Genetics* 152:401-412.

Weinhold, A.R. and Jerome, M. 1973. Initial host responses in cotton to infection by *Rhizoctonia solani*. *Phytopathology* 63:157-162.

Wen, K., Seguin, P., St.-Arnaud, M and Jabaji-Hare, S. 2005. Real-time quantitative RT-PCR of defense-associated gene transcripts of *Rhizoctonia solani*-infected bean seedlings in response to inoculation with a nonpathogenic binucleate *Rhizoctonia* isolate. *Phytopathology* 4:345-353.

Wiermer, A., Feys, B.J. and Parker, J.E. 2005. Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8:383-389.

Xue, L. 1999. Systemic acquired resistance in bean seedlings and rice protected by non-pathogenic *Rhizoctonia* species. Ph. D. Thesis McGill University, Montreal. 145 pp.

Zarate, S.I., Kempema, L.A. and Walling, L.L. 2007. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Phisiol.* 143:866-875.

Zhang, C. and Shapiro, A.D., 2002. Two pathways act in an additive rather than obligatorily synergistic fashion to induce systemic acquired resistance and *PR* gene expression. *BMC Plant Biol.* 2:9-18.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X. 2003a. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. *Plant cell* 15:2636-2646.

Zhang, Y., Tessaro, M.J., Lassner, M. and Li, X. 2003b. Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15:2647–2653.

תקציר

במחקרים שהתפרסמו בעבר נמצא כי תבדידי ריזוקטוניה, המגנים על צמחים בפני ריזוקטוניה פתוגנית ובפני פתוגנים נוספים, מעוררים בצמחים תגובות הקשורות להגנה. אולם עד כה, למיטב ידיעתנו, לא דווח מחקר שבדק ביסודיות אלו מנגנוני הגנה מושרים בצמחים שאוכלסו על ידי תבדידי ריזוקטוניה היפווירולטיים. מערכת השראת העמידות הנרכשת SAR (Systemic Acquired Resistance) פועלת ביעילות בהגנת הצמחים בעיקר כנגד פתוגנים ביוטרופיים. מערכת זו כוללת הפעלת חלבוני PR, ומעוררת ע"י פתוגנים ביוטרופיים, חומצה סליצילית (SA המתפקדת כאלמנט מפתח במערכת ה SAR), ועל ידי תרכובות כימיות כגון BTH ו Bion®. מערכת העמידות הסיסטמית ISR (Induced Systemic Resistance) פועלת ביעילות בעיקר כנגד פתוגנים נקרוטרופיים. מערכת זו מעוררת ע"י ריזובקטריה, פתוגנים נקרוטרופיים, חרקים, ומתיל ג'סמונאט (meJA המתפקד כאלמנט מפתח במערכת ה ISR, יחד עם אתילן). עירור פיטואלקסינים נמצא לעיתים בהקשר למערכת ה ISR, למרות שפיטואלקסינים יכולים גם להיות מעוררים בנפרד ממערכת זו, ולהגן על הצמחים ללא קשר עם מערכת ה ISR.

מטרת המחקר של העבודה הנוכחית הייתה לברר אלו ממערכות ההגנה הסיסטמיות מעוררות על ידי תבדידי ריזוקטוניה היפווירולנטיים ומעורבות בהגנה על צמחים מפני ריזוקטוניה פתוגנית. תוצאות הניסויים הראשונים וידאו שרמות הוירולנטיות של התבדידים ההיפווירולנטיים ששימשו במחקר היו מאד נמוכות על מגוון רחב של צמחים השייכים למשפחות בוטניות שונות. כדי לוודא שאכלוס צמחי המלפפון על ידי תבדידי ריזוקטוניה היפווירולנטיים מגן עליהם בפני ריזוקטוניה פתוגנית גם על ידי השראת עמידות, רמת ההגנה שהתבדידים ההיפווירולנטיים עוררו בצמחי מלפפון הוערכה כאשר הפתוגן הונח באותו אזור בו הונח התבדיד ההיפווירולנטי (באזור השורשים) לעומת רמת ההגנה כאשר התבדיד הפתוגן הונח על הצמח באופן שלא היה לו מגע ישיר עם התבדיד ההיפווירולנטי (היפוקוטיל ושורשים, בהתאמה), ונמצא שלמרות שהתבדידים המגנים עוררו הגנה מערכתית בצמחים - יתכן מאוד כי זו אינה דרך ההגנה היחידה המשתתפת בהגנת הצמח על ידי התבדידים. הגנה מקומית, שלא נבדקה בעבודה זו אך דווחה ממחקרים קודמים, ממלאת כנראה תפקיד חשוב בהגנת הצמחים ע"י תבדידי הריזוקטוניה ההיפווירולנטיים. מיצויים של צמחי צנונית שאוכלסו ע"י אחד

מתבדידי הריזוקטוניה ההיפווירולנטיים עיכבו גידול של תבדיד הריזוקטוניה הפתוגני. תוצאה זו מהווה אינדיקציה שפיטואלקסינים מעורבים בהגנה המעוררת ע"י תבדידים היפווירולנטיים מסויימים. מהשוואת השינויים ברמות ההגנה שהתקבלו על ידי איכלוס מוטנטים של צמחי תודרנית לבנה (*Arabidopsis thaliana*) הפגועים בגנים המעורבים במערכות ההגנה, על ידי תבדידים היפווירולנטיים, לרמות הגנה שהתקבלו על צמחי בר (wt), הסתבר שרמות ההגנה על מוטנטים הפגועים בגנים חשובים המעורבים במערכות SAR ו ISR היו נמוכות יותר מאשר רמות ההגנה על צמחי הבר. מצד שני, רמות ההגנה על צמחי הבר היו דומות לאלו שהתקבלו על מוטנט בו העיכוב במערכת ה SAR פוצה על ידי מערכת ה ISR (מוטנט *SALK-025198*). נוסף לכך, רמות ההגנה על מוטנטים שביטאו תמידית את מערכת ה SAR (*snc1, CS6571)* היו נמוכות מאלו שהתקבלו על צמחי הבר. מסיכום תוצאות אלו ניתן להסיק שעירור ההגנה הסיסטמית מהווה חלק נכבד ממנגנון ההגנה המושרה בצמחים על ידי אכלוסם בתבדידי ריזוקטוניה היפווירולנטיים, כנגד ריזוקטוניה פתוגנית. כמו כן, מערכת ה ISR היא כנראה המערכת הבולטת בהגנה המעוררת על ידי התבדידים ההיפווירולנטיים. כאשר נוטרו גנים עיקריים המשתתפים במערכות ההגנה SAR, ISR ובייצור פיטואלקסינים, בצמחי תודרנית המאוכלסים ע"י תבדידי ריזוקטוניה היפווירולנטיים, הייתה עלייה בביטוי הגנים *Pr5* (SAR), *Pdf1.2*, *Lox2*, *Lox1*, *Cori3* (ISR) ו *Pad3* (ייצור פיטואלקסינים). לפי תוצאות אלו, כל מערכות ההגנה שנבדקו עוררו על ידי אכלוס בתבדידים ההיפווירולנטיים. בעירור ע"י מערכות ההגנה SAR או ISR, בנפרד - ע"י תרכבות כימיות (Bion או meJA, בהתאמה), רק הצמחים שעורר בהם ISR הוגנו מתבדיד הריזוקטוניה הפתוגני. הגנה זו, למרות שהייתה מובהקת, לא הייתה גבוהה.

ממצאי המחקר הנוכחי מוכיחים שתבדידי ריזוקטוניה היפווירולנטיים מעוררים בצמחים ביטוי של גנים הקשורים למערכות ההגנה SAR, ISR ולייצור פיטואלקסינים, כאשר עיקר ההגנה ככל הנראה, מעירור

מערכת ה ISR ויתכן שהפיטואלקסינים. למרות שידוע ממחקרים שפורסמו בעבר שמערכת ה- SAR מגנה כנגד פתוגנים שונים, מתוצאות המחקר הנוכחי מסתבר שמערכת זו אינה ממלאת כנראה תפקיד משמעותי בעמידות המעוררת בצמחים על ידי אכלוסם בתבדידי ריזוקטוניה היפווירולנטיים כנגד ריזוקטוניה פתוגנית. בנוסף לעירור של מערכות ה SAR, ה ISR וייצור הפיטואלקסינים, ככל הנראה תבדידים היפווירולנטיים מגנים על צמחים גם על ידי הגנה מקומית. כדי להעריך את מעורבות ההגנה המקומית בהגנה של צמחים על ידי תבדידי ריזוקטוניה היפווירולנטיים דרושים מחקרים נוספים. רמות ההגנה הגבוהות של צמחים ע"י תבדידי הריזוקטוניה ההיפווירולנטיים הן ככל הנראה כתוצאה מצירוף מגוון דרכי הגנה: מקומיות ומערכתיות, מכאניות ומושרות על הצמחים. לכן לא ניתן לשחזר את תגובת ההגנה המלאה והמשולבת המתקבלת ע"י התבדידים ההיפווירולנטיים באמצעות תכשירים כימיים.

עבודה זו נעשתה בהדרכתם של:

פרופ' ברוך סנה

המחלקה לביולוגיה מולקולרית ואקולוגיה, הפקולטה למדעי החיים ע"ש ג'ורג' ס. ווייז, אוניברסיטת תל אביב, רמת אביב.

ד"ר נורית קציר

המחלקה לגידולי שדה, המכון להגנת הצומח, נווה יער, רמת ישי.

ד"ר סטנלי פרימן

המחלקה למחלות צמחים, המכון להגנת הצומח, מרכז וולקני, בית דגן.

בחינת עירור הגנה מערכתי בצמחים על ידי תבדידי ריזוקטוניה היפווירולנטיים

חיבור לשם קבלת התואר

"דוקטור לפילוסופיה"

מאת:

מיכל שרון

הוגש לסנאט אוניברסיטת תל-אביב

מאי, 2010

בחינת עירור הגנה מערכתי בצמחים על ידי תבדידי ריזוקטוניה היפווירולנטיים

חיבור לשם קבלת התואר

"דוקטור לפילוסופיה"

מאת:

מיכל שרון

הוגש לסנאט אוניברסיטת תל-אביב

מאי, 2010