

**The role of vitamin B6 as an antioxidant during the abiotic and biotic
interactions of the fungal pathogen *Rhizoctonia solani***

Jamil Samsatly

Department of Plant Science
McGill University, Montreal
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LIST OF ABBREVIATIONS

AG	Anastomosis group
Bp	Base pairs
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
ESI	Electrospray ionization
EST	Expressed sequence tag
GC/MS	Gas chromatography-mass spectrometry
GFP	Green fluorescent protein
h.p.i.	Hours post-inoculation
HR	Hypersensitive response
ITS	Internal transcribed spacer
mRNA	Messenger ribonucleic acid
LC-MS	Liquid chromatography mass spectrometry
MS	Mass spectrometry
MSMA	Minimal synthetic medium agar
NCBI	National Center for Biotechnology Information
NSERC	Natural Sciences and Engineering Research Council of Canada
PCA	Principal component analysis
PCR	Polymerase chain reaction
PL	Pyridoxal
PLP	Pyridoxal phosphate
PMP	Pyridoxamine phosphate
PN	Pyridoxine
PNP	Pyridoxine phosphate
PM	Pyridoxamine
PR	Pathogenesis related
Q2	Cumulated predictive power
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
R2X	Fraction of the sum of Squares for the <i>X</i> component
RFB	Rhizoctonia foliar blight
RNA	Ribonucleic acids
ROS	Reactive oxygen species
t(corr)	<i>X</i> and <i>Y</i> scores scaled as correlation coefficients
T-DNA	Transfer DNA
Tyr	Tyrosine
UV	Unit variance
VB6	Vitamin B6

ABSTRACT

The necrotrophic fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*, Frank, Donk) is an economically devastating plant pathogen with a wide host range. *R. solani*, and strains belonging to AG3 and AG4 are root infecting pathogens that cause damping-off and stem rot of potato (*Solanum tuberosum* L.) and soybean (*Glycine max* (L.) seedlings, respectively. Isolates of the *R. solani* AG1-IA complex can infect aerial portions of the plant as in the case of soybean leaves causing rhizocotnia foliar blight (RFB). Current cultural and chemical controls are not completely effective to manage Rhizoctonia diseases and they remain a persistent problem.

One of the earliest plant responses following pathogen recognition is the oxidative burst, leading to the production of reactive oxygen species, primarily superoxide (O_2^-) and H_2O_2 , at the site of attempted invasion. B6 is recognized as an important cofactor required for numerous metabolic enzymes, and has been shown to act as an antioxidant and play a role in stress responses. It can be synthesized through two different routes: salvage and *de novo* pathways. Using genome walking, the *de novo* biosynthetic pathway genes; *RsolPDX1* and *RsolPDX2* and the salvage biosynthetic pathway gene, *RsolPLR* were sequenced. The predicted amino acid sequences of the three genes had high degree of similarity to other fungal PDX1, PDX2, and PLR proteins and are closely related to other *R. solani* anastomosis groups. We also examined their regulation when subjected to ROS stress inducers, the superoxide generator paraquat, or H_2O_2 , and compared it to the well-known antioxidant genes, *catalase* and *glutathione-S-transferase* (*GST*). The genes were differentially regulated with substantial transcript levels depending on the gene and type of stress reflecting that differences in the type of damage induced by ROS.

Biotic stress, as a result of plant-pathogen interactions, induces the accumulation of reactive oxygen species in the cells, causing severe oxidative damage to plants and pathogens. Evidence

on the functionality of *R. solani* vitamin B6 biosynthetic pathway genes; *RsolPDX1* (KF620111.1), *RsolPDX2* (KF620112.1), and *RsolPLR* (KJ395592.1) was done using the yeast expression system. This study is the first coexpression analysis of genes encoding vitamin B6, *CAT* and *GST* in plant and fungal tissues of three pathosystems during interaction of different AG groups of *R. solani* with their respective hosts. The findings indicate that distinct expression patterns of fungal and host antioxidant genes were correlated in necrotic tissues and their surrounding areas in each of the three *R. solani* pathosystems: potato sprout-*R. solani* AG3; soybean hypocotyl-*R. solani* AG4 and soybean leaves-*R. solani* AG1-IA interactions. Levels of ROS increased in all types of potato and soybean tissues, and in fungal hyphae following infection of *R. solani* AGs as determined by non-fluorescence and fluorescence methods using H₂DCF-DA and DAB, respectively. The co-expression and accumulation of certain plant and pathogen ROS-antioxidant related genes in each pathosystem are critical during disease development from the plant's point of view, and in pathogenicity and developing of infection structures from the fungal point of view. Vitamin B6 was found to be involved during disease development of *R. solani* over different potato cultivars displaying variable vitamin B6 concentrations. A distinct pattern of expression, PN concentration, and fungal biomass was found in Russet Burbank and Chieftain. The use of *pxd1.3* Arabidopsis mutants helped to support the involvement of vitamin B6 during diseases development of *R. solani* on its plant host. We demonstrated that the regulation and production of vitamin VB6 is under tight control and is an important determinant of disease development during the interaction of *R. solani* with potato or Arabidopsis.

Overall, this manuscript offers novel insights into the biological correlation and identification of ROS antioxidant genes that can be used in soybean and potato breeding programs for resistance to *R. solani*.

RÉSUMÉ

Le champignon nécrotrophe *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*, Frank, Donk) est un phytopathogène économiquement dévastateur avec une large gamme d'hôtes. *R. solani*, et les souches appartenant à AG3 et AG4 sont des agents pathogènes infectant les racines qui provoquent respectivement la fonte des semis et la pourriture de la tige chez les plantules de pomme de terre (*Solanum tuberosum* L.) et de soja (*Glycine max* (L.). Les isolats du complexe *R. solani* AG1-IA peuvent infecter des parties aériennes de la plante, comme dans le cas des feuilles de soja provoquant la brûlure rhizoctonia foliaire (RFB). Les contrôles culturaux et chimiques actuels ne sont pas complètement efficaces pour gérer les maladies associées à *Rhizoctonia* et les maladies demeurent un problème persistant.

L'une des premières réponses des plantes après la reconnaissance de l'agent pathogène est l'explosion oxydatif, qui conduit à la production d'espèces réactives à l'oxygène, principalement le superoxyde (O_2^-) et le H_2O_2 , sur le site de la tentative d'invasion. Le B6 est reconnu comme un cofacteur important nécessaire pour de nombreuses enzymes métaboliques. Il a été démontré qu'il agissait comme un antioxydant et qu'il jouait un rôle dans les réponses au stress. Il peut être synthétisé de deux manières différentes: les voies de sauvetage et de *novo*. En utilisant le génome en marche, les gènes de la voie de biosynthèse de *novo*; *RsolPDX1* et *RsolPDX2* et le gène de la voie de biosynthèse de sauvetage, *RsolPLR*, ont été séquencés. Les séquences d'acides aminés prédites des trois gènes présentaient un degré élevé de similitude avec d'autres protéines fongiques PDX1, PDX2 et PLR et sont étroitement liées à d'autres groupes d'anastomose de *R. solani*. Nous avons également examiné leur régulation lorsqu'elles étaient soumises à des inducteurs de stress d'espèces réactives de l'oxygène (ERO), le paraquat générateur de superoxyde, ou H_2O_2 , et nous l'avons comparé aux gènes bien connus des antioxydants, la catalase et la *glutathion-S-transférase*

(*GST*). Les gènes ont été régulés de manière différenciée avec des niveaux de transcription substantiels en fonction du gène et du type de stress reflétant ces différences dans le type de dommage induit par les ERO.

Le stress biotique, résultant d'interactions plante-pathogène, induit l'accumulation d'espèces réactives de l'oxygène dans les cellules, causant de graves dommages oxydatifs aux plantes et aux agents pathogènes. Données probantes sur la fonctionnalité des gènes de la voie de biosynthèse de la vitamine B6 de *R. solani*; *RsolPDX1* (**KF620111.1**), *RsolPDX2* (**KF620112.1**) et *RsolPLR* (**KJ395592.1**) a été fait en utilisant le système d'expression de la levure. Cette étude est la première analyse de coexpression de gènes codant pour la vitamine B6, la *CAT* et la *GST* dans les tissus végétaux et fongiques de trois pathosystèmes lors de l'interaction de différents groupes d'AG de *R. solani* avec leurs hôtes respectifs. Les résultats indiquent que les profils d'expression distincts des gènes antioxydants fongiques et de l'hôte étaient corrélés dans les tissus nécrotiques et leurs zones environnantes dans chacun des trois interactions de pathosystèmes de *R. solani*: le germe de pomme de terre-*R. solani* AG3; hypocotyle de soja-*R. solani* AG4 et feuilles de soja-*R. solani* AG1-IA. Les niveaux de ERO ont augmenté dans tous les types de tissus de pomme de terre et de soja, ainsi que dans les hyphes fongiques consécutifs à une infection des AG de *R. solani*, déterminés par des méthodes de non-fluorescence et de fluorescence utilisant respectivement H₂DCF-DA et DAB. La co-expression et l'accumulation de certains gènes liés aux ROS-antioxydants chez les végétaux et les agents pathogènes dans chaque pathosystème sont essentielles lors du développement de la maladie du point de vue de la plante, ainsi que de la pathogénicité et du développement des structures infectieuses du point de vue des champignons. La vitamine B6 s'est avérée être impliquée dans le développement de la maladie de *R. solani* sur *Arabidopsis* de type sauvage et mutant *pdx1.3* ou sur différentes variétés de pommes de terre

présentant des concentrations variables en vitamine B6. Un modèle distinct d'expression, de concentration en PN et de biomasse fongique entre le russet Burbank et chieftain ou le mutant pdx1.3 Arabidopsis et le type sauvage a été trouvé dans chaque pathosystème. Nous avons démontré que la régulation et la production de vitamine VB6 sont sous contrôle strict et constituent un facteur déterminant du développement de la maladie lors de l'interaction de *R. solani* avec la pomme de terre ou Arabidopsis.

Dans l'ensemble, ce manuscrit offre de nouvelles informations sur la corrélation biologique et l'identification des gènes antioxydants des ERO pouvant être utilisés dans les programmes de sélection du soja et de la pomme de terre pour la résistance à *R. solani*.

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PREFACE AND CONTRIBUTIONS

The following thesis was prepared according to the “Guidelines Concerning Thesis Preparation” of McGill University. The thesis contains three chapters (Chapters 3-5) representing three separate research manuscripts: Chapter 3 is published in *Frontiers in Microbiology*, Chapter 4 is published to *PLoS One*, and Chapter 5 will be submitted to *Frontiers in Plant Science*. Information pertaining to authors and their contributions to each chapter are mentioned in the “Connecting Text” section before each chapter. Below is a general description of the thesis topics as well as of the contributions of each author to the thesis.

Contributions of Authors

Jamil Samsatly was the primary researcher for each chapter. He designed the experiments as well as conducted and planned all the experimental procedures and analyses with the guidance of Dr. Suha Jabaji, Department of Plant Science, Macdonald Campus of McGill University. Dr. S. Jabaji provided supervision, guidance and funding for all chapters. Dr. Rony Chamoun, Department of Plant Science, Macdonald Campus of McGill University, provided help in data analysis, and manuscript revision of Chapter 3. Dr. Tanya Copley, Department of Plant Science, Macdonald Campus of McGill University, provided help in statistical and data analysis of Chapter 4. Dr. S. Bayen, Department of Food Science supervised and aided in the vitamin B6 analyses in Chapter 5.

Contributions to Knowledge

The chapters of this thesis represent a significant contribution to knowledge on the role of vitamin B6 during the interaction of the economically important plant pathogen *Rhizoctonia solani* during abiotic and biotic stress. It offers novel insights into the biological correlation and

identification of ROS antioxidant genes that can be used in soybean and potato breeding programs for resistance to strains of *R. solani* belonging to AG3 and AG4

Chapter 3 is the first report on the characterization and identification of genes the *de novo* and salvage pathways of vitamin B6 (VB6) in *Rhizoctonia solani*. The characterization of VB6 encoding genes in fungal phytopathogens is limited. Our work represents the first report on the characterization of the most important genes of the vitamin B6 pathway in the necrotrophic plant pathogen *R. solani*: *RsolPDX1*, *RsolPDX2*, and *RsolPLR*. Also, we are the first to provide evidence for VB6 transcriptional regulation in *R. solani* in response to different abiotic stress inducers like paraquat and H₂O₂. We also extended our study to demonstrate potential role of these genes in oxidative stress protection in *R. solani*. *RsolPDX1*, *RsolPDX2*, and *RsolPLR* represent three important components of the vitamin B6 pathway in *R. solani* AG3 (strain Rs114).

Chapter 4 is the first report confirming the involvement of vitamin B6 in oxidative stress in *R. solani* using the yeast expression system. Our work provided evidence on the functionality of *R. solani* VB6 biosynthetic pathway genes; *RsolPDX1* (KF620111.1), and *RsolPDX2* (KF620112.1) by yeast complementation assays. Also, we reported on the differential expression of VB6 and several antioxidant genes in plant and fungal tissue of three pathosystems (I. potato sprouts-*R. solani* AG3; II. soybean hypocotyls-*R. solani* AG4; III. soybean leaves-*R. solani* AG1-IA) during interaction of different AG groups of *R. solani* with their respective hosts. The work of this chapter is the first to describe regulation of vitamin B6 genes, in both fungi and plants, during plant-fungus interaction. There was a unique expression pattern depending on the plant host or the different anastomosis group of *R. solani*. Most importantly, this chapter was the first to show a tissue specific response with differential transcriptional regulation of these genes in infected

necrotic tissues and their surrounding areas in potato and soybean which can be an important determinant of disease outcome and pathogenicity.

Chapter 5 presents the first validation of the functional role of vitamin B6 during disease development of *R. solani* on different potato cultivars displaying variable vitamin B6 concentrations. Our study showed a distinct pattern of expression, PN concentration, and fungal biomass between the Russet Burbank and Chieftain. Functional characterization of Arabidopsis *pdx1.3* mutants challenged with *R. solani* provided evidence into the role of the vitamin B6 pathway in the maintenance of plant tolerance during disease progression. Lastly the work in this chapter is the first to demonstrate that the regulation and production of vitamin B6 is under tight control and is an important determinant of disease development during the interaction of *R. solani* with potato.

INTRODUCTION

1.1 PROBLEM DEFINITION

To survive, plants have developed efficient defense systems against pathogenic microbes. One of the most rapid plant defense reactions after pathogen attack is the so-called oxidative burst, which constitutes the production of reactive oxygen species (ROS), primarily superoxide and H₂O₂, at the site of attempted invasion (Apostol, Heinstein et al. 1989). The produced ROS activates plant defense responses, including programmed cell death, or functions as second messengers in the induction of various plant defense-related genes (Alvarez, Pennell et al. 1998, Torres, Dangl et al. 2002, Lehtonen, Somervuo et al. 2008). Due to the toxicity of ROS molecules and their importance in plant defense responses, plants and plant pathogens have developed strategies for ROS detoxification (Apel and Hirt 2004). One strategy is the production of non-enzymatic antioxidants like ascorbate, GSH, tocopherol, flavonoids, alkaloids, and carotenoids (Molina and Kahmann 2007, Deveau, Piispanen et al. 2010, Halliwell 2011). The second strategy is enzymatic ROS scavenging through superoxide dismutase, ascorbate peroxidase and catalases, generally using NADPH as reducing equivalents (Molina and Kahmann 2007). Recently vitamin B₆, recognized as an important cofactor required for many diverse enzymes ranging from amino acid metabolism to the biosynthesis of antibiotic compounds, was proved in the last decade to have an antioxidant role (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Bilski, Li et al. 2000, Raschle, Amrhein et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009, Vanderschuren, Boycheva et al. 2013).

Fungi and plants are among many organisms that possess the ability of *de novo* biosynthesis of vitamin B₆ that involves two genes, *PDX1* and *PDX2* (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Bilski, Li et al. 2000, Benabdellah, Azcon-Aguilar et al. 2009,

Fitzpatrick, Basset et al. 2012, Vanderschuren, Boycheva et al. 2013). However, until now, identification of *de novo* vitamin B6 genes and their role have been fully characterized in only one plant pathogenic fungus, *Cercospora nicotianae* (Ehrenshaft, Bilski et al. 1999) and knowledge about the role of Vitamin B6 during the interaction of pathogens with plants is limited (Denslow, Walls et al. 2005, Zhang, Liu et al. 2014, Zhang, Jin et al. 2015).

Rhizoctonia solani Kühn [(teleomorph: *Thanatephorus cucumeris* (A.B Frank) Donk)], is an economically important soil-borne pathogen of many crops. Isolates of *R. solani* anastomosis group AG3 are identified as the etiological agents for Rhizoctonia disease of potato and black scurf affecting potato world-wide. Symptoms of the *R. solani* disease complex on potato plant parts show on upper plant parts (stem canker) and below ground level which is accompanied by formation of sclerotia on tubers (black scurf). Symptoms of stem canker occur early in the season on sprouts and stolons prior to plant emergence are characterized by brown necrotic lesions (Jeger, Hide et al. 1996, Tsrer 2010). Insights from the genome sequence and transcriptomic data of *Rhizoctonia* have revealed that *R. solani* AG3 reacts to stress by the regulation of pyridoxal reductase (*PLR*), a gene in the vitamin B6 pathway (Morissette, Dauch et al. 2008, Chamoun, Aliferis et al. 2013). Given the situation that many plant pathogens are recognized by their hosts through conserved pathogen-associated molecular patterns (Nürnberg and Brunner 2002) that elicit an oxidative burst, we reasoned that a vitamin B6-based ROS detoxification system of *R. solani* might help to overcome host responses and is crucial during its interactions with abiotic and biotic stressors. However, at present, it is not yet clear how these genes are required and whether they shield fungal hyphae or interfere with host defense responses.

Therefore, the overarching goal of this thesis is to study the role of vitamin B6 as an antioxidant during the abiotic and biotic interactions of the economically important plant pathogen *R. solani*.

1.2 OBJECTIVES AND HYPOTHESES

Objective 1. To identify and characterize the vitamin B6 biosynthesis related genes in the *de novo* pathway, *PDX1*, *PDX2* and in the salvage pathway *PLR*, in *R. solani* AG3.

Hypothesis: A number of vitamin B6 related genes (*PDX1*, *PDX2*, and *PLR*) of biosynthetic pathway are present in *R. solani* AG3.

Objective 2. Determine the relative transcript abundance of vitamin B6 related genes (*PDX1*, *PDX2*, and Pyridoxal reductase (*PLR*)) when *R. solani* is subjected to biotic and abiotic stress or during disease development on potato (*Solanum tuberosum*) and soybean (*Glycine max*).

Hypothesis a: The vitamin B6 related genes identified in *R. solani* AG3 are differentially expressed following oxidative stress as result of biotic and abiotic types of stress.

Hypothesis b: The expression of vitamin B6 related genes can be a determinant of pathogenicity on potato cultivars of various degrees of resistance.

Objective 3. Validate the functional role of the *R. solani* AG3 *PDX1* and *PDX2* genes in oxidative stress resistance using the yeast expression system.

Hypothesis: The *R. solani* AG3 *PDX1* and *PDX2* genes are involved in vitamin B6 biosynthesis and are required for resistance to oxidative stress conditions.

Objective 4. Examine phenotypes of Arabidopsis mutants with defects in the de novo pathway of VB6 biosynthesis against infection by *R. solani* AG4 and provide evidence that mutations in *PDX1.3* led to increased disease.

Hypothesis: Vitamin B6 genes are involved in vitamin B6 biosynthesis are required for resistance to oxidative stress conditions during disease development.

LITERATURE REVIEW

2.1 *Rhizoctonia solani* Kühn

The genus *Rhizoctonia* comprises a diverse group of fungi occupying different ecological niches (Ogoshi 1996). *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* Frank (Donk)) is a soil-borne fungus capable of causing diseases on field and horticultural crops. The isolates are multinucleate and genetically diverse in their cultural, morphological and physiological characteristics as well as in their pathogenic range of host plants (Sneh 1996). Strains of *Rhizoctonia* are assigned into genetically-based groups, referred to as anastomosis groups (AG) based on hyphal anastomosis reactions of compatible strains and can be separated into at least fourteen anastomosis groups (AGs) (Carling, Baird et al. 2002, Das, Plyler-Harveson et al. 2019). Comprehensive classification of *Rhizocotonia solani* was performed using the ribosomal DNA (rDNA)- internal transcribed spacer (ITS) sequences. Phylogenetic analysis of their (rDNA-ITS) confirmed that the AG-Concept is genetically based (Sharon, Kuninaga et al. 2006).

R. solani is an economically devastating plant pathogen with a wide host range. Strains belonging to AG3 and AG4 are root infecting pathogens that cause damping-off and stem rot of potato (*Solanum tuberosum* L.) and soybean (*Glycine max* L.) seedlings, respectively (Fenille, De Souza et al. 2002, Tsrer 2010, Buskila, Tsrer Lahkim et al. 2011, Sneh, Jabaji-Hare et al. 2013). Isolates of the *R. solani* AG1-IA complex can infect aerial portions of the plant as in the case of soybean leaves causing rhizoctonia foliar blight (RFB) (Fenille, De Souza et al. 2002, Stetina, Stetina et al. 2006, Ciampi, Meyer et al. 2008). The common elements in disease development of *R. solani* isolates are the close association of fungal hyphae with the host epidermis forming branches known as infection cushions or aggregates, penetration of the epidermis, inter- and intracellular colonization and breakage of plant tissue by the production of hydrolytic enzymes,

which eventually leads to the development of browning and necrosis associated with oxidative burst and death of tissue (Keijer 1996, Sneh, Jabaji-Hare et al. 2013). Current cultural and chemical controls are not completely effective to manage *Rhizoctonia* diseases and the diseases remain a persistent problem. Furthermore, resistance to *R. solani* in any plant species does not exist.

2.1.1 Fungi and reaction to stress

In the course of fungal evolution, many mechanisms have been developed to deal with the various types of abiotic and biotic stress present in its environment. Cells receive stress signals from their surrounding environment which stimulate several pathways that might form complex interactions and crosstalk with other pathways (Duffy, Schouten et al. 2003). Examples of these pathways are: kinase cascades, production of ROS, and calcium signaling (Kraus and Heitman 2003, Chamoun and Jabaji 2011, Montero-Barrientos, Hermosa et al. 2011). Different forms of environmental stress or pathogen antagonists set off a variety of defense-response genes. Fungi deal with stress by activation of a variety of pre-existing proteins (e.g., the activation of some protein kinases) (Westfall, Patterson et al. 2008, Ayer, Sanwald et al. 2013). Transcriptional responses are not in the front line of defense, instead they are strategies for ensuring that cells are able to repair damage, adapt to the changed environment and continue growth (Gasch, Spellman et al. 2000). Expression of the mitogen-activated protein kinase (MAPK) encoding gene *smkA* of the mycoparasite *Stachybotrys elegans* was up-regulated after confrontation with hyphae and sclerotia of *R. solani* (Chamoun, Aliferis et al. 2013). Increased induction of protease, cellulase, and chitinase activities were observed in the plant pathogen *Pythium ultimum* following an interaction with a *nox1*-overexpressed transformant of the biological control agent *Trichoderma harzianum* which is marked with an increase in ROS production (Montero-Barrientos, Hermosa et al. 2011).

2.1.2 *R. solani* Associated biotic and biotic stresses

Biotic stresses are associated with the interactions of *R. solani* with its hosts or antagonistic confrontation with other organisms (e.g. predation, competition, etc.). Upon the infection of potato sprouts with *R. solani* AG3, lesions are formed (Tsrer 2010). In those lesions, a hypersensitive response is developed which is the initial response of plants toward pathogen attack and it is usually accompanied by the formation of ROS (Apostol, Heinstein et al. 1989, Alvarez, Pennell et al. 1998, Torres, Dangl et al. 2002, Lehtonen, Somervuo et al. 2008). Notably, ROS was shown to play an important role in plant resistance towards pathogens. That was displayed when a double mutation in the NADPH oxidase lead to complete loss of Arabidopsis's resistance to *R. solani* AG8 (Foley, Gleason et al. 2013). Alternatively, pathogens secrete ROS-scavenging enzymes, such as superoxide dismutases (SODs) and catalases, which represses the plant's ROS-mediated defences (Jennings, Ehrenshaft et al. 1998, Molina and Kahmann 2007). In response to microbial attack, *R. solani* elaborates an array of inducible defense reactions, many of which involve the transcriptional activation of the corresponding defense genes. During mycoparasitic interaction between *R. solani* and the mycoparasite *S. elegans* (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011) several genes were highlighted upon the confrontation using suppression subtractive hybridization (SSH) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) techniques, respectively. These included genes that encode enzymes involved in vitamin metabolism [pyridoxal reductase (*PLR*)], apoptosis (proteasome subunit alpha type 3), transport proteins (purine permease), lipid metabolism (esterase/lipase), and protein degradation (polyubiquitin). On the other hand, other genes implicated in pathogenesis, energy and cellular respiration (cytochrome C oxidase subunit), biosynthesis proteins and even some stress-related genes such as thiamine and thiazole encoding genes, respectively, were down-regulated. The up-regulation of *PLR*, a member

of the vitamin B6 salvage biosynthesis pathway, in *R. solani* could be considered a plausible defense response against the mycoparasite through its antioxidative properties in ROS (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Benabdellah, Azcon-Aguilar et al. 2009).

Studies involving the effect of abiotic stresses on *R. solani* did not investigate the pattern of gene regulation. Most of these studies explored the effect of fungicides, herbicides, and metals on the growth of *R. solani* (Babich and Stotzky 1979, Weidenbörner, Hindorf et al. 1990, Black, Russin et al. 1996, Ghini and Morandi 2006, Akoijam and Dubey 2013). Some enzymatic activities were assayed following chemical treatments (Crowe and Olsson 2001). The dual effect of copper containing fungicide and heat caused a decline in the mycelial growth, confirming their potential as stressors for the pathogen (Willi, Frank et al. 2011, Orozco-Avitia, Esqueda et al. 2013). The fungus main response while facing fungicides or herbicides was increased sclerotia production that helps the pathogen to survive under unfavorable stress conditions, however this was not seen with paraquat (Black, Russin et al. 1996, Akoijam and Dubey 2013). The following abiotic variables (P, K, Ca, Mg, Na, Cu, and S) were correlated with *R. solani* growth suppression (Ghini and Morandi 2006).

2.2 Reactive oxygen species (ROS)

ROS molecules are not only known for their role as toxic metabolic products associated with the use of molecular oxygen (O₂) for respiration and energy supply by aerobic organisms, but also as modulators of cellular growth and development as well as playing an important role in synchronization of actions taken upon confrontation with biotic and abiotic stress (Bhattacharjee 2012). Most fungal processes are highly aerobic in nature, and thus there is a sufficient O₂ supply, which can lead to the production of ROS. Oxidative stress occurs where there is a relative

imbalance within cells between the generation of ROS and the intracellular anti-oxidant defenses in favor of the former (Li, Harvey et al. 2009). A common characteristic of all ROS is their ability to cause oxidative damage to DNA, lipids and proteins (Van Breusegem and Dat 2006). An oxidative stress occurs when the level of ROS inside the cell reaches beyond its antioxidant capacity [i.e. imbalance in the redox homeostasis in the cells (Fridovich 1998, Jamieson 1998, Pinheiro, Belo et al. 2002)]. ROS-dependent signaling pathways and ROS-responsive genes are activated following cellular exposure to ROS. These genes are either ROS-producing or ROS scavenging (Moradas-Ferreira, Costa et al. 1996, Mylona and Polidoros 2010). Antioxidant genes are among the most important genes implicated in control of ROS levels and their expression is affected by the levels of ROS reflecting a feedback regulation (Mylona and Polidoros, 2010). Despite studies on oxidative stress responses, in prokaryotes and eukaryotes, the information about filamentous fungi is fragmentary (Angelova, Pashova et al. 2005).

2.2.1 Chemistry of ROS

Triplet oxygen is the most stable form of molecular oxygen and does not react with most organic molecules. However, triplet oxygen can be converted into other forms of highly reactive species (ROS) (Table 2.1). Figures 2.1 and 2.2 illustrate the types of reactions leading to ROS. In reaction type I, superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) are obtained via sequential radical reduction (electron transfer) of ground state triplet oxygen by a reducing substrate. In reaction type II, singlet oxygen ($^1O_2$) is formed via energy transfer mechanism (Klotz 2002). ROS reactions and production are facilitated through the action of highly reactive mediators, such as photosensitizers (^0S) in this example, highly toxic compounds are produced only after activation by light. Figure 2.2 illustrates how photosensitizers contribute to ROS formation via reaction type I or type II. Perylenequinones belong to a chemically diverse and

large group of both natural and synthesized photosensitizers. Cercosporin, a well-studied member of perylenequinones, is produced by members of the highly successful genus of phytopathogens, *Cercospora* (Daub and Ehrenshaft 2000).

Table 2.1 The different forms of reactive oxygen species

Reactive oxygen species	Reaction Type
Singlet oxygen ($^1\text{O}_2$)	Type II (energy transfer)
Superoxide radical ($\text{O}_2^{\cdot-}$)	Type I (electron transfer)
Hydrogen peroxide (H_2O_2)	Type I (electron transfer)
Hydroxyl radical ($\text{HO}\cdot$)	Type I (electron transfer)

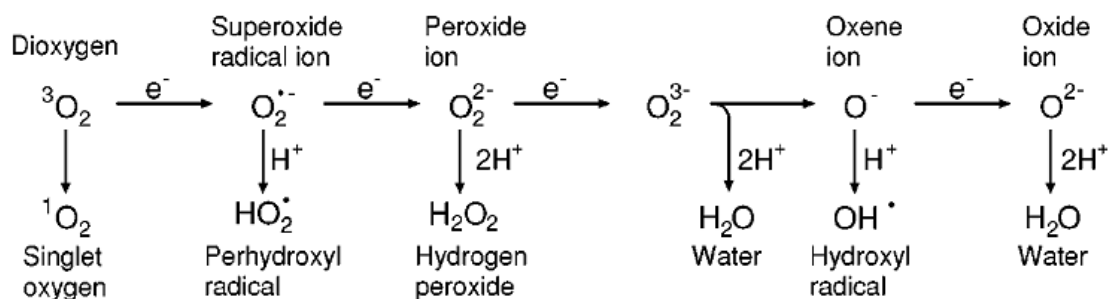


Figure 2.1 ROS production either by energy transfer or via sequential reduction of ground state triplet oxygen. Adapted from Klotz (2002).

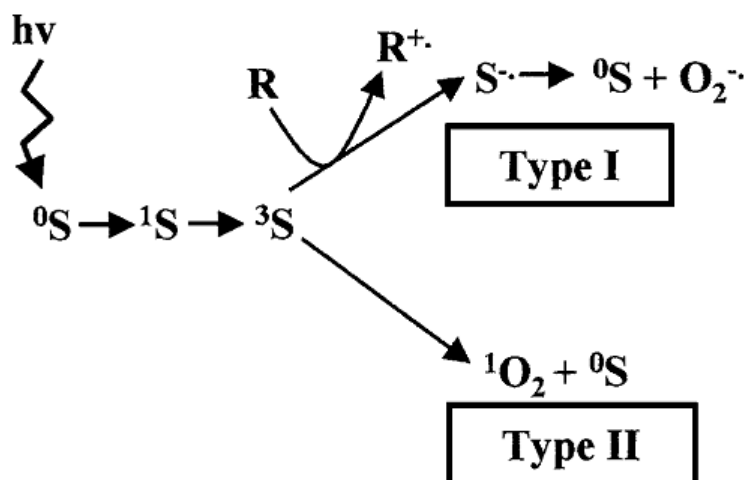


Figure 2.2 The formation of ROS from photosensitizers (^0S) is illustrated. ^0S are transformed into excited singlet (^1S) which in turn are converted to long-lived triplet state (^3S). Type I reactive oxygen species are obtained when ^3S reacts with a reducing substrate, while type II occurs when it reacts directly with ground state molecular triplet oxygen. Adapted Daub and Ehrenshaft (2000)

2.2.2 Sources of ROS

2.2.2.1 Biotic

ROS production occurs during microbe-microbe and plant-pathogen interactions (Kou, Qiu et al. 2019). Each organism exerts oxidative stress through its communication with the environment. In the plants oxidative burst, one of the most rapid defense reactions to pathogen attack is the generation of ROS, particularly superoxide and H_2O_2 at the sites of contact (Apostol, Heinsteins et al. 1989). In the pathosystem potato-*Phytophthora infestans*, tuber tissues inoculated with only avirulent, but not virulent, races of *P. infestans* provoked superoxide production (Doke 1985). During disease development of *R. solani* on its natural host potato, components of the hypersensitive reaction (e.g. oxalic acid) were manifested at higher levels in infected sprouts (Aliferis and Jabaji 2012). Formation and differentiation of sclerotia of *R. solani* is triggered by oxidative stress (Georgiou and Petropoulou 2001, Georgiou and Petropoulou 2001, Patsoukis and

Georgiou 2007) which in turn leads to the production of ROS (Georgiou, Tairis et al. 2000, Georgiou, Tairis et al. 2000, Aliferis and Jabaji 2010). The NADPH-dependent oxidase family are among the enzymes that are known to generate ROS, and are involved in fungal defense and differentiation (Takemoto, Tanaka et al. 2007). Knock-out mutations of two Arabidopsis genes, *Atrboh*d and *Atrboh*f, eliminated ROS production during disease resistance reactions of Arabidopsis to *R. solani* AG8 (Foley, Gleason et al. 2013). The same applies for fungi interacting with plants such as in the case of *M. grisea* mutants lacking NADPH oxidases, which were incapable of appressorium-mediated cuticle penetration into rice (Egan, Wang et al. 2007).

As mentioned in section 1.2, *B. subtilis* exhibited antifungal activity against *R. solani*. The inhibition was mainly due to the secretion of lipopeptides (Montealegre, Reyes et al. 2003, Gagne-Bourgue, Aliferis et al. 2013). The amphiphilic structure of lipopeptides enables them to interact with biological membranes and cause loss of integrity of the plasma membrane which in turn results in osmotic imbalance and disorganization of the cytoplasm in Bacillus-infected cells (Romero, de Vicente et al. 2007). As a result of this, ROS will be released inside the *R. solani* cells and hence oxidative stress is imposed, expressed visually as intense dark pigmentation in hyphae (Figure 2.3).

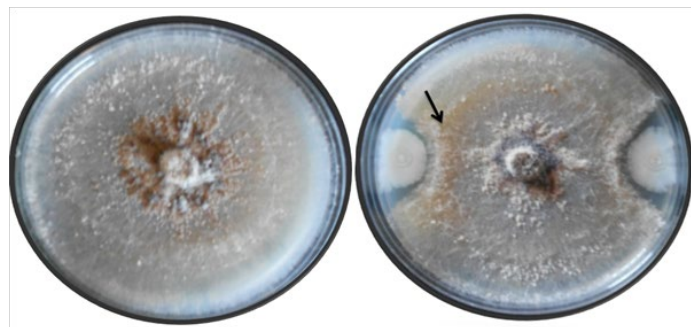


Figure 2.3 Confrontation of *Rhizoctonia solani* with *Bacillus methylotrophicus* B26 after 5 days (right photo) and *R. solani* without a confrontation (left photo). Arrow indicates dark pigmentation in hyphae of *R. solani* upon interaction.

2.2.2.2 Abiotic

It is widely accepted that the generation of ROS is enhanced under abiotic stress conditions. Factors like UV, cold, heat, salinity, toxins, metals, herbicides, and some chemicals interfere with both the metabolic balance and intracellular oxidant concentrations of cells through their ability to induce ROS generation (Mittler 2002). Oxidative stress generating chemicals (Table 2.2) are usually used in studies identifying the antioxidative role of genes (Padilla, Fuge et al. 1998, Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Benabdellah, Azcon-Aguilar et al. 2009).

Table. 2.2 List of chemicals that generate different forms of ROS and description of their mode of action

Chemicals	Released ROS	Description	Reference
Paraquat (1,1_-dimethyl-4,4_- bipyridinium dichloride)	Superoxide radical (O ₂ ⁻)	Foliar-applied and non selective herbicide. that promotes radical production in mitochondria	(Angelova, Pashova et al. 2005, Outten, Falk et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009)
Menadione (2-Methylnaphthalene- 1,4-dione)	Superoxide radical (O ₂ ⁻)	Redox cycling compound that is widely used in fungal oxidative stress studies	(Emri, Pócsi et al. 1999, Rodriguez-Navarro, Llorente et al. 2002, Pócsi, Miskei et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009).

Methylene blue (3,7-bis(Dimethylamino)- phenothiazin-5-ium chloride)	Singlet oxygen ($^1O_2$)	A photosensitizer and one of the most active ROS that generates singlet oxygen upon the activation by light	(Padilla, Fuge et al. 1998, Osmani, May et al. 1999)
Transition metals (e.g. Cu)	Hydroxyl radical ($HO^\bullet$)	ROS is released through the Fenton and metal-catalyzed Haber-Weiss reactions	(González- Guerrero, Cano et al. 2007, Benabdellah, Merlos et al. 2009)
Hydrogen peroxide (H_2O_2)	—	Direct use of H_2O_2 helps understanding its role in oxidative stress	(Bartosz 1997, Pócsi, Miskei et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009)

2.2.3 Antioxidant genes

ROS-dependent gene activation and diverse molecular mechanisms are the components of the cellular response to oxidative stress conditions. Antioxidant genes are important and central elements that maintain the redox homeostasis in the cells by controlling ROS levels. On the other hand, ROS levels regulate the expression of these genes in a feedback mechanism (Titiz, Tambasco-Studart et al. 2006, Mylona and Polidoros 2010). Antioxidant genes are divided into two groups. One group produces enzymes that directly detoxify ROS namely: superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPX*) and peroxiredoxins (*Prx*), whereas the second group acts through the production of non-enzymatic antioxidant compounds like vitamins. The cellular levels of $O_2^{\cdot -}$ and H_2O_2 are governed by the balance in the expression level of *SOD* and H_2O_2 scavenging genes. Antioxidant vitamins like ascorbic acid (vitamin C), tocopherols (vitamin E), and vitamin B6 play a major role in sustaining steady state ROS levels.

Also, they are known for their role in the cell cycle, promotion of growth and development, and responses to abiotic (oxidative) stressors and biotic stressors (Rigoni and De Tullio 2002, Traber and Atkinson 2007). Tocopherols are antioxidant molecules that react with peroxy radical and hence inhibit lipid peroxidation. In *Candida albicans*, ROS molecules which are produced by the mitochondria are detoxified by α -tocopherols (Deveau, Piispanen et al. 2010). Ascorbic acid generates ascorbate which reacts directly with $O^{\cdot-}_2$, and H_2O_2 , or reacts with other antioxidants, such as α -tocopherol (Halliwell 2011). *C. albicans* cells treated with ascorbic acid had less ROS levels (Deveau, Piispanen et al. 2010). Over the last decade, vitamin B6 has been acknowledged as a potent antioxidant, comparable to vitamins E and C, with a high ability to decrease ROS levels (Ehrenschaft, Bilski et al. 1999, Osmani, May et al. 1999, Titiz, Tambasco-Studart et al. 2006, Benabdellah, Azcon-Aguilar et al. 2009) (Figure 2.4).

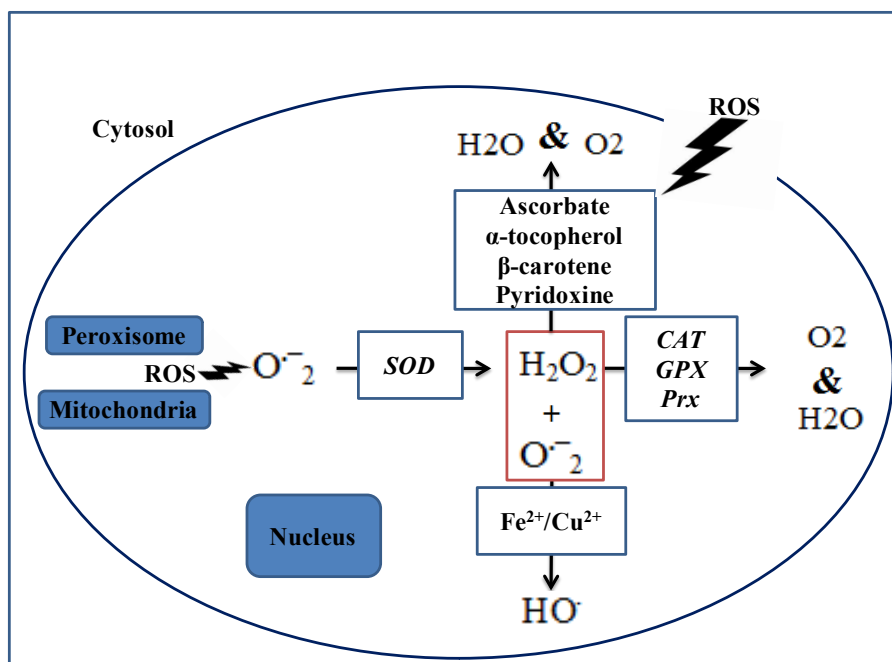


Figure 2.4 A schematic diagram showing the protective effect of antioxidants in the maintenance of the oxidant/antioxidant balance in the cellular system. SOD (superoxide dismutase) converts superoxide radical ($O^{\cdot-}_2$) to hydrogen peroxide (H_2O_2) followed by the coordinated action of a set

of enzymes namely CAT (catalase), GPX (glutathione peroxidase) and Prx (peroxiredoxins) that reduce H_2O_2 into H_2O and O_2 (Jakubowski, Bilinski et al. 2000, Mittler, Vanderauwera et al. 2004). The breakdown of H_2O_2 prevents the formation of the highly toxic hydroxyl radical through the Fenton or metal-catalyzed Haber-Weiss reactions which are mediated by $\text{Fe}^{2+}/\text{Cu}^{2+}$ (Mylona and Polidoros 2010). Antioxidant vitamins like ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids (β -carotene) and vitamin B6 play a major role in sustaining steady state ROS levels (Rigoni and De Tullio 2002, Traber and Atkinson 2007, Benabdellah, Azcon-Aguilar et al. 2009, Deveau, Piispanen et al. 2010, Halliwell 2011).

Gene expression studies focusing on oxidative stress responses from both the plant and the pathogen sides during plant-*R. solani* interactions are very limited. Foley, Kidd et al. (2016) identified a number of wheat-derived and *R. solani* AG8-derived genes involved in ROS production and redox regulation, and whose expression was affected during infection. Their study highlights the need to understand the ROS-scavenging system interplay of fungal and host derived genes involved in ROS/redox regulation at the site of infection and the surrounding areas.

2.3 Vitamin B6

Vitamin B6 is a collection of the following vitamers: pyridoxine (PN), pyridoxal (PL), pyridoxamine and their phosphorylated derivatives, and Pyridoxal-5'-phosphate (PLP) (Figure 2.5) (Snell 1944, Snell 1944) which is the active form of the cofactor (Rabinowitz and Snell 1948). The vitamers' structures were confirmed and determined by chemical synthesis (Harris and Folkers 1939, Harris, Heyl et al. 1944) and named pyridoxine to indicate its structural homology to pyridine.

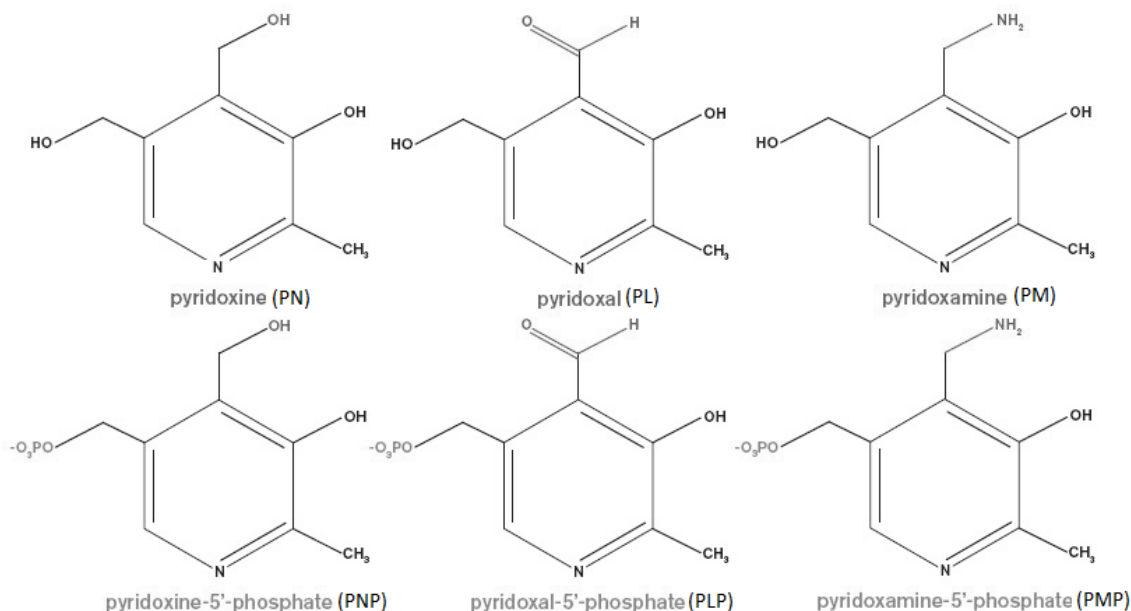


Figure 2.5 Chemical structures of the different vitamers of vitamin B6: pyridoxine (PN), pyridoxal (PL), pyridoxamine, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) (Source: http://www.lef.org/magazine/mag2009/jul2009_B6-Vitamins_02.htm).

Vitamin B6 plays a crucial role in all living organisms and is recognized as an important cofactor required for many diverse enzymes ranging from amino acid metabolism to the biosynthesis of antibiotic compounds. PLP dependent activities encompass more than 140 unique enzymatic activities -approximately 4% of classified enzyme activities- as classified by the Enzyme Commission (EC; <http://www.chem.qmul.ac.uk/iubmb/enzyme/>) (Percudani and Peracchi 2003, Hellmann and Mooney 2010). Many studies have showed that vitamin B6 can help in reduction of many important human diseases like cancer, pellagra, and neurological disease (Hellmann and Mooney 2010). Many factors can cause vitamin B6 deficiency in humans, among which is an inadequate diversity in diet. According to the National Institute of Health (NIH) of the USA, the Recommended Dietary Allowance per day is near 2 mg with an upward tolerance of 100

mg per day for adults (Morris, Picciano et al. 2008). Plants and fungi are two main sources of vitamin B6 (Di Salvo, Safo et al. 2011). For example, various cultivars of potato contain different concentrations of vitamin B6 (Mooney, Chen et al. 2013). Interestingly, potato contains the highest content of vitamin B6 compared to other staple crops like wheat, rice, maize, and cassava (Fitzpatrick, Basset et al. 2012). Thus, adopting strategies to increase the content of vitamins in plants can aid in overcoming deficiencies in human diets (Fitzpatrick, Basset et al. 2012, Mooney, Chen et al. 2013, Vanderschuren, Boycheva et al. 2013). Vitamin B6 is not only considered a vital coenzyme for numerous metabolic enzymes, but is also considered to have a contribution in cellular development of plants (Titiz, Tambasco-Studart et al. 2006, Raschke, Boycheva et al. 2011) and a role as a potent antioxidant due to its ability of quenching singlet oxygen and the superoxide anion (Ehrenschaft, Bilski et al. 1999, Osmani, May et al. 1999, Benabdellah, Azcon-Aguilar et al. 2009).

2.3.1 Vitamin B6 Pathways

Protista, fungi and plants can biosynthesize the vitamin de novo, whereas animals and humans must obtain it from dietary sources because they have lost the machinery of de novo biosynthesis (Figures 2.6 and 2.7). However, there is a commonality among these taxonomic groups since they possess the ability to interconvert the different vitamin B6 vitamers through a conserved pathway termed as the salvage pathway (Figure 2.6).

2.3.1.1 The salvage pathway

PLP is the active cofactor involved in catalysis of the various catalytic reactions (Rabinowitz and Snell 1948). Organisms convert the different vitamers: pyridoxine, pyridoxamine and pyridoxal into PLP via enzymes involved in the salvage pathway, like pyridoxal (PL)

(pyridoxine (PN), pyridoxamine (PM)) kinase, pyridoxine (PN) (pyridoxamine (PM))-phosphate oxidase (Zhao and Winkler 1995), and pyridoxal reductase (PL) (Herrero, González et al. 2011). On the other hand, phosphatases are capable of hydrolyzing the phosphate ester bond of the phosphorylated vitamers to yield the unphosphorylated derivatives (Tazoe, Ichikawa et al. 2005) (Figure 2.6).

PLR belongs to the aldo–keto reductase protein superfamily because it retains a conserved catalytic tetrad, which consists of Tyr (Y), His (H) or Ala (A), Lys (K), and Asp (D) (Barski, Tipparaju et al. 2008) and it is composed of an average 330 amino acids. Aside from its role in the NADPH-mediated reduction of pyridoxal to pyridoxine, PL reductase is involved in the transport of vitamin B6. In *Schizosaccharomyces pombe* the PL reductase encoded by the *plr1*+gene is involved in the excretion of PN following its reduction from PL as part of the maintenance of vitamin B6 levels in the cell (Morita, Takegawa et al. 2004). PLR was also characterized in *Arabidopsis* and insertion mutants of *PLR* showed significant decrease in vitamin B6 accumulation along with the significant increase in expression of salvage pathway kinases and oxidases (Herrero, González et al. 2011).

2.3.1.2 de novo pathway

Two de novo pathways exist for the synthesis of vitamin B6: 1-deoxy-D-xylulose-5-phosphate (DXP)-dependent (de novo II) and DXP-independent pathway (de novo I) (Figures 2.6 and 2.7). Importantly, studies showed that organisms which possess the de novo pathway that either one or the other pathway is present but never both (Table 2.3) (Ehrenschaft, Bilski et al. 1999, Mittenhuber 2001).

In the DXP-dependent pathway, which is heavily studied in *Escherichia coli* (Hill, Himmeldirk et al. 1996); pyridoxine 5'-phosphate (PNP) is obtained from the condensation of the non-cyclic building blocks, deoxyxylulose (DXP) and 4-phosphohydroxy-Lthreonine (4HPT) through the action of the two genes *PdxJ* and *PdxA* (Figure 2.7). That is, *PdxA* converts 4HPT to 3-amino-1-hydroxyacetone 1-phosphate (AHAP), and *PdxJ* forms PNP from AHAP and DXP. In turn, *PdxH* then oxidizes PNP into PLP (Figure 2.7). The nitrogen atom in vitamin B6 of this pathway is derived from glutamate in *E. coli* (Tanaka, Tazuya et al. 2000).

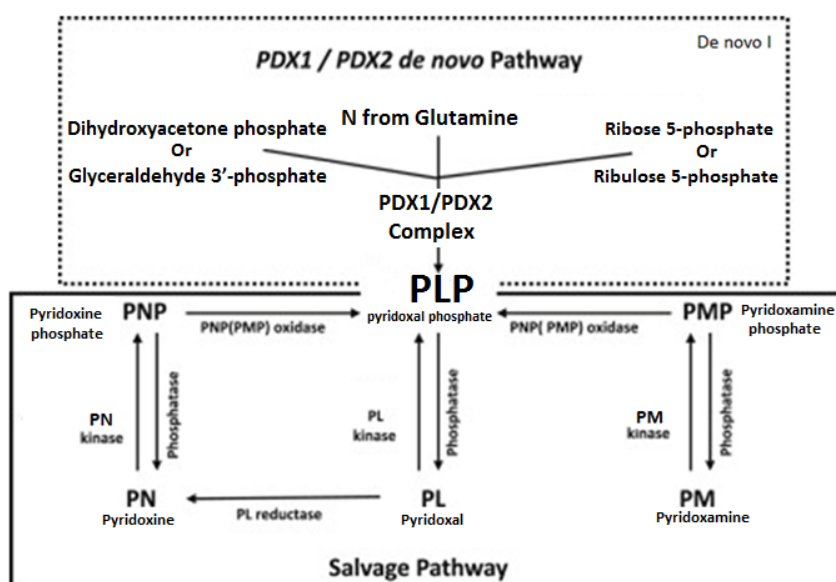


Figure 2.6 Schematic representation of the vitamin B6 de novo (upper box) and salvage pathways (lower box). **PLP**, pyridoxal phosphate; **PNP**, pyridoxine phosphate; **PMP**, pyridoxamine phosphate; **PL**, pyridoxal; **PN**, pyridoxine; **PM**, pyridoxamine **PL** (**PN**, **PM**, **PN**) kinase, **PN** phosphate oxidase (Zhao and Winkler 1995), and **PL** reductase convert pyridoxine, pyridoxamine and pyridoxal into **PLP**. Adapted and modified from Rueschhoff, Gillikin et al. (2012).

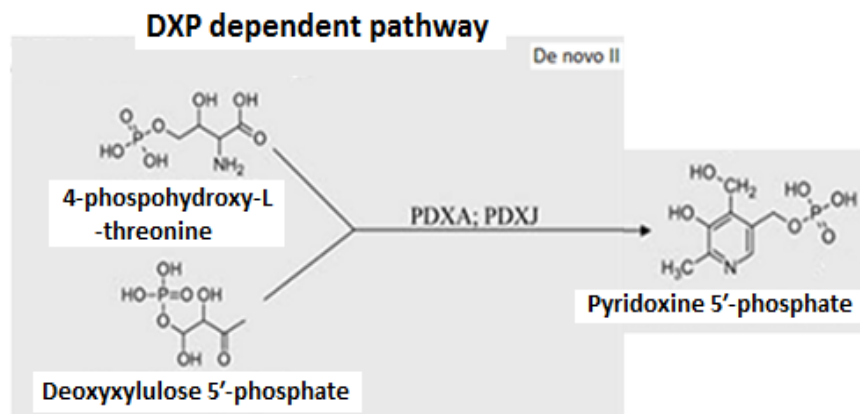


Figure 2.7 Schematic representation of the vitamin B6 de novo (Deveau, Piispanen et al.), DXP dependent pathway. Pyridoxine 5'-phosphate (PNP) is synthesized from deoxyxylulose 5'-phosphate (DXP) and 4-phosphohydroxy-Lthreonine (4HPT) through the action of the two genes *PdxJ* and *PdxA*. Adapted and modified from Mooney, Leuendorf et al. (2009).

Table 2.3 List of organisms which carry genes either homologous to *PdxA* and *PdxJ* or to *PDX1* and *PDX2*.

Organism	<i>PDX1/PDX2</i>	<i>pdxA/J</i>
Plant		
<i>Arabidopsis thaliana</i>	+	-
<i>Oryza sativa</i>	+	-
<i>Hevea brasiliensis</i>	+	-
Fungi		
<i>Aspergillus nidulans</i>	+	-
<i>Saccharomyces cerevisiae</i>	+	-
<i>Schizosaccharomyces pombe</i>	+	-
Archaea		
<i>Methanococcus jannaschii</i>	+	-
<i>Pyrococcus horikoshii</i>	+	-
<i>Methanobacterium thermoautotrophicum</i>	+	-
<i>Archaeoglobus fulgidus</i>	+	-

Eubacteria

<i>Mycobacterium leprae</i>	+	-
<i>Haemophilus influenza</i>	+	-
<i>Bacillus subtilis</i>	+	-
<i>Thermoto gamaritima</i>	+	-
<i>Mycobacterium tuberculosis</i>	+	-
<i>Escherichia coli</i>	-	+
<i>Helicobacter pylori</i>	-	+
<i>Aquifex aeolicus</i>	-	+
<i>Campylobacter jejuni</i>	-	+
<i>Vibrio cholera</i>	-	+
<i>Salmonella typhi</i>	-	+
<i>Mycoplasma pneumonia</i>	-	-
<i>Mycoplasma genitalium</i>	-	-
<i>Borrelia burgdorferi</i>	-	-

Animal

<i>Caenorhabditis elegans</i>	-	-
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Data was obtained from NCBI

Only the γ -division of the proteobacteria obtain their vitamin B6 using the former pathway whereas most other organisms (bacteria, archaea and eukarya) use the evolutionary conserved DXP-independent pathway. In this pathway, *PDX1* and *PDX2* genes contain the glutamine amidotransferase where *PDX1* functions as the synthase and *PDX2* constitutes the glutaminase domain. Recently, many research papers proved the role of PDX1 and PDX2 proteins in the de novo biosynthesis of vitamin B6, provided information about their structure and catalysis, and revealed the important steps for the formation of PLP (Ehrenshaft, Bilski et al. 1999, Osmani, May

et al. 1999, Bilski, Li et al. 2000, Raschle, Amrhein et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009).

2.3.2 On the two components of the vitamin B6 de novo I pathway

The two proteins in this pathway, PDX1 and PDX2, jointly catalyze PLP synthesis (Figure 2.6). PDX1 and PDX2 together act as a **glutamine amidotransferase** and **PLP synthase**. PDX2 hydrolyzes glutamine to glutamate and ammonia in order to supply nitrogen for the PLP heterocycle, and then PDX1 arranges the final ring closure. It was also shown that the activity of PDX2 is dependent on PDX1 (Burns, Xiang et al. 2005, Raschle, Amrhein et al. 2005). PDX2 is considered a member of the class I glutaminase family because it possesses a **Cys-His-Glu** catalytic site (Galperin and Koonin 1997). The involvement of **Cys** in catalysis was proved in *B. subtilis* when acivicin was used instead of glutamine. Acivicin has the ability to modify the **Cys** residue and render PDX2 as non-functional (Raschle, Amrhein et al. 2005). As for PDX1, the synthase domain, certain amino acids (**Lys 81** and **Lys 149**) were proposed to be involved in catalysis (Burns, Xiang et al. 2005, Raschle, Amrhein et al. 2005).

2.3.2.1 Characterization

In fungi, *PDX1* and *PDX2* were characterized and proved to be part of the DXP-independent de novo pathway through the pioneering work of Margaret Daub and colleagues on the phytopathogenic fungus *Cercospora nicotianae* (Ehrenshaft, Jenns et al. 1998, Ehrenshaft, Bilski et al. 1999). Depending on the organisms under study, *PDX1* and *PDX2* are assigned different names (Table 2.4) which creates confusion.

Genomic studies revealed that *PDX1* and *PDX2* are present in all archaea, fungi, plants and most bacteria (Mittenhuber 2001). *PDX1* has a high level of conservation among all organisms

with the signature sequence motif LPVVNFXXXGLATPADAA (www.ebi.ac.uk) (Strohmeier, Raschle et al. 2006). *PDX1* and *PDX2* don't share any homology with any of the genes involved in the *E. coli* vitamin B6 de novo pathway (Padilla, Fuge et al. 1998, Osmani, May et al. 1999, Mittenhuber 2001). In the case of *PDX2*, it shows less level of conservation when compared to *PDX1*. Depending on those conserved domains, degenerate primers were designed to identify the presence of those genes in new organisms (Ehrenshaft and Daub 2001). Sequence analysis of *PDX1* and *PDX2* genes demonstrated that the ORF average size was around 1000 bp. Some genes were shown to have introns while others were intron-less. For example, introns are present in the *PDX1* of *Cercospora nicotianae* and *Glomus intraradices*; however, they are absent the *PDX1* (*PyroA*) gene of *Aspergillus nidulans* (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Benabdellah, Azcon-Aguilar et al. 2009). Average sizes of the encoded proteins are around 300aa, and 270 aa, for *PDX1* and *PDX2* respectively (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Ehrenshaft and Daub 2001, Benabdellah, Azcon-Aguilar et al. 2009). Southern blot studies revealed that most fungi contain a single copy of those genes (Ehrenshaft, Jenns et al. 1998, Osmani, May et al. 1999, Ehrenshaft and Daub 2001, Benabdellah, Azcon-Aguilar et al. 2009) whereas in *Saccharomyces cerevisiae*, *PDX1*(*SNZ*) and *PDX2* gene families were shown to have three members (called 1, 2 and 3). However, only *SNZ1* and *SNO1* were implicated in vitamin B6 biosynthesis (Padilla, Fuge et al. 1998, Rodriguez-Navarro, Llorente et al. 2002).

Studies have demonstrated the presence of both pathways in higher plants. For the de novo VB6 pathway, many homologs for *PDX1* are present. In Arabidopsis there are three homologs: ATPDX1.1, ATPDX1.2, and ATPDX1.3 whereas in potato only two are present: STPDX1.1 and STPDX1.2 (Tambasco-Studart, Titiz et al. 2005, Tambasco-Studart, Tews et al. 2007, Moccand, Boycheva et al. 2014). ATPDX1.3 is considered to have more importance compared to ATPDX1.2

in the de novo biosynthesis of VB6 (Tambasco-Studart, Titiz et al. 2005, Titiz, Tambasco-Studart et al. 2006) and due to the its role during development of fungal diseases in Arabidopsis (Moccand, Boycheva et al. 2014, Zhang, Liu et al. 2014). PDX1.2 is a non-catalytic homolog that was found to play role as a pseudoenzyme in Arabidopsis regulating VB6 abundance during biotic or abiotic stress (Mooney, Chen et al. 2013, Moccand, Boycheva et al. 2014, Samsatly, Copley et al. 2018). Additionally, it was shown that potato STPDX2 might be regulated by STPDX1.2, due to the unique (novel) ability of the latter to interact with ATPDX2 (Mooney, Chen et al. 2013, Moccand, Boycheva et al. 2014).

Table 2.4 Characterization and nomenclature of vitamin B6 de novo I pathway genes

<i>PDX1</i> Homologue	<i>PDX2</i> Homologue	Organism	Description	Reference
<i>SOR1</i>		<i>Cercospora nicotianae</i>	The gene was firstly named as <i>SOR1</i> ; denoting singlet oxygen resistance 1, and then changed to <i>PDX1</i> when its role was confirmed in vitamin B6 de novo pathway	Ehrenshaft et al., 1998; Ehrenshaft et al., 1999
	<i>PDX2</i>	<i>C. nicotianae</i>	A second novel gene isolated in the pyridoxine biosynthesis pathway (Ehrenshaft and Daub 2001)	Ehrenshaft and Daub, 2001
<i>PYROA</i>	<i>PYROB</i>	<i>Aspergillus nidulans</i>	<i>PYROA</i> , a gene that was proved to be involved in resistance to photosensitizers and in vitamin B6 biosynthesis	Osmani et al., 1999

<i>SNZ</i>	<i>SNO</i>	<i>Saccharomyces cerevisiae</i>	<i>SNZ</i> and <i>SNO</i> genes were identified before in yeast, however, their real function (vitamin B6 biothynthesis) couldn't be determined	Braun et al., 1996; Padilla et al., 1998
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2.3.2.2 Activity

The formation of the PDX1-PDX2 complex is crucial for activation of the glutamine amidotransferase functionality. Validation of this activity was possible via enzymatic assays in which pyridoxal-5'-phosphate (PLP) confirmed as the reaction product of vitamin B6 de novo pathway (Belitsky 2004).

Proof of the interaction of PDX1 and PDX2 was first demonstrated in *Saccharomyces cerevisiae*. PDX1 (Snz1p) and PDX2 (Sno1p) were shown to interact as determined by a two-hybrid analysis (Padilla, Fuge et al. 1998). To present the evidence of a physical interaction of these two proteins, PDX1 (YaaD) and PDX2 (YaaE) from *B. subtilis* were cloned and overexpressed in *E. coli*. PDX2 was his6-tagged and both proteins were co-purified by nickel (Ni 2) affinity chromatography. PDX1, which has no affinity for Ni 2 column, co-purified with PDX2-his6 indicating that the two proteins form a complex (Belitsky 2004). A similar study was performed except that PDX1 and PDX2 were treated with acivicin that rendered PDX2 as non-functional. PDX1 was co-purified with PDX2 but its concentration was far less in the acivicin treated mixture (Raschle, Amrhein et al. 2005).

The activity of PDX1 and PDX2 was demonstrated by the incubation of PDX1 and/or PDX2 proteins in a 50 mM Tris-HCl buffer (pH 8.0) containing glutamine, either ribose 5-phosphate or ribulose 5-phosphate, and dihydroxyacetone phosphate. Increase in the absorbance

readings at 414 nm proved that activity (Braun, Fuge et al. 1996, Belitsky 2004, Raschle, Amrhein et al. 2005).

Upon the co-incubation of PDX1 and PDX2 together PLP was isolated and confirmed as the reaction product of the vitamin B6 de novo pathway using reversed-phase HPLC. Commercially available standards (pyridoxine, pyridoxal, and PLP) were used as controls (Raschle, Amrhein et al. 2005).

2.3.2.3 Functional complementation

Characterization of the *PDX1* and *PDX2* genes was made possible from cercosporin sensitive mutants of *C. nicotianae*. Strains were isolated from UV mutagenized protoplasts and were unable to grow in the presence of cercosporin-amended media and were also sensitive to other $1O_2$ generating photosensitizers. Transformation with cosmid DNA pooled from wild type *C. nicotianae* resulted in the rescue of mutant strains and rendered them resistant to both cercosporin and other photosensitizers. PDX1 was first isolated from one of these rescued strains (Ehrenshaft, Jenns et al. 1998, Ehrenshaft, Bilski et al. 1999). Two out of five mutant strains were incapable of growth on cercosporin containing media although they had wild-type *PDX1* genes. This strongly suggests the involvement of a second gene in the biosynthesis pathway. Hence, degenerate primers that amplify *SNO* homologues (Padilla, Fuge et al. 1998) were used to amplify *PDX2* in *C. nicotianae* and to test its involvement in pyridoxine metabolism. Transformation of those mutants with *PDX2* reversed the sensitivity into resistance to cercosporin (Ehrenshaft and Daub 2001). In some complementation studies transformation was performed using yeast mutants. Validation of the PDX1 role in *Glomus intraradices* in oxidative stress resistance was achieved

via complementation assays with their respective *snz1* (yeast functional *PDX1* homologue) mutant *S. cerevisiae* strains (Benabdellah, Azcon-Aguilar et al. 2009).

2.3.3 Regulation of vitamin B6 genes

The role of vitamin B6 is now considered an important element of the ROS-activated genes (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Bilski, Li et al. 2000, Benabdellah, Azcon-Aguilar et al. 2009). Vitamin B6 related genes are shown to be differentially expressed when fungi or plants are under oxidative stress (Benabdellah, Azcon-Aguilar et al. 2009). Initial studies on the regulation of *PDX1* and *PDX2* were qualitative. For example, in northern analysis assays, *SNZ1* and *SNO1* (the homologues of *PDX1* and *PDX2*) mRNA of *S. cerevisiae* accumulated at high levels during nutrient limitation and growth arrest conditions (Braun, Fuge et al. 1996). Co-regulation of *SNZ1* and *SNO1* was confirmed based on their physical proximity and because they exhibited the same pattern of expression in cells grown to stationary phase (Padilla, Fuge et al. 1998). Also, when *C. nicotianae* was grown on media containing the singlet oxygen releasing toxin cercosporin, *SOR1(PDX1)* mRNA levels were detectable by day two (Ehrenshaft, Jenns et al. 1998). As for quantitative analysis of *PDX1* and *PDX2*, qRT-PCR was used in the endomycorrhizal fungus *Glomus intraradices*. *GintPDX1* expression was substantially induced more than 2 fold depending on the type of oxidative stress and the exposure time period (Benabdellah, Azcon-Aguilar et al. 2009). In *R. solani*, only two studies have dealt with one gene of the salvage pathway of vitamin B6. Over the course of the parasitic interaction between *R. solani* and the mycoparasite *S. elegans*, there is indirect evidence that ROS were released as confirmed by substantial up-regulation of pyridoxal reductase (*PLR*), a gene in the salvage pathway of vitamin B6 (Figure 2.6) (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011).

Importantly, a recent study displayed variable expression levels of *PDX* in different genotypes of *S. tuberosum* (Mooney, Chen et al. 2013). Over expression of *PDX* in *Arabidopsis* helped in resistance to oxidative stress (Raschke, Boycheva et al. 2011). The regulation of VB6 genes and the role of its vitamer was indicated during confrontations between tobacco and *P. syringae* (Denslow, Walls et al. 2005). Recently, two studies showed the important function of VB6 during plant disease development and indicated its role in regulation of defense response against pathogen infection during confrontation between *Botrytis cinerea* and tomato or *Arabidopsis*, respectively (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). Mutations in the *Arabidopsis* *PDX1.2* and *PDX1.3* genes involved in the *de novo* pathway or Virus-induced gene silencing mediated knockdown of the tomato equivalents ended up in increased levels of diseases caused by *B. cinerea* and decreased levels of VB6 (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). Disrupting *SOS4*, an important component of the VB6 salvage pathway, reacted differently in *Arabidopsis* and tomato where it led to an increased disease susceptibility and decreased VB6 levels only in *Arabidopsis* (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). These findings imply that the VB6 vitamers might play important roles in regulating plant defense responses against different types of pathogens. However, direct molecular evidence supporting the hypothesis that VB6 as an antioxidant functions in plant defense against *R. solani* is still lacking.

2.4 CONCLUSIONS

The ability of both fungi and plants to deal with the toxicity of ROS molecules and keeping them at a tight control is possible through a diverse set of ROS detoxification systems. Vitamin B6 is proved to have an important antioxidant ability. Fungi and plants are among many organisms that possess the ability of *de novo* biosynthesis of vitamin B6 which involves two genes, *PDX1* and *PDX2*. The identification and characterization of the elements of this pathway using molecular

techniques in fungal pathogens, like *Rhizoctonia solani* is very important because of its implicated role during defense systems and disease development. Also, studying its role during the interactions of *R. solani* with various types of abiotic stressors like paraquat and H₂O₂ can help in proving the importance of vitamin B6 as an antioxidant. Validation of the functional role of the *R. solani* AG3 *PDX1* and *PDX2* genes in oxidative stress resistance would be possible through the use of yeast expression system due to the difficulty in transformation of *R. solani*. The limited knowledge on the role of vitamin B6 during disease development of fungal pathogens over their plant hosts raise the importance to study the regulation of vitamin B6 genes during the interaction of different anastomosis groups of *R. solani* with various hosts. Last, validation of the functional role of vitamin B6 during disease development of *R. solani* on vitamin B6 mutant Arabidopsis or different potato varieties displaying variable vitamin B6 concentrations would help us in proving the role in resistance to oxidative stress.

2.5 CONNECTING TEXT

Chapter 3 focuses on the characterization of the vitamin B6 biosynthesis related genes in the de novo pathway, *PDX1*, *PDX2* and the salvage pathway *PLR*, in *Rhizoctonia solani* AG3 and the regulation of these genes when *R. solani* is exposed to abiotic stress. In recent years, vitamin B6 has been identified as a potent antioxidant with a high ability to quench reactive oxygen species (ROS). Studies on vitamin B6 metabolism and regulation are limited to a few fungi including one plant pathogenic fungus, *C. nicotianae*. Necrotrophic fungi such as *R. solani* are successful pathogens that are able to overcome or suppress an array of complex ROS-mediated plant defences. However, data on ROS-scavenging systems in *R. solani* is very limited. With the aim of gaining insight into the possible implication of ROS on vitamin B6 regulation in *R. solani*, this chapter reports on the characterization of VB6 genes, *RsolPDX1* and *RsolPDX2* from the vitamin B6 *de novo* pathway and *RsolPLR* from the salvage pathway and investigates whether ROS is implicated in vitamin B6 regulation in *R. solani* during the interaction with different types of chemical stressors.

J. Samsatly, Dr. R. Chamoun, and Dr. S. Jabaji conceived, designed and executed the experiments. E. G. Thäler helped in the execution of certain experiments. J. Samsatly and Dr. R. Chamoun analyzed the data. The work was funded by a research grant to S. Jabaji from the Natural Sciences and Engineering Research Council of Canada (NSERC-Discovery, grant number 137135-13). The manuscript was written by J. Samsatly and revised by all co-authors prior to publication in Frontiers in Microbiology January 5th, 2016.

CHAPTER 3

Genes of the *de novo* and salvage biosynthesis pathways of vitamin B6 are regulated under oxidative stress in the plant pathogen *Rhizoctonia solani*

Jamil Samsatly¹, Rony Chamoun¹, Emile Glück-Thaler², Suha Jabaji^{1*}

¹ Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, QC, Canada

² Department of Plant Pathology, Ohio State University, Columbus, OH, USA

Corresponding Author:

Dr. S. Jabaji; Department of Plant Science, McGill University, 21,111 Lakeshore Rd., Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9.

Telephone: +1 (514)-398-7561

E-mail: suha.jabaji@mcgill.ca

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3.1 ABSTRACT

B6 is recognized as an important cofactor required for numerous metabolic enzymes, and has been shown to act as an antioxidant and play a role in stress responses. It can be synthesized through two different routes: salvage and *de novo* pathways. However, little is known about the possible function of the vitamin B6 pathways in the fungal plant pathogen *Rhizoctonia solani*. Using genome walking, the *de novo* biosynthetic pathway genes; *RsolPDX1* and *RsolPDX2* and the salvage biosynthetic pathway gene, *RsolPLR* were sequenced. The predicted amino acid sequences of the three genes had high degree of similarity to other fungal PDX1, PDX2, and PLR proteins and are closely related to other *R. solani* anastomosis groups. We also examined their regulation when subjected to ROS stress inducers, the superoxide generator paraquat, or H₂O₂, and compared it to the well-known antioxidant genes, *catalase* and *glutathione-S-transferase (GST)*. The genes were differentially regulated with substantial transcript levels as high as 33 fold depending on the gene and type of stress reflecting that differences in the type of damage induced by ROS. Exogenous addition of the vitamers PN or PLP in culture medium significantly induced the transcription of the vitamin B6 *de novo* encoding genes as early as 0.5 hour post treatment (HPT). On the other hand, transcription of *RsolPLR* was vitamer-specific; a down regulation upon supplementation of PN and upregulation with PLP. Our results suggest that accumulation of ROS in *R. solani* mycelia was linked to transcriptional regulation of the three genes and *R. solani* vitamin B6 biosynthesis machinery could be implicated similar to *catalases* and *GST* as an antioxidant stress protector against oxidative stress.

Keywords

Vitamin B6, *de novo* pathway, salvage pathway, *Rhizoctonia solani*, oxidative stress, abiotic stress, *PDX* genes, antioxidant genes

3.2 Introduction

Vitamin B6, a collective term that refers to a group of six vitamers: pyridoxal (PL), pyridoxine (PN), pyridoxamine and their phosphorylated derivatives (PLP, PNP, PMP) (Fitzpatrick, Basset et al. 2012, Vanderschuren, Boycheva et al. 2013). In eukaryotes and prokaryotes, vitamin B6 vitamers are produced via the *de novo* biosynthetic pathway that ultimately leads to the synthesis of the most active form pyridoxal 5'-phosphate (PLP) via a heterodimer complex made up of two pyridoxal biosynthesis proteins that belong to highly conserved protein families (PDX1 and PDX2) (Mittenhuber 2001, Raschle, Amrhein et al. 2005, Fitzpatrick, Amrhein et al. 2007). Of all the vitamers, PLP is critically important because it is essential as a cofactor for over 140 chemical reactions (Percudani and Peracchi 2003, Roje 2007, Hellmann and Mooney 2010). In addition to the *de novo* pathway, a conserved “salvage pathway” is found in all organisms (Nakano, Morita et al. 1999, González, Danehower et al. 2007, Herrero, González et al. 2011, Rueschhoff, Gillikin et al. 2012). Reactions in the salvage pathway include the reduction of PL to PN, which is carried out by pyridoxal reductase (PLR), a downstream enzyme in the vitamin B6 biosynthesis pathway, (Morita, Takegawa et al. 2004, Herrero, González et al. 2011) and the conversion of PN into PNP which is performed by PNP oxidase generating at the end of its pathway PLP (González, Danehower et al. 2007, Sang, Barbosa et al. 2007).

In recent years, vitamin B6 has been identified as a potent antioxidant with a high ability to quench reactive oxygen species (ROS), resulting in an antioxidant capacity that rivals that of

tocopherols or ascorbic acid, and may play a role in stress responses in fungi and plants (Mooney, Leuendorf et al. 2009, Vanderschuren, Boycheva et al. 2013). The antioxidant properties of vitamin B6 was originally reported in the fungal pathogen *Cercospora nicotianae*, by providing resistance to cercosporin, a singlet oxygen generating toxin (Daub and Ehrenshaft, 2000). This novel characteristic of vitamin B6 as a ROS scavenger and its ability to increase resistance to biotic and abiotic stresses have been demonstrated in plant-microbe interaction studies. Supplementation with PM could delay or decrease pathogen-induced leaf necrosis (Denslow, Walls et al. 2005) while PN could protect *Arabidopsis flu* mutant, which releases singlet oxygen in plastids, from cell death (Danon, Miersch et al. 2005).

Studies on vitamin B6 metabolism and regulation are limited to a few fungi, (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Benabdellah, Azcon-Aguilar et al. 2009) including one plant pathogenic fungus, *C. nicotianae*. Necrotrophic fungi are successful pathogens that are able to overcome or suppress an array of complex ROS-mediated plant defences (Chung 2012). The relative sensitivity of necrotrophic plant pathogens to ROS is likely determined by the effectiveness of their own ROS detoxification ability. To survive under aerobic conditions, fungi must possess detoxification systems such as NADPH oxidase (NOX) complex that can effectively scavenge ROS, maintain reduced redox states within subcellular microenvironments, and repair ROS-triggered damage (Heller and Tudzynski 2011, Chung 2012).

The soil fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*, Frank, Donk) is a generalist, necrotrophic pathogen with a wide host range, causing damping-off of seedlings, root crown, stem rots and sheath blight diseases of plants (Ogoshi 1996, Sneh 1996). Basal resistance to *Rhizoctonia* diseases in several crops is correlated with ROS-scavenging mechanisms such as hydrogen peroxide (H₂O₂) production, enhanced peroxidases (POX) and superoxide

dismutase (SOD) activities, transcriptional regulation of NOX, regulation of several metabolites in the phenylpropanoid and vitamin B6 biosynthetic pathways, accumulation of oxidized fatty acids, and increased levels of cell wall bound phenolics (Taheri and Tarighi 2011, Aliferis and Jabaji 2012, Foley, Gleason et al. 2013, Nikraftar, Taheri et al. 2013, Aliferis, Faubert et al. 2014). However, data on ROS-scavenging systems in *R. solani* is very limited. To date, evidence that *R. solani* genes of the vitamin B6 pathway are upregulated in response to biotic stress has been reported (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011). Parasitized hyphae and sclerotia of *R. solani* by the mycoparasite *Stachybotrys elegans* have displayed a substantial up-regulation in the transcription of the gene encoding PLR (Chamoun and Jabaji 2011). Other vitamin B6 biosynthetic encoding genes such as pyridoxal-5-phosphatases and transaminases were recently reported to be upregulated in *R. solani* in response to antagonistic plant associated bacteria (Gkarmiri, Finlay et al. 2015).

With the aim of gaining insight into the possible implication of ROS on vitamin B6 regulation in *R. solani*, we report on the characterization of three vitamin B6 genes, *RsolPDX1* and *RsolPDX2* from the vitamin B6 *de novo* pathway and *RsolPLR* from the salvage pathway. A significant homology between *R. solani* and other fungal vitamin B6 genes was found. *R. solani* vitamin B6 *de novo* pathway genes were up-regulated by the superoxide generator paraquat but not by H₂O₂ whereas *RsolPLR* was up-regulated by both chemicals. This displays a unique response of these genes according to the type of oxidative stress induced by ROS generating chemicals.

3.3 Materials and Methods

3.3.1 Fungal Strains, Media and Culture Conditions

Starter culture of *R. solani* Kühn AG3 (isolate Rs114, ATCC 10183) was grown on potato dextrose agar (PDA; Difco, Detroit, USA) at 24°C for five days. For the isolation of vitamin B6 related genes (*RsolPDX1*, *RsolPDX2*, *RsolPLR* genes) from *R. solani*, plugs (5 mm) from starter cultures were placed in the center of fresh PDA plates, overlaid with cellophane membranes (500 PUT; UCB, North Augusta, USA), and grown for 7 days at 24°C. Cultures of *Fusarium oxysporum* (ATCC 60860) and *Trichoderma virens* (DAOM 169262) were grown similarly to *R. solani* and used as controls.

3.3.2 Experimental Setup for Oxidative Stress

To investigate the role of *R. solani* in detoxification/homeostasis of ROS, the relative transcript abundance of vitamin B6 genes (*RsolPDX1*, *RsolPDX2*, and *RsolPLR*) was monitored over time when *R. solani* was subjected to different oxidative stress inducers or when exogenous addition of vitamin B6 vitamers were added. For this purpose, *R. solani* plugs (5 mm) were grown in Petri plates containing 15 mL of half-strength potato dextrose broth (PDB) (PDB; Difco, Detroit, USA) for 3 days at 24°C. Subsequently, the PDB media in *R. solani* cultures was removed and substituted with 15 mL of fresh PDB amended with one of the following stress inducers: 5 mM of H₂O₂ (Sigma, Toronto, ON, Canada), 7.5 mM of phenylacetic acid (PAA) (Sigma) and 4 mM of the superoxide generator paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride), (Syngenta Crop Protection, Toronto, ON, Canada), or with the vitamin B6 vitamers: 0.01g L⁻¹ pyridoxine (PN) (Sigma), and 0.01g L⁻¹ pyridoxal-5'-phosphate (PLP) (Sigma). No amendment was added to the half-strength PDB media in the control plates. Oxidant sensitivity and effective concentrations (EC₅₀) for H₂O₂ and paraquat were determined by obtaining dose-response curves and the concentration which resulted in near 50% inhibition of *R. solani* growth at 72 HPT (Wang et al.,

2011), whereas the concentration of applied PAA was selected based on previous reports (Bartz et al., 2012). The vitamin B6 vitamers were chosen based on optimized concentrations that do not inhibit the growth of *R. solani*.

Comparison of the relative transcript abundance of *RsolPDX1*, *RsolPDX2*, and *RsolPLR* genes with the well-established antioxidants *glutathione S-transferase (GST)* and *catalase* genes was performed using specific primers designed in this study (Table 3.1). All experiments were conducted with three biological replicates and two technical replicates per treatment or control. Mycelia from treatments and controls were harvested at different hours post treatment (HPT) that spanned from 0.5 hour (hr) to 72 hr depending on the type of the stress inducer, flash-frozen in liquid nitrogen, and processed for RNA extraction. Additional culture plates of *R. solani* exposed to H₂O₂ and paraquat were kept for light and fluorescence microscopy.

3.3.3 Nucleic Acids Extraction

Total genomic DNA was isolated from 100 mg of ground tissue of *R. solani*, *F. oxysporum*, and *T. virens* using the DNeasy Plant Mini Kit™ (Qiagen, Toronto, ON, Canada). Total RNA was isolated from 100 mg of ground *R. solani* mycelia exposed to various stress treatments and from the control using the RNeasy Plant Mini Kit™ (Qiagen) and treated with RNase-free DNaseI™ (Qiagen) according to the manufacturer's recommendations. The concentration and purity of RNA were assessed by spectrophotometry with ND1000 (NanoDrop, Wilmington, Delaware), while RNA quality was verified on 1.2% (w/v) formaldehyde-agarose gel electrophoresis. A total of 500 ng RNA was reverse transcribed using the Quantitect Reverse transcriptase kit™ (Qiagen).

3.3.4 Genome Library Construction and Manipulation of the Vitamin B6 Biosynthesis Genes *RsolPDX1*, *RsolPDX2*, and *RsolPLR*

Degenerate primer pairs, DegF-(*PDX1*)/DegR-(*PDX1*) and DegF-(*PDX2*)/DegR-(*PDX2*), were designed from the alignment of eight protein sequences of *PDX1* (**Figure 3.1A**) and eleven protein sequences of *PDX2* (**Figure 3.1B**) belonging to different fungi, respectively. Primers design was based on the conserved domains in *PDX1* and *PDX2* (**Figures 3.1A, 1B**). The primer pairs were also tested on the genomic DNA of *F. oxysporum* (ATCC 60860), and *T. virens* (DAOM 169262) since both fungi are known to harbor both genes, *PDX1* (*F. oxysporum* Genbank accession number ENH63312) and *PDX2* (*T. virens* Genbank accession number EHK18113), respectively. The degenerate primers were used in PCR reactions to amplify putative products of *PDX1* and *PDX2* from genomic DNA of *R. solani*.

For the construction of Genome-walking libraries, *R. solani* AG3 (Rs114) genomic DNA was digested with four restriction enzymes (EcoRV, DraI, PvuII, and StuI) following the manufacturer's recommendations (Clontech, CA, USA). For Genome-walking PCR reactions, a set of gene-specific primers (*PDX1*-GSP, *PDX2*-GSP, *PLR*-GSP: 5'GSP1, 5'GSP2, 3'GSP1, and 3'GSP2) (Table 3.1) were designed based on the genomic DNA sequence of the putative products for *PDX1* and *PDX2*, and on *R. solani* mRNA sequence (Genbank accession number EU008744.1) for *PLR*. Specific PCR fragments were obtained, purified using the QIAquick PCR Purification Kit (Qiagen), and sub-cloned in pDrive cloning vector (Qiagen). Positive clones were sequenced and assembled in a contig using BioEdit v7.2.0 (Hall, 1999) and then blasted on NCBI for homology and identity confirmation. The full sequences of *RsolPDX1*, *RsolPDX2*, and *RsolPLR*

genes were annotated and submitted to GenBank database (KF620111, KF620112, and KJ395592), respectively.

3.3.5 Genomic Southern Hybridization

Specific probes for each of the *RsolPDX1*, *RsolPDX2*, and *RsolPLR* genes were constructed by amplifying genomic DNA fragments of *R. solani* using an appropriate set of primer pairs (Table 3.1). The amplified products were DIG-labeled using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, QC, Canada). The genomic DNA of *R. solani* was digested with an appropriate set of restriction enzymes [(EcoRI, PstI, and HindIII for *PDX1*), (EcoRI, PstI, and KpnI for *PDX2*), (EcoRI, PstI, HindIII, and XhoI for *PLR*)], electrophoresed on a 1 % agarose gel, transferred onto Nylon Hybond N⁺ membrane (Roche Applied Science), and UV cross-linked. Hybridization was performed following the procedure of Chamoun et al. (2013).

3.3.6 Phylogenetic Analysis

Multiple sequence alignments of *RsolPDX1*, *RsolPDX2*, and *RsolPLR* cDNA with other members of *PDX1*, *PDX2* and *PLR* from selected fungi (Ascomycetes and Basidiomycetes) and plants were conducted using Clustal Omega (Sievers and Higgins, 2014). The tree was constructed using MEGA 6.0 and generated by the maximum likelihood method based on the General Time Reversible model with 1000 Bootstrap replicates (Tamura, Peterson et al. 2011).

3.3.7 Quantitative RT-PCR

QRT-PCR assays were conducted on five target genes *RsolPDX1*, *RsolPDX2*, *RsolPLR*, *GST* and *catalase*, and three reference genes *G3PDH*, *Histone-3*, and β -*Tubulin* (Table 3.1) using Stratagene Mx3000 (Stratagene, Cedar Creek, USA). Each template and negative control had three biological replicates with two technical replicates included in each run. PCR assays were performed as previously described (Chamoun, Aliferis et al. 2013) using the appropriate annealing temperature for each primer pair (Table 3.1). The relative transcript abundance levels of the genes were calculated according to (Zhao and Fernald 2005) and normalized against the reference genes showing the lowest minimal variation and coefficient of variation (CV) across all treatments. The relative transcript abundance of the genes was tested for significance between treatments and controls at each harvesting time point by two-way analysis of variance (ANOVA) and, when appropriate, to least significant differences (LSD) at $P < 0.05$ using the SPSS statistical package v. 22.0, (IBM Corp., Armonk, NY, USA).

3.3.8 Optical and Fluorescence Microscopy

To associate changes in the transcript abundance of the genes with the ROS accumulation in *R. solani* to the different stress inducers, control and treated-mycelia with H₂O₂ (5 mM) or paraquat (4 mM) were viewed under a light and fluorescent microscope at 3 and 24 HPT. Images were digitally documented with the Moticam 2300 digital camera (GENEQ Inc. Montreal, Quebec) for light microscopy. For ROS detection, *R. solani* mycelia were incubated with 10 μ M of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), a specific ROS molecular-detection probe, in half-strength PDB for 30 min. Mycelia were then washed with pre-warmed (28°C) half-strength

PDB for 30 min to remove the non-internalized probe and were examined under Zeiss SteREO Discovery.V20 fluorescence microscopes (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) at 3 and 24 HPT. Fluorescence intensity emitted from individual cells was measured using an excitation wavelength of 470 nm.

3.4 Results

3.4.1 *R. solani* Genes *RsolPDX1*, *RsolPDX2*, and *RsolPLR* are Members of the Vitamin B6 Biosynthetic Pathway

The designed primer pairs DegF/R-(*PDX1*) and DegF/R-(*PDX2*) successfully amplified PCR products of 671 bp and 263 bp in size, respectively which were confirmed by sequencing to be *PDX1* and *PDX2*. Subsequently, the gene specific primers (*PDX1*-GSP, *PDX2*-GSP, *PLR*-GSP) (Table 3.1) were used to obtain the whole sequences of *RsolPDX1*, *RsolPDX2* and *RsolPLR* using the Genome-walking technique. Initially, only partial sequence was obtained for *PLR*, hence, additional *PLR*-GSP primers (Table 3.1) were designed based on genome sequence of *R. solani* AG3 (strain Rhs1AP) to get the complete sequence. PCR products for each of the three genes were assembled into contigs and their sequence analysis led to the identification of the vitamin B6 genes belonging to the *de novo* pathway in *R. solani*, *RsolPDX1* (accession number KF620111) and *RsolPDX2* (accession number KF620112), and to a identification of *RsolPLR* (accession number KJ395592) belonging to the vitamin B6 salvage pathway. The organization of the three genes and their features are depicted in **Figures 3.2-3.4**.

The full length of the *RsolPDX1* and *RsolPDX2* sequences obtained during our analyses are 1640 bp and 1178 bp, respectively. The open reading frame of *RsolPDX1* consists of 1402 nucleotides

encoded from 61 to 1463 bp, while the ORF of *RsolPDX2* consists of 980 nucleotides encoded from 99 to 1079 bp. Both genes are interrupted with several introns (**Figures 3.2, 3.3**). The predicted protein sequences of *RsolPDX1* and *RsolPDX2* consist of 322 and 248 amino acid residues with a molecular weight of 33.65 or 26.78 kDa and a calculated isoelectric point (pI) of 6.625 or 5.815, respectively. The signature motif of the PDX1 protein family (LPVVSFAAGGVATPADAA) and conserved amino acids (D, M, K, and GTG) were identified in PDX1 (**Figure 3.2**). Similarly, the signature motif of the PDX2 protein family (C-H-E) which is essential for the protein activity in addition to other conserved amino acids (i.e., PGGEST, FIRAP and FHPE) are highlighted in PDX2 (**Figure 3.3**).

The obtained full length of the *RsolPLR* sequence is 1837 bp and its sequence analysis revealed that it belongs to the aldo_keto_reductase family. *RsolPLR* ORF is encoded from 50 to 1627 bp and is interrupted by several introns whereas conserved amino acids (i.e. AR, SE, E, and YSPLG) in PLR proteins are highlighted (**Figure 3.4**). *RsolPLR* gene encodes a putative polypeptide of 337 amino acid residues with a molecular weight of 36.92 kDa and a calculated isoelectric point (pI) of 5.89 (**Figure 3.4**).

3.4.2 Gene Copies of *RsolPDX1*, *RsolPDX2* and *RsolPLR*

Gene copies of *RsolPDX1*, *RsolPDX2* and *RsolPLR* in the genome of *R. solani* were estimated by Southern blot analysis (**Figure 3.5**). Under high stringency conditions of hybridization, *RsolPDX1* and *RsolPLR* probes hybridized to more than one high molecular fragment, indicating that two copies are present for each of the genes. On the other hand, *RsolPDX2* is shown to be present as single copy as revealed by the hybridization of its corresponding probe to a single fragment (**Figure 3.5**).

3.4.3 *RsolPDX1*, *RsolPDX2*, and *RsolPLR* are Phylogenetically Related to Homologs of the Vitamin B6 genes

The phylogenetic tree of *PDX1*, *PDX2*, and *PLR* shows a complete separation between the three families of vitamin B6 biosynthesis genes. Within each gene family, Basidiomycete and Ascomycete sequences have clustered in separate clades. *Candida albicans* and *Saccharomyces cerevisiae* were clustered in a separate clade for each gene. *PDX1*, *PDX2*, and *PLR* sequences of different *R. solani* anastomosis groups are consistently clustered together in a separate clade (**Figure 3.6**). Within each of the two families, *RsolPDX1/2* are most closely related to *PDX1/2* of Rhs1AP, another strain of *R. solani* AG3. Finally, within the aldo-keto reductase superfamily, *RsolPLR* gene is closely clustered to the *PLR* gene of *R. solani* AG1-IA, the rice sheath blight pathogen (**Figure 3.6**).

3.4.4 *R. solani* Genes of the Vitamin B6 *de Novo* and Salvage Pathways are Differentially Regulated by Different Stress Inducers

To provide insight into the regulation of *R. solani* vitamin B6 biosynthesis genes exposed to different stress inducers, and investigate whether they could play a role in homeostasis of ROS, the relative transcript abundances of *RsolPDX1*, *RsolPDX2*, and *RsolPLR* genes were assessed in *R. solani* at 24 and 72 HPT with paraquat and PAA and also at 0.5 and 3 HPT with H₂O₂.

3.4.4.1 Paraquat

Compared to control cultures, the relative transcript abundance of *RsolPDX1* and *RsolPDX2* has significantly increased at both time points with a higher fold increase of 6.0 and 9.9 in *RsolPDX1* and *RsolPDX2*, 24 HPT, respectively (**Figures 3.7A,D**). Likewise, there was a significant increase in relative transcript abundance level of *RsolPLR* at both HPT with a notable fold increase of 32.8 (**Figure 3.7G**).

3.4.4.2 H₂O₂

Exposure of *R. solani* mycelia to H₂O₂ had no effect on *RsolPDX1* (**Figure 3.7B**) but an effect on *RsolPDX2* at 3 HPT with a slight fold increase (1.68 fold) (**Figures 3.7B,E**). Notably, H₂O₂ caused a notable increase in *RsolPLR* relative transcript abundance at 0.5 and 3 HPT with a substantial fold increase of 27.8 fold at 3 HPT (**Figure 3.7H**).

3.4.4.3 PAA

To obtain further insights into the putative activities of vitamin B6, we also assessed whether the relative transcripts abundance of *RsolPDX1*, *RsolPDX2*, and *RsolPLR* are regulated by PAA, an antimicrobial and antioxidant compound produced by *R. solani* AG3 (Bartz, Glassbrook et al. 2012). The three genes were mostly down-regulated in the range of 5.0-1.5 fold at 24 and 72 HPT, respectively (**Figures 3.7C,F,I**).

3.4.5 The Antioxidant Encoding Genes *GST* and *catalase* are Transcriptionally Regulated in *R. solani* by Abiotic Stress

To confirm the presence of oxidative stress in *R. solani*, the activity of other well-known antioxidant genes like *GST* and *catalase* was compared to the activity of the vitamin B6 genes under the same abiotic stress conditions (**Figure 3.8**). *GST* was significantly induced by paraquat and H₂O₂ (**Figures 3.8A,B**) with an 8.6 fold and 4.2 increase at 24 and 72 HPT, respectively. Paraquat significantly upregulated *catalase* at both time periods, with a highest increase of 3.9 fold at 72 HPT (**Figure 3.8C**). However, exposure to H₂O₂ caused a downregulation of 5.9 fold decrease as compared to the control at 3 HPT (**Figure 3.8D**).

3.4.6 *R. solani* Vitamin B6 Genes are Regulated by External Supplementation of Vitamin B6 Vitamers

To understand the role of vitamin B6 vitamers on the transcriptional regulation of vitamin B6 genes, *R. solani* was grown in PDB amended with either PN or PLP. No growth differences were observed between the control and the treatments (data not shown). Exogenous addition of PN, induced the regulation of both *RsolPDX1* and *RsolPDX2* (6.1 and 7.7 fold, respectively) at 3 HPT (**Figures 3.9A,C**), caused a drop to near basal level values at 24 HPT (1.7 and 1.1 fold, respectively), followed by significant down-regulation (16.7 and 9.1 fold, respectively) at 72 HPT (**Figures 3.9A,C**). PN caused a decrease in *RsolPLR* relative transcript abundance at all time points (**Figure 3.9E**). The amendment of growth medium with PLP, induced the expression of *RsolPDX1* and *RsolPDX2* at 24 HPT followed by a drop to the basal level at 72 HPT (**Figures 3.9B ,D**). There was a substantial increase in transcript relative abundance of *RsolPLR* 3 HPT (2.5 fold) and 24 HPT (6.7 fold) when PLP was added exogenously (**Figure 3.9F**).

3.4.7 Accumulation of ROS in *R. solani* Mycelia is Linked to Transcriptional Regulation of *RsolPDX1*, *RsolPDX2* and *RsolPLR*

To determine whether the increase in vitamin B6 transcripts in the paraquat or H₂O₂-treated mycelia are linked to an accumulation of ROS in the fungal hyphae, the presence of these chemical species was estimated by adding H₂DCF-DA to the growing media in which the fungus was exposed to 3 and 24 hr under H₂O₂ and paraquat stress, respectively. An intense green fluorescence, corresponding to the oxidative form of H₂DCF-DA, was detected in both treatments (**Figures 3.10G-L**). Hyphae of the untreated control plates did not display any green fluorescence (**Figures 3.10A-F**). *R. solani* growth was reduced by the addition of paraquat and H₂O₂ as compared to the control treatment (**Figures 3.11A,C,D**). Additionally, H₂O₂-treated hyphae showed increased levels of vacuolarization and compaction (**Figures 3.11D,F**) when compared to the control (**Figure 3.11B**) leading to loss of viability. These data confirm that exposure of the fungus to paraquat or H₂O₂ induces an oxidative stress in the fungal hyphae and could be related to the oxidative status of the fungus.

3.5 Discussion

In this study, we characterized for the first time the *PDX1/PDX2* genes of the *de novo* component and the *PLR* gene of the salvage component of the vitamin B6 biosynthetic pathway in the plant pathogenic fungus *Rhizoctonia solani* AG3 (Rs114) and provided the evidence for their transcriptional regulation under different stress inducers. We also show that these genes might play a role in oxidative stress protection in *R. solani*.

Sequences of the vitamin B6 genes are similar to those found in other organisms. The predicted amino acid sequences of the three genes showed high degree of similarity to other fungal PDX1, PDX2, and PLR proteins, and their close relatedness to other fungal homologs was confirmed (Ehrenshaft, Bilski et al. 1999, Osmani, May et al. 1999, Bilski, Li et al. 2000, Raschle, Amrhein et al. 2005, Benabdellah, Azcon-Aguilar et al. 2009). The release of the draft genome sequence of *R. solani* AG3 (strain Rhs1AP) (Cubeta, Thomas et al. 2014) further confirmed the genes' annotations. The presence of conserved amino acids suggests the ability of PDX1-PDX2 complex formation that is crucial for vitamin B6 biosynthesis (Belitsky 2004, Raschle, Amrhein et al. 2005). Interestingly, there is variation in the number of introns present in various vitamin B6 *de novo* biosynthesis genes. As opposed to the *PDX1* or *PDX2* homologs identified in *Cercospora nicotianae* and *Glomus intraradices* which are intronless (Ehrenshaft, Bilski et al. 1999, Ehrenshaft and Daub 2001), the three vitamin B6 genes in *R. solani* AG3 (Rs114) contain introns similar to *PyroA* (*PDX1*) gene of *Aspergillus nidulans* (Osmani, May et al. 1999). From an evolutionary stand point of view, the presence of introns is advantageous because it provides organisms with diversity of proteins through alternative splicing or because introns might possess elements that are involved in regulation of gene expression (Kalsotra and Cooper 2011, Yang, Zhu et al. 2013).

Southern blot analysis indicated that two copies are present for *RsolPDX1* and *RsolPLR*, most likely as a result of gene duplication, and a single copy is present for *RsolPDX2* in *R. solani* AG-3 (Rs114). While most other fungi contain a single copy of those genes (Ehrenshaft, Jenns et al. 1998, Osmani, May et al. 1999, Ehrenshaft and Daub 2001, Benabdellah, Azcon-Aguilar et al. 2009), *Saccharomyces cerevisiae*, *SNZ* (*PDX1* homolog) and *SNO* (*PDX2* homolog) genes were shown to have three members each with only *SNZ1* and *SNO1* being implicated in vitamin B6

biosynthesis (Padilla, Fuge et al. 1998, Rodriguez-Navarro, Llorente et al. 2002). Only one copy of each of the three genes was found in the draft genome sequence of *R. solani* AG3 (strain Rhs1AP). This could be an indication that the *PDX1*, *PDX2* and *PLR* gene copy numbers differ among the various *R. solani* strains, although this will require subsequent validation once higher quality genome assemblies are released. Our Southern hybridization provides only preliminary evidence of gene copy number. We don't exclude the possibility of having additional copies due to high molecular size of the fragments obtained. Much remains to determine whether the two copies of *RsolPDX1* and *RsolPLR* are both functional or one of them is a pseudogene.

To date, evidence that *R. solani* genes of the vitamin B6 pathway are upregulated in response to biotic stress has been reported (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011, Gkarmiri, Finlay et al. 2015). However to the best of our knowledge, little is known of the physiological responses of *R. solani* to various oxidative stress and also no studies exist on transcriptional regulation of genes in the vitamin B6 biosynthetic pathway, nor on their role in oxidative stress alleviation in *R. solani* experiencing abiotic stress.

The increased formation of ROS in hyphal cells can induce oxidative stress and damage to DNA, RNA, protein, and lipids, leading to the loss of cell viability (Sharma, Jha et al. 2012). Our results clearly showed that paraquat and H₂O₂ induced ROS formation in hyphal cells of *R. solani* leading to growth reduction and loss of viability of hyphal cells. These observations were linked to the regulation of vitamin B6 encoding genes and their collective possible role as oxidative stress alleviation. Our findings indicate exposure of *R. solani* to paraquat upregulates *RsolPDX1* and *RsolPDX2* expression possibly suggesting a role of vitamin B6 in protection of *R. solani* against superoxide anions. In other organisms, gene regulation studies have continually connected vitamin B6 to oxidative stress and noted an increased transcriptional activity of vitamin B6 *de novo* pathway genes in response to oxidative stress. As examples, *GintPDX1*, the *Glomus intrarradices*

and SNZ1, the *Saccharomyces cerevisiae* PDX1 homolog and PDX2 of *S. pombe* showed increased transcript accumulation upon treatments with Paraquat and H₂O₂, respectively (Lee, Dawes et al. 1995, Chen, Toone et al. 2003, Benabdellah, Merlos et al. 2009).

Furthermore, we show evidence that the vitamin B6 salvage pathway appears to be involved in the oxidative stress response in *R. solani*. *RsolPLR* transcript levels were up-regulated by both paraquat and H₂O₂ at all time points. This finding is in agreement with the transcriptional increase of *PLR* in *S. pombe* after 1 hr of H₂O₂ treatment (Chen, Toone et al. 2003) and with the role of *PLR* in PN catalysis (Morita, Takegawa et al. 2004, Sang, Barbosa et al. 2007). The reason for the differential transcriptional regulation of *RsolPDX1* and *RsolPDX2* and *RsolPLR* by H₂O₂ and superoxide radicals is unknown, and may reflect differences in the type of damage induced by ROS.

To overcome oxidative damage, all living organisms have developed antioxidant systems to efficiently quench ROS excess and to keep ROS production and scavenging systems in check (Sharma, Jha et al. 2012). Maintaining cellular ROS homeostasis is achieved by the production of non-enzymatic antioxidants such as *GST* and enzymatic antioxidants such as catalases. Therefore, studying the regulation of *GST* and *catalase* in stressed cultures of *R. solani* is direct confirmation that these genes play a role in oxidative stress protection. Similar to vitamin B6 encoding genes, differential upregulation of *GST* and *catalase* was observed in response to the type of stressor, although the fold increase in vitamin B6 encoding genes was substantially higher. These results may indicate that vitamin B6, via the expression of *RsolPDX1* and *RsolPDX2* and *RsolPLR*, has better ability than glutathione to decrease ROS levels (Ehrenshaft, Bilski et al. 1999).

The role of *RsolPDX1* and *RsolPDX2* and *RsolPLR* in vitamin B6 biosynthesis suggests a possible regulation of their expression by vitamin B6 and the expression of the homologs is

dependent on vitamin B6 availability. Surprisingly in other biological systems, regulation of *PDX1* expression was not affected by the addition of vitamin B6 (Rodriguez-Navarro, Llorente et al. 2002, Benabdellah, Azcon-Aguilar et al. 2009). The authors claimed that this independence is related to the high constitutive expression levels that may mask the effect of the absence of the vitamin. In our study, both PN and PLP vitamers up-regulated the vitamin B6 *de novo* genes, *RsolPDX1* and *RsolPDX2*, in *R. solani*. Given that the involvement of the pyridoxine encoding *de novo* genes in vitamin B6 biosynthesis, and that the central role of PLP as a cofactor hold the presence of a regulatory switch based on the amount of PN and PLP available is inevitable (Mooney, Leuendorf et al. 2009), it is very plausible that vitamin B6 vitamers may act as regulators. Alternatively, the addition of vitamers might be perceived by *R. solani* as signal indicating stress leading to upregulation of the *de novo* vitamin B6 genes on the onset of treatment. In a similar fashion, plants supplemented with the well-known antioxidants riboflavin (vitamin B2) or thiamine (vitamin B1) developed systemic resistance to bacterial or fungal infections along with increased transcription of PR related genes (Dong and Beer 2000, Ahn, Kim et al. 2005). As for the decrease in the transcript abundance levels of both *RsolPDX1* and *RsolPDX2* at 72 HPT might be due to conversion of PN into PNP and eventually to PLP, resulting in decrease of substrate availability (Zhao and Winkler 1995, Rueschhoff, Gillikin et al. 2012). In this regard mechanisms of how vitamers regulate the *de novo* vitamin B6 genes remain to be explored further.

Contrary to *RsolPDX1* and *RsolPDX2*, the transcriptional response of *RsolPLR* was vitamer specific; a down regulation in response to PN and up regulation in response to PLP. These results were expected since PN is the product of the reaction catalyzed by *PLR* (Herrero, González et al. 2011) causing a decrease in relative transcript abundance of *RsolPLR*. Increase in transcript levels

may be due to the conversion of PLP to PL, the substrate for PLR (Zhao and Winkler 1995, Herrero, González et al. 2011).

3.6 Conclusion

RsolPDX1, *RsolPDX2*, and *RsolPLR* represent three important components of the vitamin B6 pathway in *R. solani* AG3 (Rs114). These genes are differentially regulated upon various types of oxidative stress, such as paraquat and H₂O₂. Taken together, the indirect participation of the *de novo* (*RsolPDX1* and *RsolPDX2*) and salvage (*RsolPLR*) vitamin B6 genes in the oxidative stress response of the plant pathogenic fungus *R. solani* is strongly suggested. In yeasts, gene disruption and over expression have been valuable in delineating the biological role of *PDX1* from fungi. Comparable approaches should prove equally valuable to define the role of *R. solani* vitamin B6 genes.

3.7 Author Contributions

JS, RC, and SJ conceived, designed and executed the experiments. EGT helped in the execution of certain experiments. JS and RC analyzed the data. JS, RC, and SJ contributed to the writing of the manuscript.

3.8 Acknowledgements

The work was supported by a research grant to SJ from the Natural Sciences and Engineering Research Council of Canada (NSERC-Discovery, grant number 137135-13). The authors thank J.-B. Charron for the use of Zeiss SteREO Discovery.V20 microscope.

Table 3.1. List of primers of *R. solani* used in this study.

Primer	Sequence (5' → 3')	Annealing temperature (°C)	Amplicon size (bp)	Method	Reference	
Vitamin B6 <i>de novo</i> pathway genes						
DegF-(<i>PDX1</i>)	AAGGTACCTGTNACVATYCCNGTBATGG	54	670	Degenerate PCR	This study	
DegR-(<i>PDX1</i>)	TTCTGCAGAGCNGCRTCVGCVGGNGTVGC					
5'GSP1-(<i>PDX1</i>)	TGACGAACAGCCTCGACAACATTTCC	62	219	Genome walker & Real time-PCR	This study	
3'GSP2-(<i>PDX1</i>)	TCCGTTTGTTCTGTGGGGCTACATCTCTC					
5'GSP2-(<i>PDX1</i>)	ATCATGGCTGCGCCTTCGGAATAACG	62	—	Genome walker	This study	
3'GSP1-(<i>PDX1</i>)	TCTCACCCCTGCTGACGAACAGCATC	62	300	Genome walker & Southern blot	This study	
<i>PDX1</i> probeR	TTGCGAATCTCGGCGTTGACCG	65				Southern blot
DegF-(<i>PDX2</i>)	AACTGCAGTTGGGGNACHTGYGCNGG	51	260	Degenerate PCR	This study	
DegR-(<i>PDX2</i>)	CCTCTAGAGACNGGNGCNCKDATRAA					
3'GSP2-(<i>PDX2</i>)	AAGAAGGGTGGTCAAGAGGTTTTTG	72	—	Genome walker	This study	
5'GSP2-(<i>PDX2</i>)	CGAAAGATTCAAGCTTCAATGGTGCTTA					
3'GSP1-(<i>PDX2</i>)	AATCTTGCTTGCCTCTGGTGGTGTG	72	225	Genome walker, Real time-PCR PCR	This study	
5'GSP1-(<i>PDX2</i>)	ATCCCATTAATGGTCGGTCCTCATCA	72	640	Genome walker, Real time-PCR & Southern		
<i>PDX2</i> probeF	ATGACTAGAACTGAAACGGAAC	62				Southern blot
Vitamin B6 salvage pathway genes						
<i>PLR</i> AKR8 F	GAAAGCCTCCTCTTGAATCT	58	300	Real time-PCR & Southern blot	Chamoun and Jabaji, 2011	
<i>PLR</i> AKR8 R	GGGTAAGATTGGATCGATTGGG					
5'GSP1-(<i>PLR</i>)	GGCGATGATCTTTAATGCGTCCACTAG	67	—	Genome walker	This study	
5'GSP2-(<i>PLR</i>)	TTTGAAAGCCTCCTCTTGAATCTGG					
3'GSP1-(<i>PLR</i>)	AAGTTTCATTCTGGAGCTACGAGGAAG	52	837	Sequencing	This study	
3'GSP2-(<i>PLR</i>)	TAAAGTGATTGCCAAGGCTGCTGAAATTG					
5'GSP3-(<i>PLR</i>)	TAATAGAAAGCAAGAAATCGC	57	791	Sequencing	This study	
3'GSP3-(<i>PLR</i>)	GCTCAAATAAATCAACCTTC					
5'GSP4-(<i>PLR</i>)	AAGAGACTCGTAAAGGTGCG	57	791	Sequencing	This study	
3'GSP4-(<i>PLR</i>)	ATGCCACCAATTTCGTTTCAG					
Antioxidant genes						
<i>RS-GST^a -F</i>	AGAAGACGAGGCAAAATGCGA	57	256	Real time-PCR	gi 576992090	
<i>RS-GST^a -R</i>	ATCTCTTCAACCGCCTTCCAGT					
<i>RS-Catalase-F</i>	ACCAGAAGTGTTAGTCCAGCGG	56	190	Real time-PCR	gb JATN01000310.1	
<i>RS-Catalase-R</i>	CATCCGGTCACAGCAGCGTAA					
Reference genes						
<i>Tubulin-F</i>	GTTGATTTCGAAGATCCGTG	55	139	Real time-PCR	FJ392707	
<i>Tubulin-R</i>	CGAGTTCTCGACCAACTGAT					
<i>Histone 3-F</i>	AAGTCTGCACCCGTAAGTTC	55	289	Real time-PCR	M. Cubeta ^b	
<i>Histone 3-R</i>	AACAACGAGACGAGGTAAGC					
<i>G3PDH^c -F</i>	GGTATTATTGGATACACTGA	55	129	Real time-PCR	Chamoun and Jabaji, 2011(Chamoun and	
<i>G3PDH^c -R</i>	TTAAGCCTCAGCGTCTTTCT					
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	55	Variable	cDNA quality	Gardes and Bruns, 1993	
ITS4-R	TCCTCCGCTTATTGATATGC					

^a Glutathione S-Transferase

^bSequence of Histone 3 gene was provided by Marc Cubeta, North Carolina State University

^c *G3PDH*: Glyceraldehyde-3-phosphate dehydrogenase gene

highlighted DegF: Degenerate forward primer, DegR: Degenerate reverse primer. Boxes represent the location of degenerate primers used in PCR.

```

1   CTATAGGGCACGCTGCTCGACGGCCCGGGCTGGTAAATTCGGATTCGGG
51  CCACTCGATTCTCAACTACGACTGTGACTCGATCTCCTTGCTATCATGTC
    M S

101 TGCTCCTGTGTGCTCTCTGCCAACGGAACCTTCTACCCCTACTCCTGCTG
    A P V S S L P N G T S T P T P A

151 CGACTCAAGCAGCTGCTGCGGGTGTCTAATGGTCTGCTGCGCCACGC
    A T Q A A A A G A A N G P A A P R

201 TCCGCTCAGAACAGGATGGGAGCAGTAGCTTGGGCACTTCCAGGTCAA
    S A Q N K D G S S S L G T F Q V K

251 ATCTGGTCTGCGCCAGATGCTCAAGGTAAGAGTTGTTTCATAAAATACGC
    S G L A Q M L K

301 ACACCTGGCTGATATGAGAATAGGTGGTGTGATCATGGACGTCGTCAAC
    G G V I M D V V N

351 GCCGCGCAAGCACGATCGCTGAAGAGGCCGCGCCTGCGCTGTCATGGC
    A A Q A R I A E E A G A C A V M A

401 TCTAGAGCGCGTTCCGCCGATATCAGGAGAGAGGGCGGAGTCGCACGTA
    L E R V P A D I R R E G G V A R

451 TGGTATGACCTGGACTCGTTAAATTTTACGGTGAGACTAACAGTTTGC
    M

501 GGTACAATAGAGCGATCCTAAATGATCAAAGAGATTGTGGACGCCGTGA
    S D P K M I K E I V D A V

551 CCATTCCCGTGATGGCCAAAGTCCGCATTGGCCATTTTGTGAGGCCCGAG
    T I P V M A K V R I G H F V E A Q

601 GTATGTGTGACCTTTTTTGTCTGTGCTCCGGCCCGCCGACTCATACTT

651 CACGTGCTTTGATAGATTCTCCAGGCGATCGGGATCGACTATATCGACGA
    I L Q A I G I D Y I D E

701 GTCCGAGGTTCTCACCCTGCTGACGAACAGCATCACATTAACAACACT
    S E V L T P A D E Q H H I N K H

751 GCTTCGGTATTCGGTTTCTCTGGGGGCTACATCGCTCGGTTAGTTTTC
    S F R I P F V C G A T S L

801 ACACAATGTATGCGTGTGATTGATTGACTGATCTCGATCTTTTGGCGCC

851 TTGTGACTTGTGATTGTTTTCAATATTTCCCCACAAGCCGAGGCGCTTC
    G E A L

901 GTCGTATCTCCGAAGCGCGACCCATGATTCGCACCAAGGGCGAGGCTGGA
    R R I S E G A A M I R T K G E A G

951 ACTGGAAATGTTTTCGAGGCTGTTGCTCATCGCGGGCGGTCACACGCGGA
    T G N V V E A V R H A R A V N A E

1001 GATTCGCAAGCCGCTAGTATGACCGATGAGGAGCTTTACTCTTATGCGA
    I R K A A S M T D E E L Y S Y A

1051 AGGAGTGGCTGCTTCTTTTCATCTTCGAGACGAGATAGGTTCTTAATATT
    K E

1101 TTATGAACAGGATTCAAGCACCCCTCCACCTTCTCAAGGAGACGGCCCGC
    I Q A P F H L L K E T A R

1151 CTGAAGCGTTTGCCAGTTGTGACGTTTCCGCCGAGGTTGTTGCTACCCC
    L K R L P V V S F A A G G V A T P

1201 CGCTGACGCTGCCATGATGATGCAACTCGGTTGCGACGGAGGCAAGTGT
    A D A A M M M Q L G C D G

1251 CATATCATGAATCTGCAGGCTAGCTCATAATATACATATAGTCTTTGTCG
    V E V

1301 GATCTGGCAITTTTCTTGTGCGTTCGGCCGATGATCTCCACATTCATTC
    G S G I F L

1351 TTATTGATCAATTCGATTTTAACAGTCGGGAGACCCAGCTAAGCGTGCTC
    S G D P A K R A

1401 GTGCGATCGTCCAGTCCGTTACGCACTACAATAACCCCAAGGTTTTGGCC
    R A I V Q S V T H Y N N P K V L A

1451 GAGGTTTCCGAAGATCTAGGAGAGGCCATGGTTGGGCTGACTATGTGAGT
    E V S E D L G E A M V G L T M *

1501 GCCTTGCGAGACGCATATACCCACTCTTTGCCCTCCTTATGTCAGCCGCT
1551 GACCAAGCTTTTGTCTTACTCAGCGACAAAGACCTCAAGGGCGGACGTAT
1601 GGCCGGAAGAGGCTTGTAACATACACTGCAGCCATCTGTGAATGATCGT
1651 GAAGTGAAGACATATTGTTGATT

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Figure 3.2. Complete sequence of *RsolPDX1* (gene accession number KF620111). Introns are written in italic in the gene sequence. The signature motif of the PDX1 protein family is underlined. Amino acids known to be essential for enzyme activity are shadowed. Asterisk indicates end codon. Dotted line indicates southern blot probe.

```

1   CGACGGCCCGGGCTGGTATCGCATAGTACATTTTGTAAACCGAAATCCAT
51  CCGAGCTTACCACTTTTACTTTTGGACCTCAACTGCGACCAGCCTCGAT
    M
101 GACTAGAACTGAACCGAAGCCCGACTATGCGGATGCTACTGTCGSCG
    T R T E T E R P T M A M A T V G
151 TCCTAGGTTGTCGCTCCGTCACCTCATGCGCCCATTTTAGACATCGTA
    V L
201 ATTCTTCCCATACTCGCTCGCTTATTGTCTTGCCCGTTGATCATCGAAAC
251 CATATCGTGTGACTTACATCTATCTATCCCACAGCACTTCAGSGAGCATT
    A L Q G A F
301 TGCAGAACACCCAGGAGATTATATCACGCGTGACACCATGTGTACATGCCA
    A E H Q E I I S R V T P C V H A
351 CCCTCGTACGGAATCCGCGAGAACTCGCACTCTGCGACGGGCTGATCATC
    T L V R T P A E L A L C D G L I I
401 CCGGGCGGAGAGAGTACGTCAATTGTGCTCGTGACGATTGGCAATCT
    F G G E S T S I V L L A R L A N L
451 AATAGACCCCTTGCAGAGATTGTGAGCGAGAACCCGTTTGGGGAACGT
    I E P L R E F V R T K P V W G T
501 GTGCTGGGGCAATCTTCTTCCCTCTGGTGGTGTGAAGGCCCAAGAAG
    @ A G A I L L A S G G V E G A K K
551 GGTGGTCAAGAGGTTTTTGGAGGTGTCGGGCTCCGGATCGGACGAAACGG
    G G Q E V F G G V G V R I G R N G
601 ATTTGGCTCGCAGGTAAGTTATTGGTCCTTTAAGTTCAAACTAGGTCATA
    F G S Q
651 CTTAAGCACCATTGAAGCTTGAATCTTTCGAAGCGAGCTGAGCTCAAC
    L E S F E A E L E L N
701 GGCCTTGTGATGAGGACCGACCATTTAATGGGATATTTCAGAGCGCC
    G L A D E D R P F N G I F I R A P
751 CGTGAGTCTCATAGACCTACGAGTTGAGGTACCGACTGAATAATAGACTG
    G
801 GTCTAGATAATTCTGTCGTTTACGAAGCCCGACCCCAAGCGCCCTATAGA
    I I L S F T K P D P K R P I E
851 ATACCTAGCTCGATTACCCGATCATTACTCCCGAGGACCCACCCACA
    Y L A R L P D H L L P E D P P H
901 CCGGTTCGGGCGACATCACCTCAGGTGATCCAGCATCGACCCCGTACC
    T G S G D I T S G D P S I D P R T
951 GTGGTGGCACTGCGGCAAGGACGAACACTGCTCACGACATCCACCCTGA
    V V A L R Q G R T L L T T F H P E
1001 GCTCACGCGTGACATCGATTCCATGAGTATTTTGTTCGGAGCTGTGTAC
    L T R D D R F H E Y F V R S C V
1051 TTGGGCTTCTCCGAGCTTTGCCAAGTGAACCGATGGTGGCACCGGATT
    L G L S P S F A K *
1101 GGCTAGATGTTACGAAAGCACAGATGGAGCAATAGAAATCTAGACATAGT
1151 TTGTTACAGGACCAGCCCGGGCCGTCGA

```

Figure 3.3. Complete sequence of *RsolPDX2* (accession number KF620112). Introns are written in italic in the gene sequence. The signature motif of the PDX2 protein family C-H-E (known to be essential for enzyme activity) is underlined. Conserved amino acids are shadowed. Asterisk indicates end codon. Dotted line indicates southern blot probe.

```

1   TTAGCCCTAATTTTTGTGATCTAAAAACACACCCGGGCCAGAA
51  TGGTCCAAACAACACTATATTCGGAGTTCTATTGTTAAGATTGGCCAT
    M V Q T T T I F G V P I V K I G H

101 GGTTTGATGTGGGAGAGTAATTGCGATTTCGGAGTGTGAGCTTGCCTGATT
    E L M W

151 TTACCGGATAAACCTACACAGATGCTCTGCGTTCAGAACCCCTTTTGA
    M S C V P E P P F D

201 TGAACAGTGCCTTGTAGTTGATTATGTCAGGTATCAACTTTGCTCCTCCCG
    E Q C F E L I I A G I N F A P P

251 GAGTCAAAGTATTTTTGAATGGTGGTAAGTAGCAAAAAAACACCTTAAT
    G V K V F L N G

301 ACGGGTAAATGCTGACTTCTATATAGGGGAATTCATGGCACAGCTCGA
    G E F M G T A S

351 ACATAAATGCCAATCTAGAACTCCTGAACAGATTTTTTACCAAGTATCCG
    N I N A N L E L L N R F F T K Y P

401 CAGTACGCTGAACGTACTTTTCTTCCGTCAGGTGATACCAAGCAATGG
    Q Y A E R T F L S V K

451 ATAAGGTTTCGAGCATAGCTGACAACTCAATTAGGGGCGGTTGTTGTTT
    G A V V V

501 CTGAACACGGCTTTGCTTGGGACAGTTCGTAAGTGGAGTCAGATATCTCC
    S E H G F A W D S

551 CAATTGAGTTGACATCAACCGTCACTATGAAGTCCCGCAGGTTTAGAGCG
    S P A G L E R

601 TAGTATCAACAACATCATTTCAAAGCTGGGCCCAAACAAGAAGTTGATT
    S I N N I I Q K L G P N K K V D

651 TATTGAGCCCGCTCGGTATGTATCGATTTTGACATTAACTGTCTATGTA
    L F E P A R

701 CGGGTCAGACTGATAGGATTGGTGCAATTACAGTGTGATCCCAAAGTTC
    V D P K V

751 CTATTGAAGAAACCATGGCTGACTCAGTAAATATGGTGAAGCTTAGGAA
    P I E E T M A V L S K Y

801 AATTCATATCTGACTGAGAGGTTCACTTGACTGCAACAGTTGAATC
    V E S

851 GGGTAAGATTGGATCGATTGGGCTGTCGGAATGTTCTGCGGCTACACTTG
    G K I G S I G L S E C S A A T L

901 AACGGCAAGCAAGGATTCATATACTATTGATATACCTGGGCGGAA
    E R A S K

951 GCTGACAAATGTAGAGCTAGGTCGGCAAGTGGCGCCGTTGAAATAGAAG
    V G K V A A V E I E

1001 TTTCAATCTGGAGCTACGAGGAAGAGACTCGTAAAGTGCCTATTTAATA
    V S F W S Y E E E T R K

1051 TTCATGGGCCCCCGGACGCTTCAAGTGGATTGCGAGTATTCGCAAGG
    V I A K

1101 CTGCTGAAATTGGGCGGTAGTTGCTGGATATAGCCCCCTTGGACAAGGA
    A A E I G A V V A A Y S P L G Q G

1151 GTATTGACTGGAAGCCTAAATCCACACAGCTGCATAGGATGATTAATCG
    V L T G S L N P T Q L H K D D Y R

1201 CAATGACTTCCCGAGATTTTCAAGAGGAGGTAAACGAAGCTTGATTCTGATT
    N H F P R F Q E E

1251 GGGCCTCACTTGCTGATGAGTGAATCAACAAATAGGCTTTCAAAAACAA
    A F K N N

1301 TATGAAGCTAGTGGACGCATTAAAGATCATCGCCGAGAAAAAGAAATATTA
    M K L V D A L K I I A E K K N I

1351 CACCCGCTCAGCTCTGTCTGGCCTGGGTATCCTCTCTCGCACCACACGTG
    T P A Q L C L A W V S S L A P H V

1401 GTTCCAAATCCCAAGTCAACGTGAGTATGAAAGCTATTTCATGTTTCAAAC
    V P I P S S T

1451 GAGATAACTGAATCTTTTGTGTCGTAGACGTGCGGCTCGTACATTGGAG
    R A A R T L E

1501 AACGTGTCTGCAGCTTCGGTTGAGCTGAGCGAACAAGACCATGAGGAAAT
    N V S A A S V E L S E Q D H E E I

1551 CAAGCATGCAATGGAGTTGAATCCAGTATCTGGCGATCGATATGCTAAGG
    K H A M E L N P V S G D R Y A K

1601 AAATCATGAAGTCAGTCTGGGGGTGATTATCTCGTGGCTCTTGATGTGCA
    E I M K S V W G *

1651 TATATTCAGGGACTTGGAGTATGCAAAGTGTGTCTATAGAAGTTGAAGGA
1701 AATATAGCACTTTTATGTTTCAGCGGAATATTCTAACCATATAAACTTG
1751 CTTCAAGCCAGCTCAAGCCTGAGCTAACGAGTGGAGCAATAGCTAAACGA
1801 AATTGGTGGCATGGAGCGCCACGTACTCTCCTGGGC

```

Figure 3.4. Complete sequence of *RsolPLR* (gene accession number KJ395592). Introns are written in italic in the gene sequence. The four residues that compose the Aldo_keto_reductase conserved catalytic tetrad are underlined. Conserved amino acids are shadowed. Dotted line indicates southern blot probe.

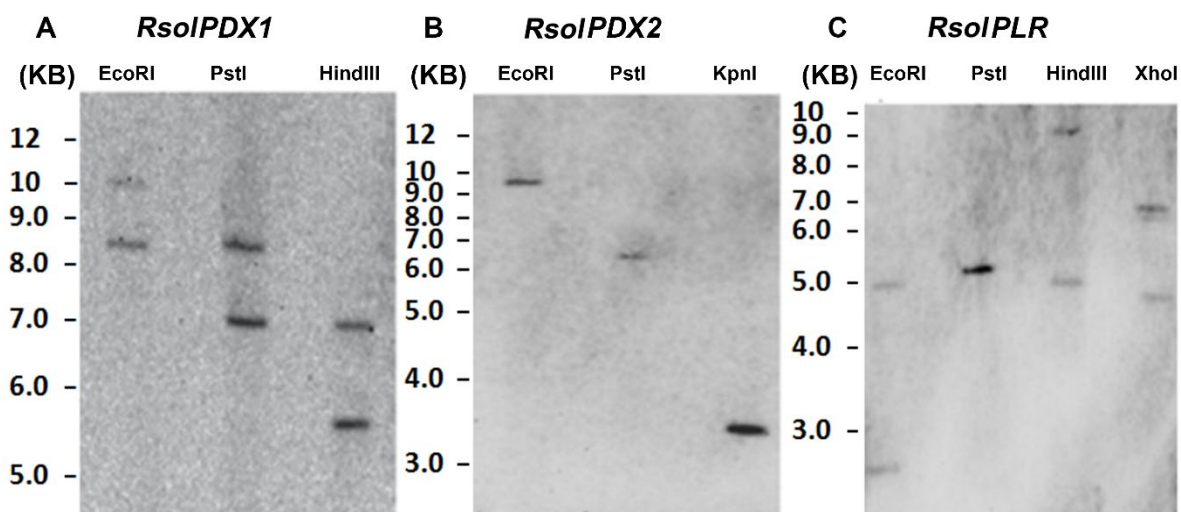


Figure 3.5. Southern blot analysis showing gene copy number of *RsolPDX1* (A), *RsolPDX2* (B), and *RsolPLR* (C) in *R. solani* AG3.

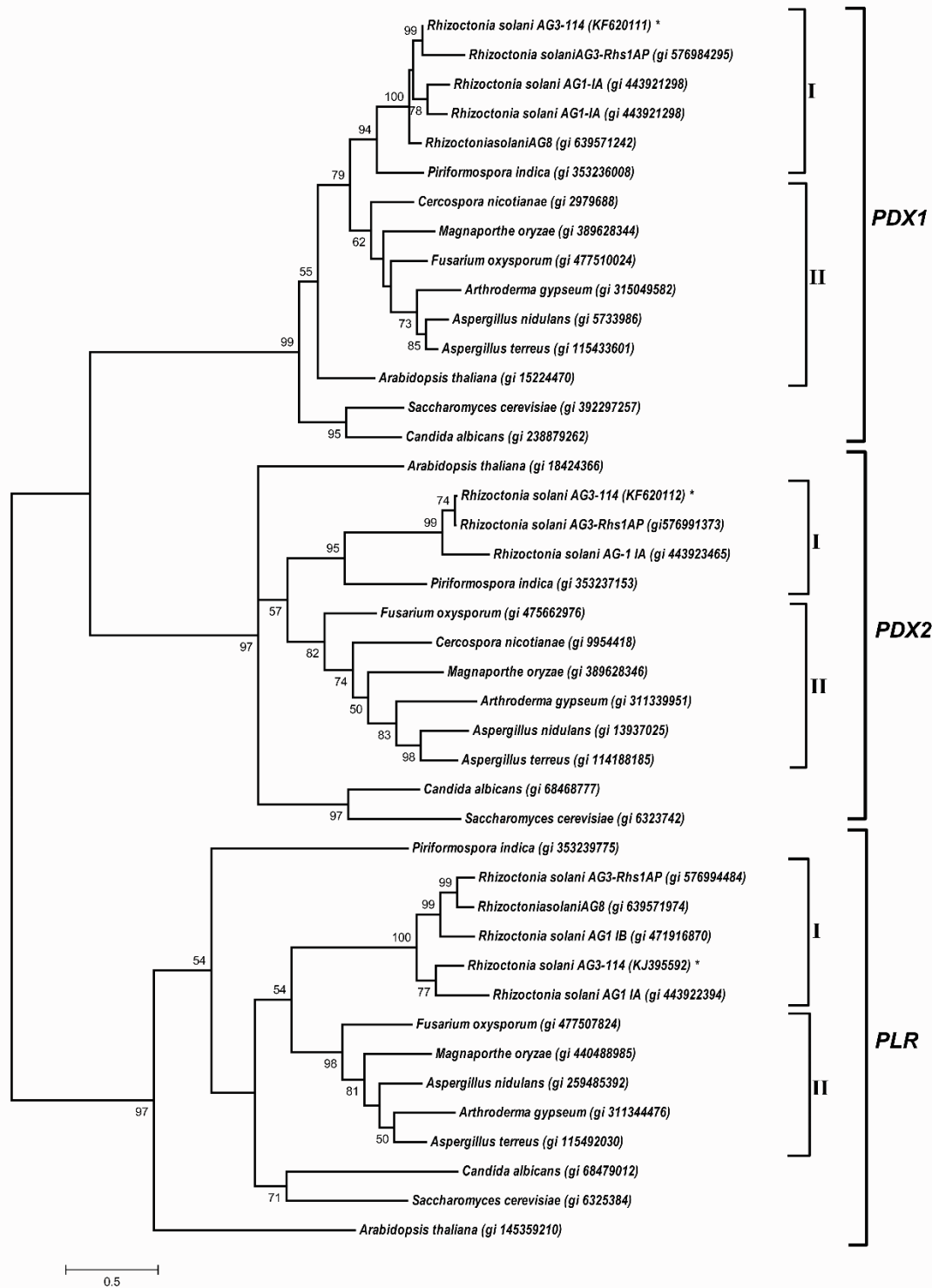


Figure 3.6. A phylogenetic tree based on the similarity among cDNA sequences of *PDX1*, *PDX2*, and *PLR* from selected fungi and plants. The phylogenetic relationship was generated from multiple sequence alignments using Clustal Omega and the tree was constructed using the program MEGA 6.0, and generated by the maximum likelihood method (Tamura et al., 2011). NCBI accession numbers are listed next to the species names. Asterisk indicates *R. solani* AG3- 114 cDNA sequences of *RsolPDX1*, *RsolPDX2*, and *RsolPLR*. Bootstrap scores (1000 replicates) are shown at nodes only when they are higher than 50 %. I: Basidiomycetes, II: Ascomycetes.

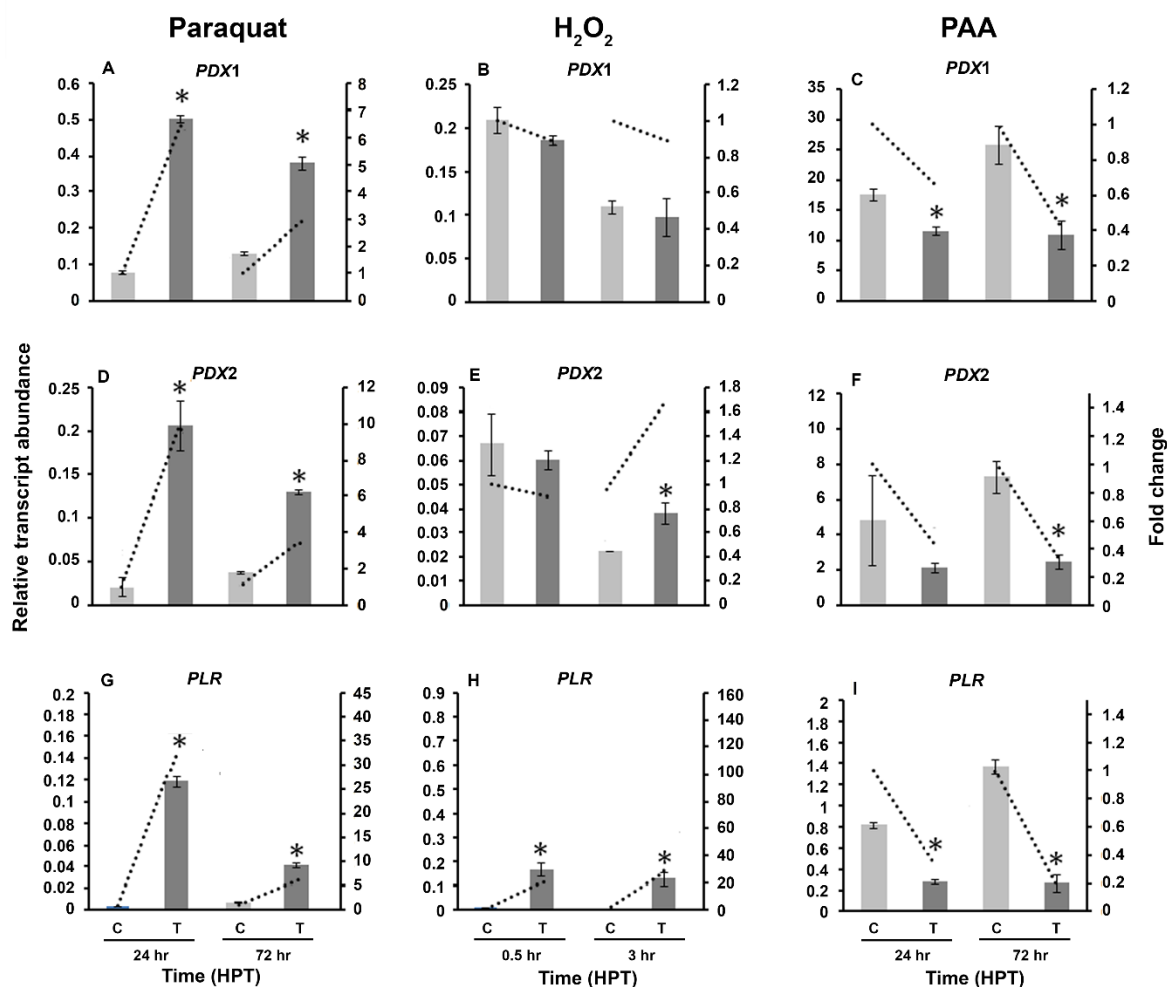


Figure 3.7. Effect of abiotic stress on *R. solani* AG3 vitamin B6 genes. Regulation of *RsolPDX1* (A, B, C), *RsolPDX2* (D, E, F), and *RsolPLR* (G, H, I) transcripts when grown in PDB amended with paraquat (4 mM) (A, D, G), hydrogen peroxide (H₂O₂) (5 mM) (B, E, H) or phenylacetic acid (PAA) (7.5 mM) (C, F, I). The relative transcript abundance of gene expression was normalized with appropriate housekeeping genes (G3PDH for paraquat and H₂O₂; Histone and Tubulin for PAA). C: control; *R. solani* grown without stress inducers. T: treatment with stress inducer. Asterisk indicates significant relative transcript abundance between the control and interaction at each time point using Least Significant Difference test ($P < 0.05$). Bars represent the

average relative transcript abundance of three biological replicates $\pm$ standard deviation. Dotted line represents fold change which was calculated by normalization of treatment samples with appropriate controls at each corresponding time point.

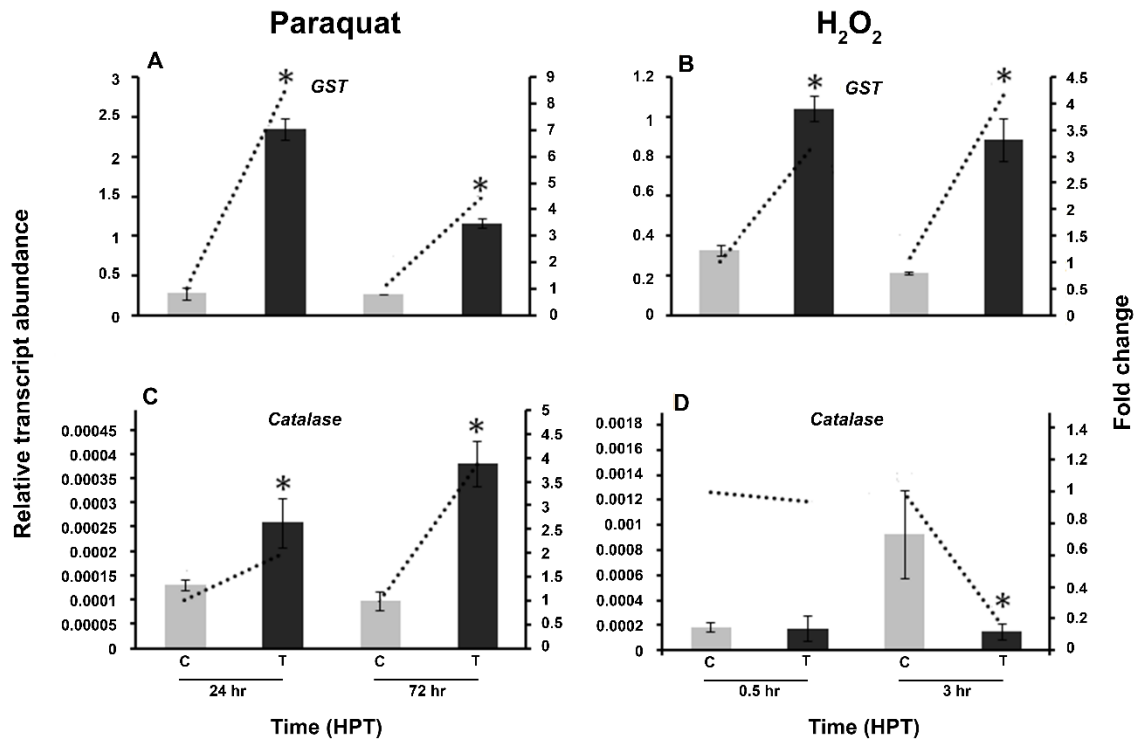


Figure 3.8. Effect of abiotic stress on *R. solani* AG3 glutathione *S*-Transferase (*GST*) and *catalase* genes. Regulation of *R. solani* AG3 *GST* (A, B), and *catalase* (C, D) transcripts when grown in PDB amended with paraquat (4 mM) (A, C) or hydrogen peroxide (H₂O₂) (5 mM) (B, D). The relative transcript abundance of gene expression was normalized with G3PDH. C: control; *R. solani* grown without stress inducers. T: treatment with stress inducer. HPT: hours post treatment. Asterisk indicates significant relative transcript abundance between the control and interaction of each time point using Least Significant Difference test ($P < 0.05$). Bars represent the average relative transcript abundance of three biological replicates $\pm$ standard deviation. Dotted line represents fold change which was calculated by normalization of treatment samples with appropriate controls at each corresponding time point.

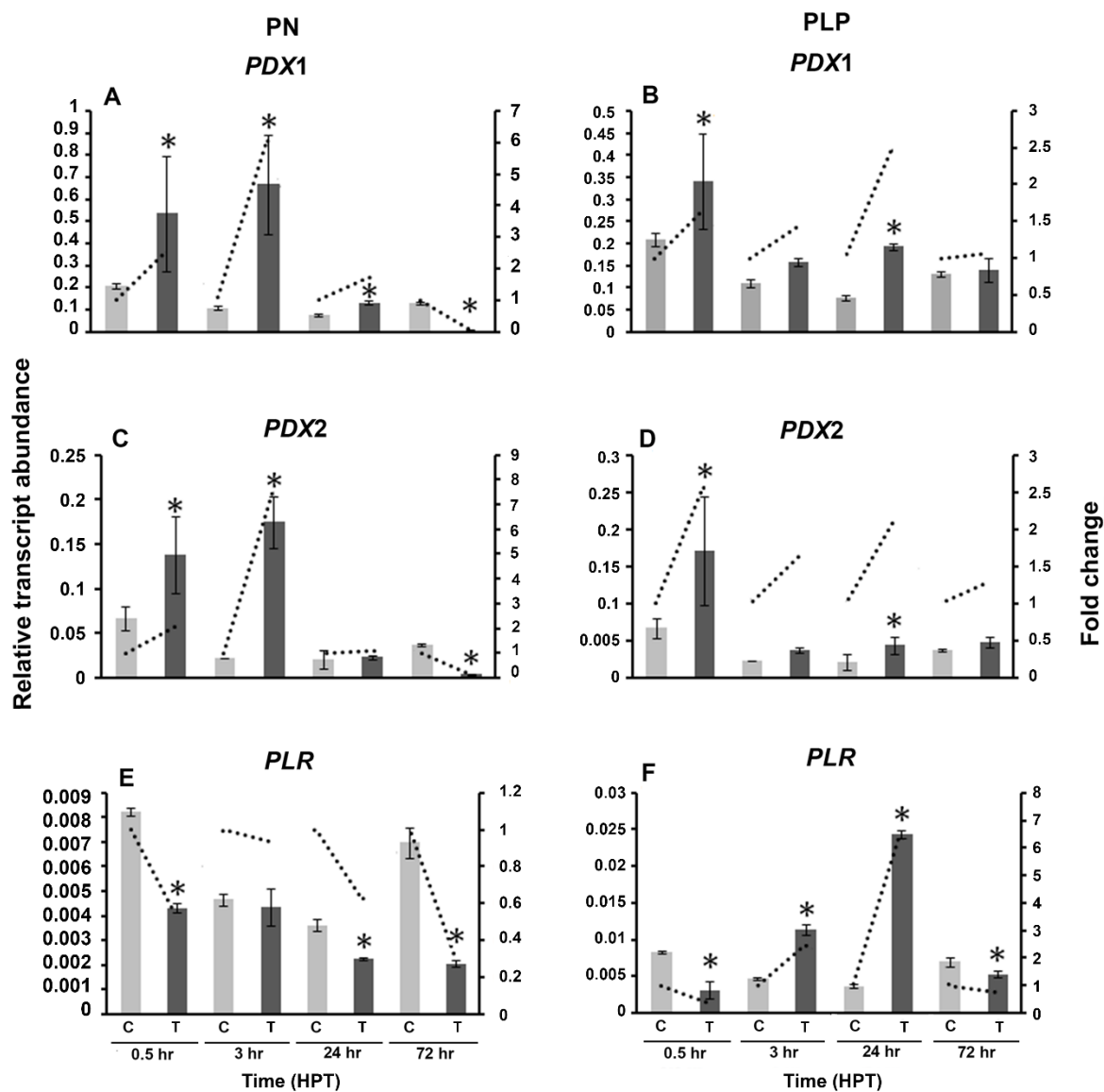


Figure 3.9. Effect of Vitamin B6 vitamers (PN: pyridoxine or PLP: pyridoxal 5'-phosphate) on *R. solani* AG3 vitamin B6 genes. Regulation of *RsolPDX1* (A, B), *RsolPDX2* (C, D), and *RsolPLR* (E, F) transcripts when grown in PDB amended with PN (0.01g/L) (A, C, E) or PLP (0.01g/L) (B, D, F). The relative transcript abundance of gene expression was normalized with G3PDH. C: control; *R. solani* grown without stress inducers. T: treatment with vitamers.

HPT: hours post treatment. Asterisk indicates significant relative transcript abundance between the control and interaction of each time point using Least Significant Difference test ($P < 0.05$). Bars represent the average relative transcript abundance of three biological replicates $\pm$ standard deviation. Dotted line represents fold change which was calculated by normalization of treatment samples with appropriate controls at each corresponding time point.

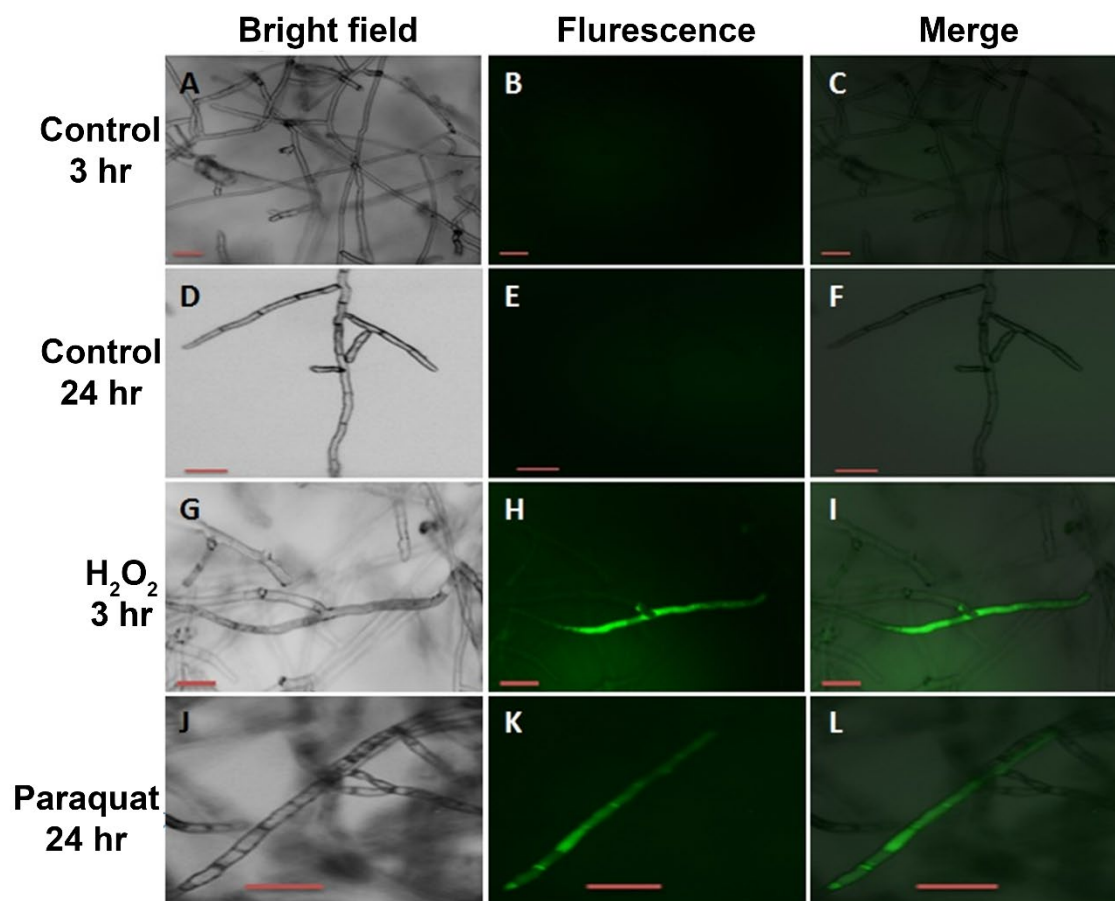


Figure 3.10. Intracellular production of ROS in *R. solani* AG3 mycelia grown for three days before exposure to 4 mM paraquat or 5 mM H₂O₂. The production of ROS was visualized by fluorescence microscopy using the ROS-sensitive probe H₂DCF-DA. Control mycelia with no evidence of endogenous ROS production at 3 HPT (**A–C**) or at HPT 24 (**D–F**). Mycelia grown in PDB amended with 5 mM H₂O₂ at 3 HPT (**G–I**) or 4 mM paraquat at 24 HPT (**J–L**). Bar = 50 μ m.

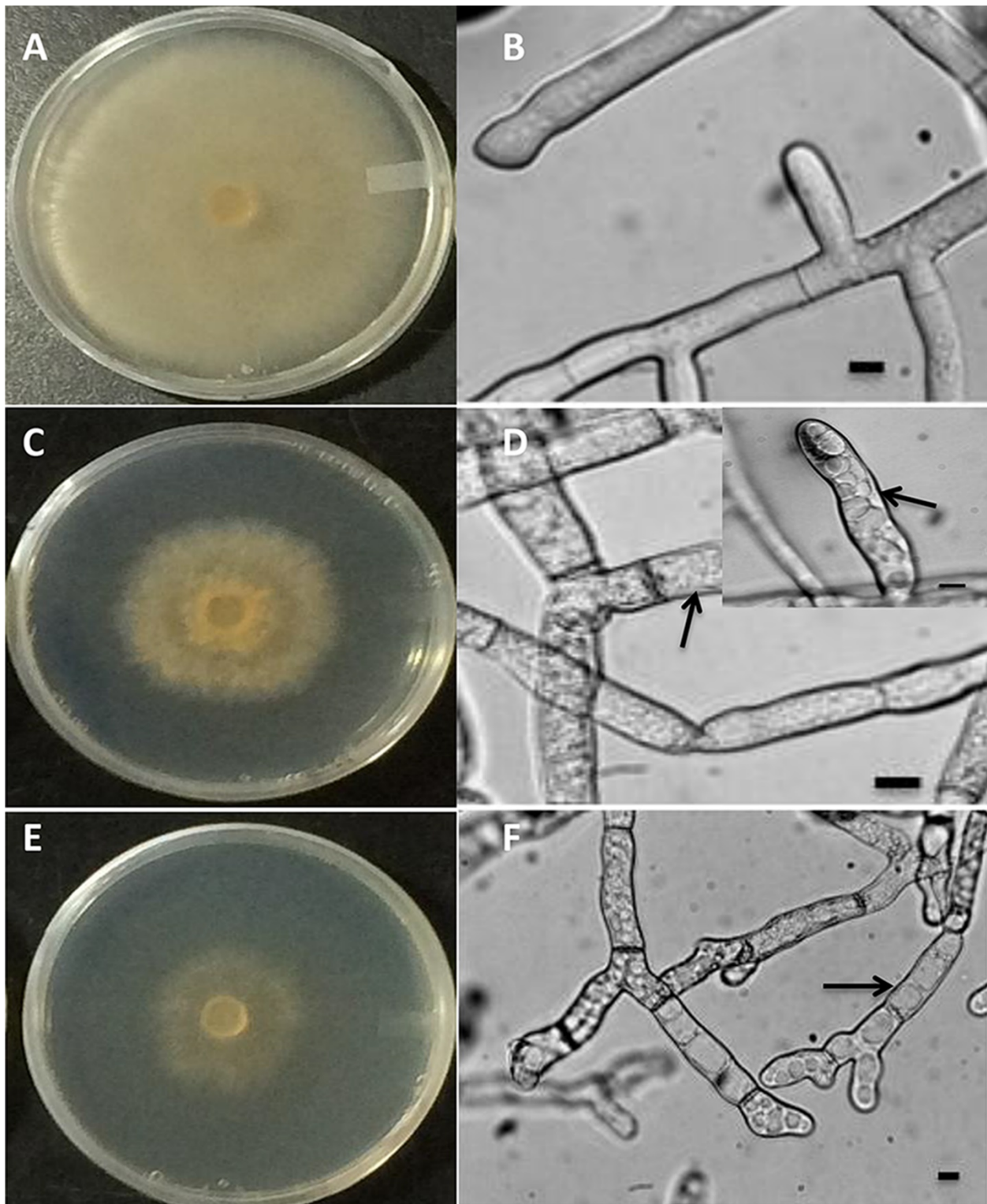


Figure 3.11. Macroscopic (**A, C, E**) and microscopic (**B, D, F**) images of *R. solani* AG3 mycelia grown for three days before exposure to abiotic stress. Control mycelia in PDB at 3 HPT or at 24 HPT showing normal hyphal cellular organization (**A, B**). Mycelia grown in PDB amended with 5mM H₂O₂ at 3 HPT (**C, D**) or 4mM paraquat at 72 HPT (**E, F, inset**) displaying intracellular disorganization, vascularization and loss of cytoplasm (**arrows**). Bar = 10 µm.

3.9 CONNECTING TEXT

Chapter 4 examines the role of vitamin B6 pathway during the interaction of different *R. solani* anastomosis groups with their natural hosts like potato and soybean. Biotic stress that arises as a result of plant-pathogen interactions induces the accumulation of reactive oxygen species in the cells, causing severe oxidative damage to plants and pathogens. The identification of the vitamin B6 in *R. solani* as a powerful antioxidant due to its ability to quench ROS, lead us to further investigate the functionality of *R. solani* vitamin B6 biosynthetic pathway genes by yeast complementation assays. Since gene expression studies focusing on oxidative stress responses of both the plant and the pathogen following *R. solani* infection are very limited, the aim of this chapter is to study the regulation of genes encoding vitamin B6, *CAT* and *GST* in plant and fungal tissues during interaction of different AG groups of *R. solani* with their respective hosts.

J. Samsatly and Dr. S. Jabaji conceived and designed the experiments. J. Samsatly performed the experiments. J. Samsatly and Dr. T. Copley analysed the data. Dr. S. Jabaji contributed reagents and materials and analysis tools. The manuscript was written by J. Samsatly and revised by all co-authors prior to publication in Published in PLOS ONE February 21st, 2018.

CHAPTER 4

Antioxidant Genes of Plants and Fungal Pathogens are Distinctly Regulated during Disease

Development in Different *Rhizoctonia solani* Pathosystems

Jamil Samsatly, Tanya R. Copley, Suha H. Jabaji

Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, QC, Canada

Corresponding Author:

Dr. S. Jabaji; Department of Plant Science, McGill University, 21,111 Lakeshore Rd., Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9.

Telephone: +1 (514)-398-7561

E-mail: suha.jabaji@mcgill.ca

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4.1 Abstract

Biotic stress, as a result of plant-pathogen interactions, induces the accumulation of reactive oxygen species in the cells, causing severe oxidative damage to plants and pathogens. To overcome this damage, both the host and pathogen have developed antioxidant systems to quench excess ROS and keep ROS production and scavenging systems under control. Data on ROS-scavenging systems in the necrotrophic plant pathogen *Rhizoctonia solani* are just emerging. We formerly identified vitamin B6 biosynthetic machinery of *R. solani* AG3 as a powerful antioxidant exhibiting a high ability to quench ROS, similar to *CATALASE* (*CAT*) and *GLUTATHIONE-S-TRANSFERASE* (*GST*). Here, we provide evidence on the functionality of *R. solani* vitamin B6 biosynthetic pathway genes; *RsolPDX1* (**KF620111.1**), *RsolPDX2* (**KF620112.1**), and *RsolPLR* (**KJ395592.1**) by yeast complementation assays. Since gene expression studies focusing on oxidative stress responses of both the plant and the pathogen following *R. solani* infection are very limited, this study is the first coexpression analysis of genes encoding vitamin B6, *CAT* and *GST* in plant and fungal tissues of three pathosystems during interaction of different AG groups of *R. solani* with their respective hosts. The findings indicate that distinct expression patterns of fungal and host antioxidant genes were correlated in necrotic tissues and their surrounding areas in each of the three *R. solani* pathosystems: potato sprout-*R. solani* AG3; soybean hypocotyl-*R. solani* AG4 and soybean leaves-*R. solani* AG1-IA interactions. Levels of ROS increased in all types of potato and soybean tissues, and in fungal hyphae following infection of *R. solani* AGs as determined by non-fluorescence and fluorescence methods using H₂DCF-DA and DAB, respectively. Overall, we demonstrate that the co-expression and accumulation of certain plant and pathogen ROS-antioxidant related genes in each pathosystem are critical during disease development from the plant's point of view, and in pathogenicity and developing of infection structures from the fungal point of view.

4.2 Introduction

The necrotrophic fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*, Frank, Donk) is an economically devastating plant pathogen with a wide host range. *R. solani* is classified into fourteen anastomosis groups (AGs) based on hyphal fusion (García, Onco et al. 2006), and strains belonging to AG3 and AG4 are root infecting pathogens that cause damping-off and stem rot of potato (*Solanum tuberosum* L.) and soybean (*Glycine max* (L.) seedlings, respectively (Fenille, De Souza et al. 2002, Tsrör 2010, Buskila, Tsrör Lahkim et al. 2011, Sneh, Jabaji-Hare et al. 2013). Isolates of the *R. solani* AG1-IA complex can infect aerial portions of the plant as in the case of soybean leaves causing rhizoctonia foliar blight (RFB) (Fenille, De Souza et al. 2002, Stetina, Stetina et al. 2006, Ciampi, Meyer et al. 2008). The common elements in disease development of *R. solani* isolates are the close association of fungal hyphae with the host epidermis forming branches known as infection cushions or aggregates, penetration of the epidermis, inter- and intracellular colonization and breakage of plant tissue by the production of hydrolytic enzymes, which eventually leads to the development of browning and necrosis associated with oxidative burst and death of tissue (Keijer 1996, Sneh, Jabaji-Hare et al. 2013). Current cultural and chemical controls are not completely effective to manage Rhizoctonia diseases and the diseases remain a persistent problem. Furthermore, resistance to *R. solani* in any plant species does not exist.

One of the earliest plant responses following pathogen recognition is the oxidative burst, leading to the production of reactive oxygen species, primarily superoxide (O_2^-) and H_2O_2 , at the site of attempted invasion (Torres 2010). The produced ROS activates plant defense responses, including programmed cell death, or functions as second messengers in the induction of various plant defense-related genes (Alvarez, Pennell et al. 1998, Barna, Fodor et al. 2012). In the case of

necrotrophic fungi, ROS plays a central role during their interaction with their plant hosts by stimulating the plant's basal defense responses (Apel and Hirt 2004, van Kan 2006, Asai and Yoshioka 2009, Pietrowska, Rozalska et al. 2015). Several studies showed that the onset of basal resistance in plants to *R. solani* is tied with ROS-scavenging mechanisms, accumulation of metabolites related to vitamin B6 biosynthetic pathway, oxylipins production and cell wall bound phenolic compounds (Taheri and Tarighi 2011, Aliferis and Jabaji 2012, Foley, Gleason et al. 2013, Nikraftar, Taheri et al. 2013, Aliferis, Faubert et al. 2014, Keshavarz-Tohid, Taheri et al. 2016).

Different strategies of ROS scavenging are deployed by biotrophic and necrotrophic fungi during the infection process. Biotrophic pathogens such as rust fungi respond to oxidative stress by containing and suppressing the oxidative burst, while necrotrophic pathogens such as *Botrytis cinerea* rely on the exploitation of the oxidative burst in plants to its own advantage and in some cases contribute to it (Heller and Tudzynski 2011). Hence, the ability of necrotrophic fungi to surpass or manipulate ROS-related plant defenses is crucial for disease progression, and ROS detoxification is essential to the sensitivity of the necrotrophic fungus while encountering its host. Detoxification systems, such as the NOX complex is a good example of how necrotrophic fungi such as *Botrytis cinerea* and *Alternaria alternata* can sustain a reduced redox states within subcellular microenvironments (Heller and Tudzynski 2011). In recent years, vitamin B6 was recognized as a strong antioxidant displaying a great capacity to quench ROS matching that of tocopherols or ascorbic acid, and may have a function in stress alleviation in fungi and plants (Mooney, Leuendorf et al. 2009, Vanderschuren, Boycheva et al. 2013). Data on ROS-scavenging systems in *R. solani* are just emerging. To date, the upregulation of *R. solani* genes particularly, *PYRIDOXAL REDUCTASE (PLR AKR8 ;DW520695)*, and *PYRIDOXAL-5-PHOSPHATASES* and

TRANSAMINASES of the vitamin B6 salvage biosynthetic pathway, was reported in *R. solani* hyphae in association with a mycoparasite or an antagonistic bacteria, respectively (Chamoun and Jabaji 2011, Gkarmiri, Finlay et al. 2015).

We recently fully characterized two genes of the *de novo* vitamin B6 biosynthetic pathway ; *RsolPDX1* (KF620111.1) and *RsolPDX2* (KF620112.1) genes, and one gene *RsolPLR* (KJ395592.1) of the vitamin B6 salvage biosynthetic pathway of *R. solani* AG3. Upon exposure to the ROS-generating chemicals, paraquat and H₂O₂, the vitamin B6 genes exhibited differential regulation and their transcript abundance levels were higher than levels to the well-recognized antioxidant genes, *CATALASE* (*CAT*) and *GLUTATHIONE-S-TRANSFERASE* (*GST*) (Samsatly, Chamoun et al. 2015). These results clearly implicate vitamin B6 genes of the *de novo* and the salvage biosynthetic pathways function as antioxidants against oxidative stress (Samsatly, Chamoun et al. 2015).

Gene expression studies focusing on oxidative stress responses from both the plant and the pathogen sides during plant-*R. solani* interactions are very limited. Foley, Kidd et al. (2016) identified a number of wheat-derived and *R. solani* AG8-derived genes involved in ROS production and redox regulation, and whose expression was affected during infection. Their study highlights the need to understand the ROS-scavenging system interplay of fungal and host derived genes involved in ROS/redox regulation at the site of infection and the surrounding areas. However, our knowledge on the role of vitamin B6 genes as antioxidants in other AGs of *R. solani* is limited.

In this study, we first provide evidence on the functionality of *RsolPDX1* and *RsolPDX2* of *R. solani* AG3, and their involvement in vitamin B6 *de novo* biosynthesis pathway via

heterologous complementation of the yeast, *Saccharomyces cerevisiae* strains $\Delta snz1$ and $\Delta sno1$. Second, we showed that the antioxidant genes encoding vitamin B6 (i.e., *PDX*, *PLR*), *CAT* and, *GST* of *R. solani* AG3 and potato are differentially induced and transcriptionally regulated at the site of infection (i.e., necrotic tissues, and in the surrounding areas) during *R. solani* AG3-potato sprout interaction (pathosystem I). Third, we extended our study to investigate whether differential and spatial expression patterns and transcriptional regulation of vitamin B6 genes and other antioxidant genes also occur in the pathogen and host upon *R. solani* AG4 infection of soybean hypocotyl (pathosystem II) and also upon *R. solani* AG1-IA infection of soybean leaves (pathosystem III).

4.3 Materials and Methods

4.3.1 Yeast complementation assays- heterologous expression of *R. solani* AG3

***PDX1* and *PDX2* and growth assays**

Full-length clones of *RsolPDX1*-AG3 and *RsolPDX2*-AG3 were obtained by primer pairs (*RsolAG3-PDX1-CDS-F/R* and *RsolAG3-PDX2-CDS--F/R*) (Table 4.1) from cDNA of *R. solani* AG3. The PCR products of the two genes were purified using the QIA quick PCR Purification Kit (Qiagen, Toronto, ON, Canada), and sub-cloned into the pDrive cloning vector (Qiagen) to obtain pDrive-*PDX1*-AG3 and pDrive-*PDX2*-AG3. The clones were digested with NotI and ligated into a NotI-digested yeast shuttle vector pFL61 (Addgene, Cambridge, MA, USA) (Minet, Dufour et al. 1992) to obtain pFL61-*PDX1*-AG3 and pFL61-*PDX2*-AG3. To determine whether *PDX1*-AG3 and *PDX2*-AG3 encode functional proteins, the *Saccharomyces cerevisiae* mutant strains ($\Delta snz1$:disrupted *pdx1* and $\Delta sno1$:disrupted *pdx2*), obtained from the European *Saccharomyces*

cerevisiae Archive for functional analysis (EUROSCARF; <http://www.rz.uni-frankfurt.de/FB/fb16/mikro/EUROSCARF/>), were transformed using a lithium acetate-based method (Schiestl and Gietz 1989) with the construct pFL61-expression vector or with pFL61 alone (negative control), and tested for their capacity to complement the defect function of the *snz1* and *sno1*.

For vitamin B6 growth-dependence assays: Yeast cultures grown to exponential phase (2×10^7 cells mL⁻¹) followed by two serial 10-fold dilutions corresponding to the wild-type BY4742, and transformants were spotted on minimal selective SC (Difco Laboratories, MI, USA) medium lacking vitamin B6. The mutants, Δ *snz1* and Δ *sno1* are sensitive to the superoxide generator menadione, and this sensitivity is corrected by the addition of vitamin B6 (Rodriguez-Navarro, Llorente et al. 2002). Transformed yeast cells were grown on minimal SC medium to the post-diauxic phase for 20h followed by a treatment with 40 mM menadione (Sigma, Address) for 3 h in the presence or absence of 2 μ g mL⁻¹ of vitamin B6 and spotted on SC medium lacking Uracil and vitamin B6. Pictures were captured from plates that were incubated for 3 d at 30°C (Benabdellah, Azcon-Aguilar et al. 2009).

4.3.2 Biological material and inoculation

4.3.2.1 Fungal material

Starter cultures of *R. solani* AG3 isolate Rs114 (ATCC 10183), AG4 isolate A76 obtained from M. Cubeta, North Carolina University, USA, and AG1-IA strain ROS-2A4 obtained from P. Ceresini, University of São Paulo State (UNESP), Brazil were recovered from cryogenic storage

at -80°C on potato dextrose agar (PDA; Difco Laboratories) and grown for 3 days at 24°C in the dark and used as a source of inoculum for plant infection. Cultures of *R. solani* strains grown on half-strength PDA overlaid with cellophane membrane were used for the visualization of ROS in their hyphae.

4.3.2.2 Plant material

Certified potato tubers (*Solanum tuberosum* var. Russet Burbank, susceptible to *R. solani* AG3), obtained from the Potato Research Center (NB, Canada) and propagated at the Macdonald Campus Farm (Ste. Anne de Bellevue, Canada), were kindly provided by D.J. Donnelly (McGill University). Potato tubers were grown without light at 4°C to induce sprout formation. For sprout induction, uniform-sized tubers were surface sterilised and sprouted for 12 days under controlled conditions following the methods of Aliferis and Jabaji (2012), Chamoun et al. (2015). Prevention of browning of the sprout tips was achieved by applying 1.5 mL solution of CaNO_3 (0.5M) to sprouts of 3 cm in length. Inoculation of sprouts was performed when sprouts were 8 cm in length.

Soybean (*Glycine max*) cv. Williams 82 seeds, a susceptible cultivar to *R. solani* AG-4, were sterilized according to Aliferis et al. (2014). Pre-germinated seeds were planted in Cone-tainers® (Stuewe & Sons, Inc., Oregon, USA) filled with 130 mL of sterile turf:perlite (1:1, v/v) and incubated in the dark in a growth chamber in order to produce etiolated seedlings with longer lengths of hypocotyls (approximately 15 cm in height). Hypocotyls were inoculated with AG4 isolate A76 as previously published (Aliferis et al. 2014).

For unifoliate leaf inoculation with *R. solani* AG1-IA, pre-germinated soybean seeds in Cone-tainers® as described above were grown under controlled conditions of temperature, humidity and light following the method of Copley et al. (2017). Fully expanded soybean unifoliate leaves were detached from 2 week-old plants, and used for inoculation.

4.3.3 Inoculation of potato sprouts, soybean hypocotyls and soybean leaves

The goal of this study is to examine the transcriptional regulation of antioxidant genes from both the plant and pathogen side in necrotic tissues and their surrounding areas in three pathosystems: I. potato sprouts-*R. solani* AG3; II. soybean hypocotyls-*R. solani* AG4; III. soybean leaves-*R. solani* AG1-IA.

Excised sprouts and etiolated hypocotyls were arranged horizontally in sterile Pyrex trays (40 cm × 26 cm) lined with wet sterile Whatman No. 1 paper. Each treatment replicate consisted of 4 excised potato sprouts or 4 etiolated hypocotyls. There were 3 biological replicates per treatment. Inoculation was performed by sandwiching horizontally the sprouts (8 cm in length) or hypocotyls (15 cm in length) of each treatment replicate between two PDA strips (2 cm × 8 cm) of a 3-day-old *R. solani* AG3 Rs114 or *R. solani* AG4 isolate A76 cultures, respectively, with the bottom edge of the strips placed at 4 cm from the basal part of the potato sprouts or hypocotyl sprouts according to the method of Aliferis and Jabaji (2012), Chamoun, Samsatly et al. (2015). Potato sprouts or soybean hypocotyls sandwiched with sterile PDA strips served as controls. Moist, sterile absorbent cotton wool was placed at the base of the sprouts or the hypocotyls of each replicate treatment to maintain humidity. Pyrex trays were sealed with Saran Wrap, and placed in a growth chamber under photosynthetically inactive black light (365 nm) at 24°C.

Fully expanded unifoliate soybean leaves were placed into sterile Pyrex trays as described above. Inoculation was performed by placing PDA plugs (5 mm) of 3-day-old *R. solani AGI-1A* culture disc, or sterile PDA alone (control), at the center of leaves. Each replicate treatment consisted of 5 unifoliate leaves. Trays were wrapped with plastic membrane, placed in growth chambers under conditions of day/night temperatures, light cycles and humidity as we previously described in Copley, Aliferis et al. (2017).

PDA strips or plugs were removed 120 h, 36h and 18h post-inoculation (HPI) of plant tissue to reveal visible necrotic lesions with infection zones on sprouts, hypocotyls and leaves, respectively. These time points were selected in order to capture the onset of infection cushions and the development of mycelial aggregates that were stereoscopically confirmed (**Figure 4.1**). Necrotic tissues containing infection zones plus a one-cm area away from necrotic tissues were harvested. Similar areas and amounts of tissues were harvested from control samples. Harvested tissues were flash-frozen with liquid nitrogen and stored at -80°C . Each treatment (infected or control) consisted of three biological replications.

4.3.4 RNA extraction, primer design and quantitative RT-PCR

Total RNA was isolated from 100 mg of flash frozen pulverized tissue obtained from the necrotic lesions and their surrounding areas of respective treatments and corresponding areas of control soybean hypocotyls, leaves, or potato sprouts. Extraction was done using the TRIZOL reagent (Generay Biotech, Shanghai, China) following the manufacturer's instructions. RNA concentration and purity was spectrophotometry measured using ND1000 (NanoDrop, Wilmington, Delaware), and RNA quality was verified by gel electrophoresis. RNA (500 ng) was reverse transcribed using the Quantitect Reverse transcriptase kitTM (Qiagen). QRT-PCR assays

were conducted on fungal and plant target antioxidant genes encoding *GST*, *CAT*, the *de novo* vitamin B6 biosynthesis genes; a synthase (*PDX1*) and a *glutaminase*, (*PDX2*), and the vitamin B6 salvage pathway encoding gene, *PYRIDOXAL REDUCTASE* (*PLR*) and appropriate internal reference genes for the three pathosystems (Table 4.1) using Stratagene Mx3000 (Stratagene, Cedar Creek, USA). Primer names were preceded by the plant or fungus abbreviation: *GM*, *Glycine max*, *ST*, *Solanum tuberosum*, or *Rsol*, *R. solani*. Primers were designed based on sequences from NCBI, and were checked for specificity to amplify only their target gene. QRT-PCR conditions were optimized for each primer set, and products were confirmed by sequencing. For primer pairs that were used on more than one AG, they were checked for their ability to amplify with comparable efficiencies in the different pathosystems. Reverse transcription PCR assays were performed on three biological replicates and two technical replicates. PCR assay conditions were performed as previously described (Chamoun, Aliferis et al. 2013, Samsatly, Chamoun et al. 2015) using suitable annealing temperature for each primer pair (Table 4.1). The relative transcript abundance levels of the plant and fungal-derived genes were estimated and normalized against their respective reference genes according to Zhao and Fernald (2005). In the case of *R. solani* AG-3, expression of the antioxidant genes was normalized using G3PDH. This choice was based on the lowest coefficient variation when compared to Tubulin and Histone using the statistical tool Bestkeeper (<http://www.gene-quantification.info>).

4.3.5 Statistical analyses

Significance of the relative transcript abundance between treatments and controls were analyzed by two-way analysis of variance (ANOVA), and when necessary by least significant differences (LSD) at $P < 0.05$ using the SPSS statistical package v. 22.0, (IBM Corp., Armonk, NY, USA).

In order to find potential correlations and trends between relative abundance of plant and fungal genes expressed in necrotic tissues and their surrounding areas of each pathosystem, a multivariate analysis of the data was performed using the SIMCA-P+ v.12.0 software (Umetrics, MKS Instruments Inc., Andover, MA, USA). The data matrix consisted of biological replicates of control plant tissues, and tissues of necrotic lesions and areas surrounding them (columns; X variables) and the variables for relative transcript abundance of antioxidant genes (rows; Y variables). Data were obtained from the analysis of relative transcript abundance of 11 genes for potato *sprouts-R. solani* AG3, soybean leaves-*R. solani* AG1-IA and soybean hypocotyls-*R. solani* AG4 [11 rows x 9 columns]. For the evaluation of data and detection of outliers, principal component analysis (PCA) was performed. To determine which of the genes were most affected in each pathosystem, PCA loading coefficient plot for the effect of the relative transcript abundance of genes on tissue type, and a loading biplot was built for the visualization of correlations between the X and Y variables using the pc (corr) (i.e., correlation scaled loadings of the correlation between Y variables and X scores based on the variable importance of X as scores and loadings) setting in SIMCA-P+. For data normalization, mean-centering and Pareto [PAR] scaling were used.

4.3.6 Optical and fluorescence microscopy

Association of the detected ROS that accumulated during disease development, with changes in the transcript abundance of the genes was visualized during disease development in the three pathosystems. Cellular and extracellular ROS accumulation was visualized by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) and 3,3'-diaminobenzidine (DAB) staining methods in control and infected soybean leaves and hypocotyls, and potato sprouts, along with mycelia of control *R. solani* AG1-IA, AG4, AG3 grown on half-strength PDA overlaid with

cellophane membrane. For ROS detection, *samples* were incubated with 10 μ M H₂DCF-DA, a specific ROS molecular-detection probe, in H₂O for 30 min. Samples were then washed with pre-warmed (28°C) H₂O for 30 min to remove the non-internalized probe. To visualize H₂O₂ accumulation *in situ*, DAB staining was performed, by treating the samples DAB as described in Pogany, von Rad et al. (2009) with some modifications. Whole leaves of soybean were treated with DAB for 24h, whereas thin shavings (approximately 2 x 0.5 cm) of soybean hypocotyls and potato sprouts were obtained with a blade and treated for 4h. All samples were cleared with saturated 15.1 M of chloral hydrate solution, and examined under Zeiss SteREO Discovery.V20 microscope (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada). Fluorescence detection from plant and fungal tissues were read at an excitation wavelength of 470 nm.

4.4 Results

4.4.1 Functional characterization of *RsolPDX1* and *RsolPDX2* by yeast

heterologous complementation provided final proof of *R. solani* AG3 autotrophy to vitamin B6

To determine whether *RsolPDX1* and *RsolPDX2* encode functional enzymes, the full coding sequence of each gene was cloned into the yeast expression vector pFL61 and transformed into *S. cerevisiae* strains defective in either *snz1* (yeast functional *PDX1* homolog; Δ *snz1*) or *snol* (yeast functional *PDX2* homolog; Δ *snol*) and therefore unable to grow in media lacking vitamin B6 (Rodriguez-Navarro, Llorente et al. 2002, Benabdellah, Azcon-Aguilar et al. 2009). Thus, growth of the transformed mutant *S. cerevisiae* cells on media lacking vitamin B6 will confirm the complementarity of the *R. solani* homologs to yeast vitamin B6 genes in addition to their role in vitamin B6 biosynthesis. On media not amended with pyridoxine, growth of *S. cerevisiae*

strains $\Delta snz1$ and $\Delta sno1$ expressing *RsolPDX1* and *RsolPDX2*, was evident (**Fig 4.2A and Fig 4.2B**). In contrast, the non-transformed strains or the empty vector-transformed $\Delta snz1$ or $\Delta sno1$ mutant cells did not grow on media lacking pyridoxine. Complementation of the $\Delta snz1$ mutant by *Rsol-PDX1* and $\Delta sno1$ mutant by *Rsol-PDX2* demonstrated that *Rsol-PDX1* and *Rsol-PDX2* are functional, and that they can replace the yeast SNZ1 and SNO1, respectively, implying that they are involved in *de novo* vitamin B6 biosynthesis

$\Delta snz1$ and $\Delta sno1$ display sensitivity to the superoxide generator menadione, and this sensitivity is corrected by the addition of vitamin B6 (Rodriguez-Navarro, Llorente et al. 2002). Thus, it is expected that vectors harbouring *Rsol-PDX1* or *Rsol-PDX2*, but not the mutants transformed with the empty vector, are resistant to menadione (**Fig 4.2C and Fig 4.2D**). Unsurprisingly, growth defect restoration of the empty vector-transformed mutants (*pFL61- $\Delta snz1$* , *pFL61- $\Delta sno1$*) was achieved by the addition of 2 $\mu\text{g ml}^{-1}$ of pyridoxine. In conclusion, these data indicate that that *Rsol-PDX1* and *Rsol-PDX2* confers resistance to ROS to the cells.

4.4.2 Distinct expression of host and pathogen antioxidant genes in necrotic tissues and surrounding areas

For the discovery of trends of plant and fungal antioxidant encoding genes, in the three pathosystems, PCA revealed tight groups with no outliers ($P < 0.05$) (**Appendix I**). Antioxidant genes that influenced the separation of treatments (i.e., plant control, infected plant necrotic tissues and surrounding tissues) for each pathosystem were selected based on strong loading coefficients obtained from the loadings plots (**Fig 4.3**) and designated to have an effective antioxidant role in plant-pathogen interactions of each pathosystem. The strength of the correlations was

pathosystem-dependent. Additionally, selected genes were highly associated with the distinct plant tissue type as revealed by the PCA- loading biplots (**Fig 4.3**).

4.4.3 *R. solani* AG3 infection of potato sprouts dramatically activates the *de novo* vitamin B6 genes of both the pathogen and the host

A remarkable gene upregulation of the fungal and plant vitamin B6 *de novo* pathway genes was observed during disease development of *R. solani* AG3 on potato sprouts. Based on PCA loading biplots and coefficients, the model showed that the loading coefficient values (p) of *RsolAG3-PDX1* (p, 54%) *ST-PDX1.1* (p, 40%), *RsolAG3-PDX2* (p, 35%), and *RsolAG3-GST* (p, 25%), were tightly linked with tissues of surrounding areas of necrotic lesions. On the other hand, the potato *CATALASE*, *ST-CAT* (p, -45%) was negatively associated with necrotic tissues and their surrounding areas (**Figs 4.3A and Fig 4.3B**). Fold increases of transcript abundances of the above fungal genes ranged from 1.6 to 4.7 depending on the gene and the plant's area sampled (i.e, necrotic tissue vs surrounding or adjacent tissue) for *RsolAG3-PDX1* and *RsolAG3-PDX2* (**Fig 4.4**). Interestingly, the plant homologue *ST-PDX1.1* was differentially upregulated and tissue-dependent (**Fig 4.4**). *ST-PDX1.1* had an increase (1.9 folds) in only the tissues surrounding the necrotic area.

4.4.4 Fungal-derived vitamin B6 genes and *RsolAG4-GST* are differentially regulated during soybean root and stem rot disease development caused by *R. solani* AG4

The fungal vitamin B6 genes along with *RsolAG4-GST* play a significant role in soybean-*R. solani* AG4 pathosystem compared to their plant's counterpart during disease development (**Fig**

4.3D). A strong positive loading coefficient value (p, 80%) was observed for *RsolAG4-PLR* (**Fig 4.3D**) with significant upregulation of transcript abundance in necrotic tissues and surrounding areas by 38.1 fold and 34.4 fold, respectively (**Fig 4.5**). *RsolAG4-PDX1* was moderately linked (p, 20%) with necrotic tissue showing an increase of transcript abundance by 1.8 fold (**Fig 4.5**). However, *RsolAG4-GST* (p, -35%) and *RsolAG4-PDX2* (p, -20%) were negatively associated with necrotic tissue and surrounding areas. (**Figs 4.3C and Fig 4.3D**).

Among the plant-derived genes, *GM-GST* (p, 25%) and *GM-PLR* (p, 15%) were moderately linked with necrotic tissue (**Figs 4.3C and Fig 4.3D**). *GM-PLR* and *GM-GST* showed an increase of 1.5 and 1.4 folds, respectively, in necrotic tissue (**Fig 4.5**). The soybean *GM-CAT* encoding gene was positively upregulated in both the necrotic tissues and surrounding areas with 2.8 and 2.2 fold increase, respectively (**Fig 4.5**). The *de novo* plant vitamin B6 genes showed an interesting pattern where both *GM-PDX2* and *GM-PDX1.1* were significantly downregulated by 1.6 and 1.5 fold, respectively (**Fig 4.5**) while *GM-PDX1.2* was significantly upregulated in areas surrounding to necrotic tissues (1.6 fold) (**Fig 4.5**).

4.4.5 Major plant-derived vitamin B6 genes and *GM-GST* play a prominent role during soybean RFB disease development caused by *R. solani* AG1-IA

Upon fungal challenge, the relative transcripts abundance of the soybean *PYRIDOXAL REDUCTASE* (*GM-PLR*) and *GLUTATHIONE-S-TRANSFERASE* (*GM-GST*) was highly upregulated whereas their corresponding fungal genes were notably downregulated (**Fig 4.6**). PCA analysis showed that leaf soybean *GM-GST* (p, 95%), and *GM-PLR* (p, 20%), were closely linked with the necrotic tissue, with significant increased abundance levels of *GM-GST* and *GM-PLR* by 5.3 and 4.6 fold, respectively (**Fig 4.6**). On the other hand, *GM-PDX1.1* (p, -20%) was negatively

linked with necrotic tissue and surrounding areas (**Fig 4.3E, and Fig 4.3F**), and displayed a significant downregulation with fold changes of 3.4 and 1.8 fold, respectively (**Fig 4.6**). Although not supported by high loading coefficient values in the PCA loading analysis, the fungal genes *RsolAG1-IA-PDX1* (one component of *de novo* vitamin B6 biosynthesis genes) and *RsoAG1-IA-GST* exhibited significant down-regulation with fold changes of 2.6 and 1.5, respectively, in necrotic tissues, and with fold changes of 3.2 and 5.6, respectively, in the surrounding areas (**Fig 4.6**).

4.4.6 Accumulation of ROS in *R. solani* mycelia is related to transcriptional regulation of vitamin B6 machinery and other antioxidant genes

The presence of ROS in the fungal hyphae and plant tissues was detected by fluorescent H₂DCF-DA and non-fluorescent DAB methods. Absence of fluorescence was observed in control hyphae grown on half-strength PDA (**Fig 4.7B, Fig 4.7H and Fig 4.7N**), while an intense green fluorescence was detected in *R. solani* AG3, AG4, and AG1-IA, hyphae during disease development (**Fig 4.7F, Fig 4.7L, Fig 4.7R**). Tissues of control and infected potato sprouts, soybean hypocotyls and leaves did not display any green fluorescence (**Fig 4.7D, Fig 4.7J, Fig 4.7P**) and (**Fig 4.7F, Fig 4.7L, Fig 4.7R**), respectively.

To demonstrate whether the generation of ROS was promoted by *R. solani* infection, plant tissues were assayed *in situ* for the production of H₂O₂ in response to infection, using DAB staining. DAB oxidizes in the presence of H₂O₂ generating a dark brown precipitate in plant tissues (Thordal - Christensen, Zhang et al. 1997). Tissues of infected potato sprouts and soybean hypocotyls and leaves displayed a strong brown precipitate specifically at necrotic and surrounding

areas (**Fig 4.8B, Fig 4.8D and Fig 4.8F**). However, control uninfected tissues did not show any browning (**Fig 4.8A, Fig 4.8C and Fig 4.8E**).

4.5 Discussion

It is widely known that ROS management through the use of antioxidants is important for pathogenic microorganisms to prevent excessive oxidative stress (Alvarez, Pennell et al. 1998, Barna, Fodor et al. 2012). This research further supports our preceding study on the novel function of vitamin B6 genes in *R. solani* as an antioxidant stress protector against ROS that prevents expansion of oxidative stress (Samsatly, Chamoun et al. 2015). Necrotrophic fungi can regulate intracellular levels of ROS for developmental and virulence purposes while the host uses ROS to hinder disease progression. A comprehensive understanding of plant responses to fungal pathogens exists (Glazebrook 2005, Conrath 2009). Gene expression studies focusing on oxidative stress responses from both the plant and the pathogen side during plant-*R. solani* interactions are very limited (Foley, Kidd et al. 2016, Copley, Aliferis et al. 2017). This study is the first analysis of gene expression encoding vitamin B6 and several antioxidant genes in plant and fungal tissue of three pathosystems during interaction of different AG groups of *R. solani* with their respective hosts.

In a previous study, we fully characterized two vitamin B6 *de novo* biosynthetic pathway genes; *RsolPDX1* (**KF620111.1**) and *RsolPDX2* (**KF620112.1**) genes, and one gene, *RsolPLR* (**KJ395592.1**) of the vitamin B6 salvage biosynthetic pathway of *R. solani* AG3 (Samsatly, Chamoun et al. 2015). The use of mutant yeasts and complementation with *PDX1* or *PDX2* proved to be a valuable tool in fungi (Rodriguez-Navarro, Llorente et al. 2002, Benabdellah, Azcon-

Aguilar et al. 2009). In this study, we confirmed that *R. solani* AG3 possesses a functional vitamin B6 biosynthesis pathway via successful complementation of the *S. cerevisiae* null mutant $\Delta snz1$ phenotype with *RsolAG3-PDX1*, and the null mutant $\Delta sno1$ with *RsolAG3-PDX2*. Complementation of each of the *S. cerevisiae* null mutants ($\Delta snz1$ and $\Delta sno1$) with its respective *R. solani* AG3 gene restored their function implying their ability of *de novo* production of vitamin B6 in yeast. This also suggests that *RsolAG3-PDX1* and *RsolAG3-PDX2* can interact with their respective yeast counterpart to form the PDX1–PDX2 complex (Gengenbacher, Fitzpatrick et al. 2006). Similar to other fungi, our complementation assays validated the role *RsolAG3-PDX1* or *RsolAG3-PDX2* in oxidative stress resistance through the reversion of the menadione sensitivity of the $\Delta snz1$ or $\Delta sno1$ mutant strains, respectively (Rodriguez-Navarro, Llorente et al. 2002, Benabdellah, Azcon-Aguilar et al. 2009). Taken together, our results provide the proof of *R. solani* AG3 autotrophy to vitamin B6. Complementation assays for *PDX1/PDX2* of *R. solani* AG4 and AG1-IA were not attempted, as their respective genes are not characterized.

It is common knowledge that ROS is produced at the site of attempted invasion through an oxidative burst during disease development (Alvarez, Pennell et al. 1998, Lehtonen, Somervuo et al. 2008, Torres 2010, Foley, Kidd et al. 2016). Oxidative damage can be prevented in both the host and the pathogen when their antioxidant machinery is efficiently used (Apel and Hirt 2004, Sharma, Jha et al. 2012). Both plants and fungi have ROS scavenging activities that detoxify ROS either directly through the action of enzymes such as; superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPX*) and peroxiredoxins (*Prx*), or indirectly via vitamins like ascorbic acid (vitamin C), tocopherols (vitamin E), and vitamin B6 (Mooney, Leuendorf et al. 2009, Mylona and Polidoros 2010, Sharma, Jha et al. 2012, Breitenbach, Weber et al. 2015) .

To describe trends (positive or negative) between the plant's and fungal antioxidants genes

(total 11 antioxidant/pathosystem) in response to stress during plant-pathogen interactions of each pathosystem, it necessitates the use of multivariate analysis. The application of PCA revealed tight groups with no outliers. PCA loading biplots demonstrated that several trends could be observed between the relative transcript accumulation of antioxidant genes and the plant's areas sampled (necrotic tissues or surrounding areas) in each pathosystem. These results indicate the distinctive differences in regulation of fungal-derived and plant-derived antioxidant genes of each pathosystem in response to *R. solani* attack of different AGs.

Our results clearly showed that ROS formation was induced in hyphal cells of *R. solani* AG1-IA, AG3 and AG4 during disease development on their respective hosts. ROS production was also reported in *R. solani* AG 2-2 IV and AG-8, serious pathogens of sugar beet and wheat, respectively (Taheri and Tarighi 2011, Foley, Kidd et al. 2016). These findings support the notion that several AGs produce ROS. The induced ROS were also linked to the regulation of antioxidant genes and their collective possible role in oxidative stress alleviation. A strong brown precipitate was detected in tissues of infected potato sprouts and soybean hypocotyls and leaves. Accumulation of H₂O₂ in single cells followed by their death was reported in various plant-fungus interactions (Asselbergh, Curvers et al. 2007, Pogany, von Rad et al. 2009, Laluk and Mengiste 2010, Chung 2012). These data confirm that *Rhizoctonia* disease development in potato sprouts and soybean hypocotyls and leaves provokes an oxidative stress in fungal hyphae and plant tissues specifically in necrotic areas and could be related to the oxidative status of the fungus as well as the plant.

The differential expression of antioxidant genes in the host and the pathogen is an important determinant of disease outcome and pathogenicity. ROS is under tight control during disease progression where sometimes it can lead to cell death of fungal and plant tissues.

Alternatively, pathogen-induced ROS themselves can act as signalling molecule promoting development of fungal structures or resistance in plant cells (Barna, Fodor et al. 2012, Foley, Kidd et al. 2016). In our study, comparison of the differential gene expression across the three pathosystems was not attempted, because the method of inoculation and plant tissue types are different in each pathosystem, resulting in variable infection intensities and different gene expression. Instead, we focused on understanding the expression patterns of fungal and host antioxidant genes in each pathosystem. Our results showed that distinct expression patterns of fungal and host antioxidant genes were associated in necrotic tissues and their surrounding areas for each of the three *R. solani* pathosystems.

During *R. solani* AG3 infection of potato sprouts, significant gene expression of both fungal and plant vitamin B6 *de novo* pathway genes was observed. Genes of the vitamin B6 *de novo* pathway of *R. solani* and potato (homolog *ST-PDX1.1*) were highly induced in the surrounding tissue and to a lesser extent in the necrotic tissue. Interestingly, the potato *ST-PDX* genes (i.e., *ST-PDX1.1* and *ST-PDX 1.2* homologs of *ST-PDX1* and *ST-PDX 2*) are characterized according to their catalytic ability (Moccand, Boycheva et al. 2014) and were expressed in all potato tissues (Mooney, Chen et al. 2013). The catalytic homolog *ST-PDX1.1*, but not the non-catalytic homolog *ST-PDX 1.2*, was induced in response to biotic and abiotic stress (this study, Moccand, Boycheva et al. (2014)). These results strongly indicate that *ST-PDX1.1* plays a role in disease development. It has been reported that *Arabidopsis* (*AT-PDX1.2*) acts as a pseudoenzyme that can have a role of a positive regulator of vitamin B6 abundance during abiotic stress (Moccand, Boycheva et al. 2014). Additionally, it was shown that potato *ST-PDX2* might be regulated by *ST-PDX1.2*, due to the unique (novel) ability of the latter to interact with *AT-PDX2* (Wagner, Bernhardt et al. 2006, Mooney, Chen et al. 2013). In our study, the upregulation of *ST-*

PDX2 was not coupled by the upregulation of *ST-PDX1.2* during disease development. Whether *ST-PDX1.2* acts as a positive regulator of vitamin B6 abundance during potato interaction with *R. solani* merits further studies.

Activation of some of the antioxidant genes was tissue specific. Higher expression of host genes (*ST-PDX2*, *ST-PLR* and *ST-GST*) compared to their counterpart in the pathogen in necrotic tissues was detected. Tissue specificity response is supported by the findings of Denslow, Walls et al. (2005). The increase in the transcript abundance of *PDX1* was evident upon infection of tobacco leaves with *P. syringae* pv. *phaseolicola* at the region surrounding the infiltration area. However, levels of *PDX1* were lower in the infiltration area where hypersensitive response has developed (Denslow, Walls et al. 2005).

In Pathosystem II, both fungal- and plant-derived antioxidant genes played a significant role during disease development where some of them were substantially induced while others were downregulated. Interestingly, fungal and host genes encoding *PLR*, a member of the vitamin B6 salvage biosynthetic pathways, were among the driving antioxidant force during soybean hypocotyl-*R. solani* interaction. Both fungal-derived and soybean-derived *PLR* encoding genes were expressed in the necrotic region as well as in the surrounding tissue, however *RsolAG4-PLR* was highly expressed. Similarly, in other studies, transcriptional regulation of *PLR* was substantially induced in response to accumulation of ROS in *R. solani* mycelia parasitized by a mycoparasite or exposed to different abiotic stressors (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011, Samsatly, Chamoun et al. 2015). The results of our study clearly indicate the involvement of *RsolAG4-PLR* as an efficient quencher of ROS. On the other hand, the upregulation of soybean *PLR* (*GM-PLR*), but not at the same extent as that of the pathogen, in the same areas may imply that the plant may also be limiting ROS damage to the soybean tissue.

The interplay of ROS antioxidant genes produced by fungi and their hosts during their interaction was evident in *R. solani* AG1-IA-soybean leaves interaction (pathosystem III). Major plant-derived antioxidant genes of soybean were upregulated in necrotic leaf tissues whereas the corresponding *R. solani* AG1-IA antioxidant genes showed notable downregulation. These results may possibly indicate that the elevation of antioxidant capacity of two of the three vitamin B6 genes, *GM-PDX2* and *GM-PLR* as well as the enzymatic antioxidant, *GLUTATHIONE TRANSFERASE* (*GM-GST*) in soybean leaves against *R. solani* AG1-IA increased their tolerance to the development of necrosis and suppressed the spread of the pathogen. These results are supported by the over expression of the antioxidant VB6 genes (i.e., *PDX*) in *Arabidopsis* that led to increased tolerance to oxidative stress (Raschke, Boycheva et al. 2011, Vanderschuren, Boycheva et al. 2013). Equally *Arabidopsis* mutants with defects in vitamin B6 *de novo* biosynthetic pathway (*PDX1.2* or *PDX1.3*) showed increased levels of disease of gray mold caused by *Botrytis cinerea* (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). Taken together, these results support the novel function of vitamin B6 genes as antioxidant stress protector against ROS to prevent oxidative stress.

In plant-necrotroph systems, the downregulation of antioxidant genes appears to play an important role during infection and initiation of the oxidative burst, leading to an overflow of ROS in infected areas. For example, in response to infection with *Botrytis cinerea*, leaves of ivy pelargonium (*Pelargonium peltatum*), showed a strong nitric oxide (NO) burst and H₂O₂ accumulation to arrest disease progression through NO-dependent reversible inhibition of catalase (Floryszak-Wieczorek, Arasimowicz et al. 2007). In our study, the plant's *GmPDX1.1*, and *ST-CAT* in pathosystems I and III were substantially suppressed, respectively, which may lead to

enhanced reactive oxygen species generation that typically accompanies infections caused by necrotrophs (Able 2003, Unger, Kleta et al. 2005).

4.6 Conclusions

In conclusion, we confirmed the role of *R. solani* AG3 de novo vitamin B6 genes through yeast complementation assay. In our previous study [27], we provided evidence that vitamin B6 genes of the *de novo* and the salvage biosynthetic pathways function as antioxidants against oxidative stress induced by ROS chemical inducers (Samsatly, Chamoun et al. 2015). In this study, we extend our research to examine whether genes of vitamin B6 machinery are also implicated as antioxidants in response to oxidative stress formed during plant-pathogen interactions. Our findings present new evidence on: (i) the co-expression of VB6 genes along with other well-known antioxidant genes (i.e., *CAT* and *GST*) in both the plant and pathogen side during their interaction, and (ii) the differential transcriptional regulation of the genes in infected necrotic tissues and their surrounding areas of three pathosystems involving different plants and different AGs of *R. solani*.

It is also likely that the requirement of Vitamin B6 genes of the salvage pathway differs among different plant species in response to pathogen infection. It appears that both the pathogen and host employ unique expression strategy depending on the type of interaction as well as the type of tissue affected. Hence, the study offers novel insights into the biological correlation and identification of ROS antioxidant genes that can be used in soybean and potato breeding programs for resistance against *R. solani*. Overall, we demonstrate that the co-expression and accumulation of certain plant and pathogen ROS-antioxidant related genes in each pathosystem are critical

during disease development from the plant's point of view, and in pathogenicity and developing of infection structures from the fungal point of view. Future studies are underway to test whether different potato cultivars containing variable amounts of vitamin B6 (Mooney, Chen et al. 2013) exhibit differential degrees of susceptibility to *R. solani* AG 3, and that this susceptibility is correlated with content and differential gene expression of vitamin B6.

4.7 ACKNOWLEDGEMENTS

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4.8 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments by SJ, JS. Performed the experiments by JS. Analysed by JS and TC. Contributed reagents and materials and analysis tools by SJ. Wrote the paper by JS, SJ.

4.9 SUPPORTING INFORMATION

Appendix I. Principle component analysis score plots (PC1/PC2) for the effect of 11 antioxidant genes relative transcripts abundance on control, necrotic lesions and surrounding areas of necrotic lesions of Pathosystem I (A), II (B) and III (C).

Table 4.1. List of primers used in this study

Target organism	Primer	Sequence (5' → 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
PDX1 (Pyridoxine biosynthesis gene)					
Soybean	GM ^a -PDX1.1-F	TACAGTGTACGGCAACGGTGCA	59	235	XM_003542937.3
	GM-PDX1.1-R	GTCCTTGATGAGCTGCGGG			
	GM-PDX1.2-F	TACAGTCTACGGCAACGGCGCC	54	235	NM_001251112.2
	GM-PDX1.2-R	ATCCTTGATGAGCTGCGGA			
Potato	ST ^b -PDX1.1-qF	CTGTGACTATTCCTGTAAT	55	83	(Mooney, Chen et al. 2013)
	ST- PDX1.1-qR	GTAATCTACTCCGATAGC			
	ST-PDX1.2-qF	TGCTCTAATCCTTACAAG	55	162	
	ST-PDX1.2-qR	GTAGGTCTCATCACTAAC			
R. solani AG3	5'GSP1-(PDX1)	TGACGAACAGCCTCGACAACATTTC	55	219	(Samsatly, Chamoun et al. 2015)
	3'GSP2-(PDX1)	TCCGTTTGTCTGTGGGGCTACATCTCTC			
R. solani AG4	RsoFAG8-PDX1- F	ATGATCAAAGAGATCGTGGAC	53	243	AVOZ01001318.1
	RsoLAG8-PDX1-R	GATCATTGCTGCACCTTCGGA			

<i>R. solani</i> AG1-IA	<i>RsolAG1-IA-PDX1-F</i>	ATCTCTGCCTACTACCAATG	51	131	AFRT01001225.1
	<i>RsolAG1-IA-PDX1-R</i>	TGCCCCCATCCGTGTTCTGAG			
<i>PDX2</i> (Pyridoxine biosynthesis gene)					
Soybean	<i>GM-PDX2-F</i>	TTAGGAGTGAAAGGTGTGGAG	53	328	XM_014774652.1
	<i>GM-PDX2-R</i>	TTTGGAGACGAGCTCTGGCAC			
Potato	<i>ST-PDX2-qF</i>	ATTCCAATCCTGCTATTC	55	185	(Mooney, Chen et al. 2013)
	<i>ST-PDX2-qR</i>	CACAATATCAGAAGTTCCT			
<i>R. solani</i> AG3	<i>3'GSP1-(PDX2)</i>	AATCTTGCTTGCCTCTGGTGGTGTG	55	225	(Samsatly, Chamoun et al. 2015)
	<i>5'GSP1-(PDX2)</i>	ATCCCATTAATGGTCGGTCCTCATCA			
<i>R. solani</i> AG4& <i>R. solani</i> AG1-IA	<i>RsolAG8-PDX2-F</i>	ATTATATCACGCGTGACACC	53	207	JATN01000314.1
	<i>RsolAG8-PDX2-R</i>	GGCGCCTTCAACACCACCAGA			
<i>PLR</i> (PYRIDOXAL REDUCTASE)					
Soybean	<i>GM-PLR-F</i>	TCCTCAAAGCCTGAACCCGA	53	115	XM_003527250.3

	<i>GM-PLR-R</i>	TAGAAGGAGTTCGTTGGTGT			
Potato	<i>ST-PLR-F</i>	GCCGCCCAAACCCGAACCCGA	58	118	XM_006358747.2
	<i>ST-PLR-R</i>	AAGGAGGATCTCGTTAGTGT			
<i>R. solani</i> AG3	<i>PLR AKR8 F</i>	GAAAGCCTCCTCTTGAATCT	58	300	(Chamoun and Jabaji 2011)
	<i>PLR AKR8 R</i>	GGGTAAGATTGGATCGATTGGG			
<i>R. solani</i> AG4 & <i>R. solani</i> AG1-IA	<i>RsolAG1-IA-PLR-F</i>	AAGGGGCACTAACCGGAAAGC	58	194	AFRT01000983.1
	<i>RsolAG1-IA-PLR-R</i>	ATGTTGCCCCGAGAGAGGAGAC			
	<i>CATALASE</i>				
Soybean	<i>GM-CAT^d-F</i>	ACATGTTCACCTTTTATTTG	47	149	NM_001253092
	<i>GM-CAT-R</i>	CTTTATAACCACTAGTGGTCTT			
Potato	<i>ST-CAT-F</i>	ATGTTCACCTTTCCTCTTCGAC	52	147	AY442179.2
	<i>ST-CAT-R</i>	CTTGACACCACATGTGGGCTT			
<i>R. solani</i> AG3	<i>RsolAG3-CAT-F</i>	ACCAGAAGTGTTAGTCCAGCGG	56	190	(Samsatly, Chamoun et al. 2015)

	<i>RsolAG3-CAT-R</i>	CATCCGGTCACAGCAGCGTA			
<i>R. solani AG4& R. solani AG1-IA</i>	<i>RsolAG1-IA-CAT-F</i>	TGCGGGACTCATGCTGCTCTC	55-54	114	AFRT01002805.1
	<i>RsolAG1-IA-CAT-R</i>	TCCTTGGTGCCTTTCCTATCC			
	<i>GLUTATHIONE S-TRANSFERASES</i>				
Soybean	<i>GM-GST^e-F</i>	TCCATTTGGGATGAGGGTCAG	53	208	NM_001251762
	<i>GM-GST-R</i>	ATTTCTGTCAATCCAAACCTCC			
Potato	<i>ST-GST-F</i>	TCCTTTTAGCCATAGAGTTGA	51	200	POTPR1A
	<i>ST-GST-R</i>	CTTCAAATGCCTCATCAATGC			
<i>R. solani AG3</i>	<i>RsolAG3-GST-F</i>	AGAAGACGAGGCAAATGCGA	57	256	(Samsatly, Chamoun et al. 2015)
	<i>RsolAG3-GST-R</i>	ATCTCTTCAACCGCCTTCCAGT			
<i>R. solani AG4 & R. solani AG1-IA</i>	<i>RsolAG1-IA-GST-F</i>	TACAAACGCATACCTTATCGC	53	217	ELU40094.1
	<i>RsolAG1-IA-GST -R</i>	TTGGAGCCGGGTAAGTTTTG			
	<i>Reference genes</i>				

Soybean	<i>UKN2^f_F</i>	GCCTCTGGATACCTGCTCAAG	58	79	(Hu, Zhou et al. 2009)
	<i>UKN2_R</i>	ACCTCCTCCTCAAACCTCTCTG			
Potato	<i>ST-Actin7-qF</i>	GGCTATGTATGTTGCTAT	55	186	(Mooney, Chen et al. 2013)
	<i>ST-Actin7-qR</i>	ATCTTCATCAGGTTATCAG			
<i>R. solani</i> AG3	<i>G3PDH^g -F</i>	GGTATTATTGGATACACTGA	55	129	(Chamoun and Jabaji 2011)
	<i>G3PDH -R</i>	TTAAGCCTCAGCGTCTTTCT			
	<i>Tubulin-F</i>	GTTGATTTCGAAGATCCGTG	55	139	FJ392707
	<i>Tubulin-R</i>	CGAGTTCTCGACCAACTGAT			
	<i>Histone 3 -F</i>	AAGTCTGCACCCGTAAGTTC	55	289	M.Cubeta ^h
	<i>Histone 3 -R</i>	AACAACGAGACGAGGTAAGC			
<i>R. solani</i> AG1-IA	<i>RsolH3ⁱ_F</i>	CTTCCAATCATCGGCAGTCCTC	52	76	(Copley, Aliferis et al. 2017)
	<i>RsolH3_R</i>	ATTGGTATCTTCGAACAAAGACACGAG			
<i>R. solani</i> AG4	<i>GM-RS-4^j</i>	CGGTTCATCTGCATTTACCTT	55	88	(Johanson, Turner et al. 1998)
	<i>GM-RS3-R</i>	AGTGTTATGCTTG GTTCCACT			

<i>Transformation</i>					
<i>R. solani</i> AG3	<i>RsolAG3-PDX1-CDS-F</i>	GCGGCCGCATCATGTCTGCTCCTGTGTC	55	969	KF620111.1
	<i>RsolAG3-PDX1-CDS-R</i>	GCGGCCGCCACTCACATAGTCAGCCCAA			
<i>R. solani</i> AG3	<i>RsolAG3-PDX1-CDS-F</i>	GCGGCCGCTCGATGACTAGAACTGAAAC	55	747	KF620112.1
	<i>RsolAG3-PDX1-CDS-R</i>	GCGGCCGCGGTTCACCTGGCAAAGCTC			

a Glycine max

b Solanum tuberosum

c Rhizoctonia solani

d Catalase

e Glutathione S-Transferase

f Hypothetical protein unknown 2

g G3PDH: Glyceraldehyde-3-phosphatedehydrogenase

h Sequence of Histone3 gene was provided by Marc Cubeta, North Carolina State University

i Histone 3

j ITS1 of 5.8S rRNA

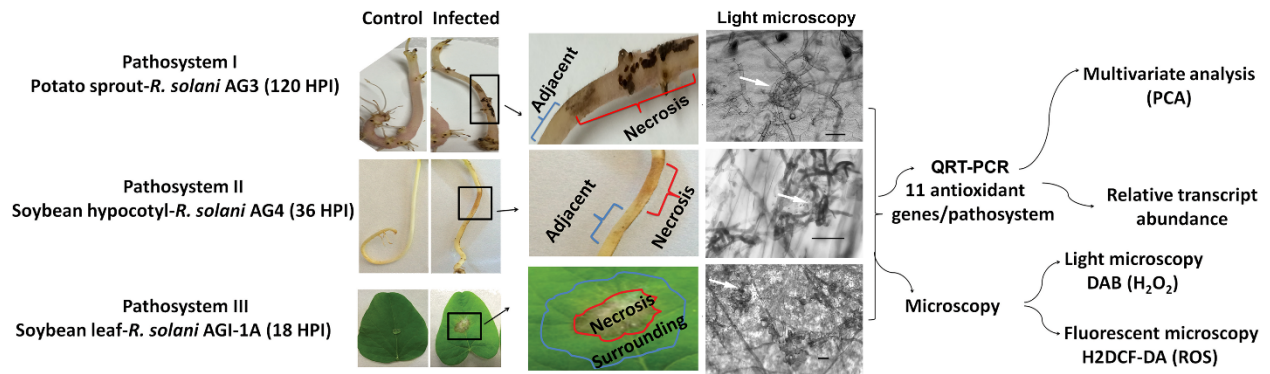


Figure 4.1. Flow chart of the experimental set up for disease development in the three pathosystems: I. potato sprouts-*R. solani* AG3; II. soybean hypocotyls-*R. solani* AG4; III. soybean leaves-*R. solani* AG1-1A. Sprouts and hypocotyls in pathosystems I and II, were sandwiched between two PDA strips of a 3-day-old *R. solani* AG3 or AG4 cultures, respectively. Sprouts or hypocotyls sandwiched with sterile PDA strips served as controls. Leaves in pathosystem III were inoculated with 3-day-old plugs of *R. solani* AG1-1A. Samples were taken at 120 HPI (Hour post-inoculation) (pathosystem I), 36 HPI (pathosystem II), and 18 HPI (pathosystem III) from necrotic lesions and adjacent or surrounding areas. Corresponding segments were taken from controls. Typical initials of infection cushions with mycelial mass aggregates are clearly visible under the light microscope (white arrows). Transcriptional abundance of 11 antioxidant genes from both the plant and pathogen of each pathosystem was analyzed in necrotic tissues and their surrounding areas followed by multivariate analysis (PCA). The three pathosystems were examined for ROS accumulation using fluorescent (H₂DCF-DA) and DAB staining methods in control and infected sprouts, hypocotyls and leaves along with mycelia of control *R. solani*. Bar = 50 μ m.

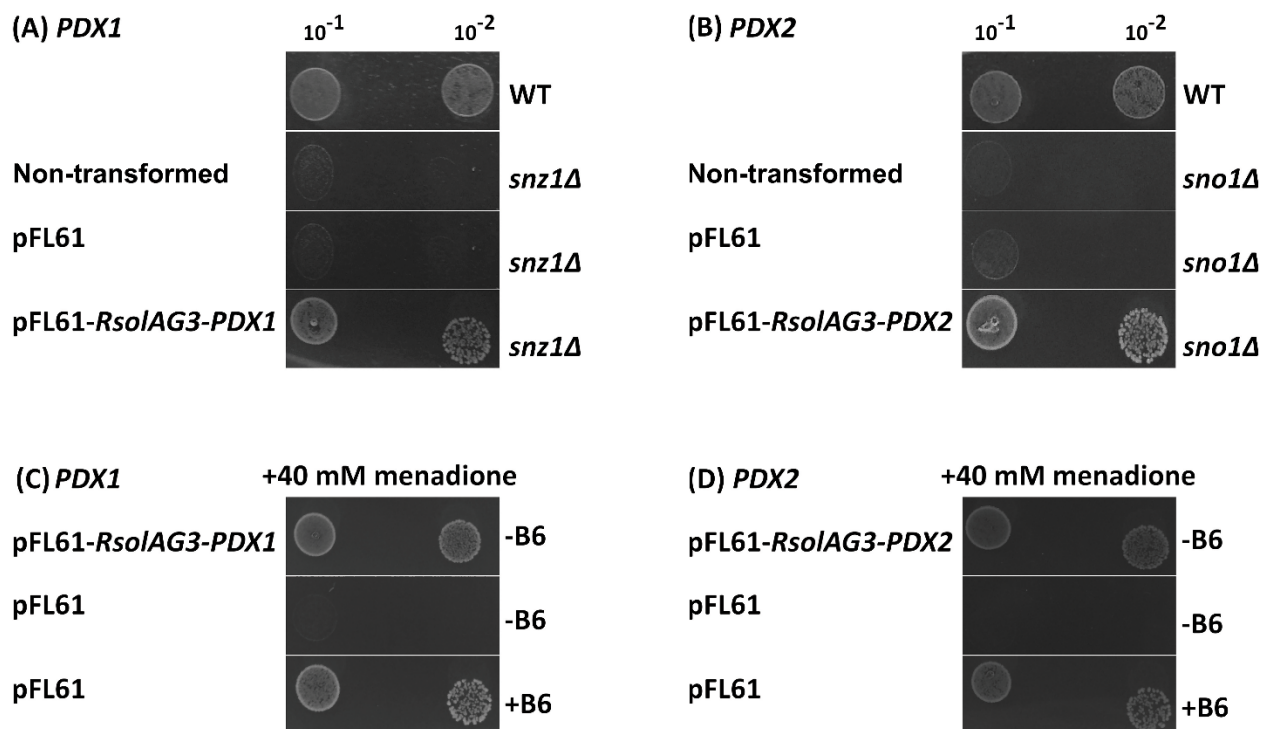


Figure 4.2. Complementation of the yeast *Δsnz1* and *Δsno1* mutants by *RsolPDX1* and *RsolPDX2*, respectively. Yeast cultures grown to exponential-phase followed by two serial 10-fold dilutions were placed onto vitamin B6-deficient SC (SC-B6) agar plates composed of (A) wild-type (WT), *Δsnz1* mutant, *Δsnz1* transformed with either pFL61 alone or the *RsolPDX1* expression vector (pFL61-*RsolPDX1*), (B) wild-type (WT), *Δsno1* mutant, *Δsno1* transformed with either pFL61 alone or the *RsolPDX2* expression vector (pFL61-*RsolPDX2*) (left colonies: 10⁻¹ ml⁻¹ right colonies: 10⁻² ml⁻¹). (C) *Δsnz1* mutant yeast transformed either with pFL61 alone or with the *RsolPDX1* expression vector (pFL61-*RsolPDX1*), and (D) *Δsno1* mutant yeast transformed either with pFL61 alone or with the *RsolPDX2* expression vector (pFL61-*RsolPDX2*) were both

grown to the post-diauxic phase in SC-Uracil medium followed by a 180 min treatment with 40 mM menadione in the presence (+B6) or absence (-B6) of $2 \mu\text{g ml}^{-1}$ of vitamin B6. Following menadione treatment, two serial 10-fold dilutions using SC-Uracil medium (left colonies: 10^{-1}ml^{-1} , right colonies: 10^{-2}ml^{-1}) were placed in SC-Uracil-B6 plates. Pictures were obtained after 3 d incubation at 30°C .

second component. Outer ellipses represent the Hotelling's T^2 at a 95 % confidence interval. R^2X represents the fraction of the sum of squares of the two principal components.

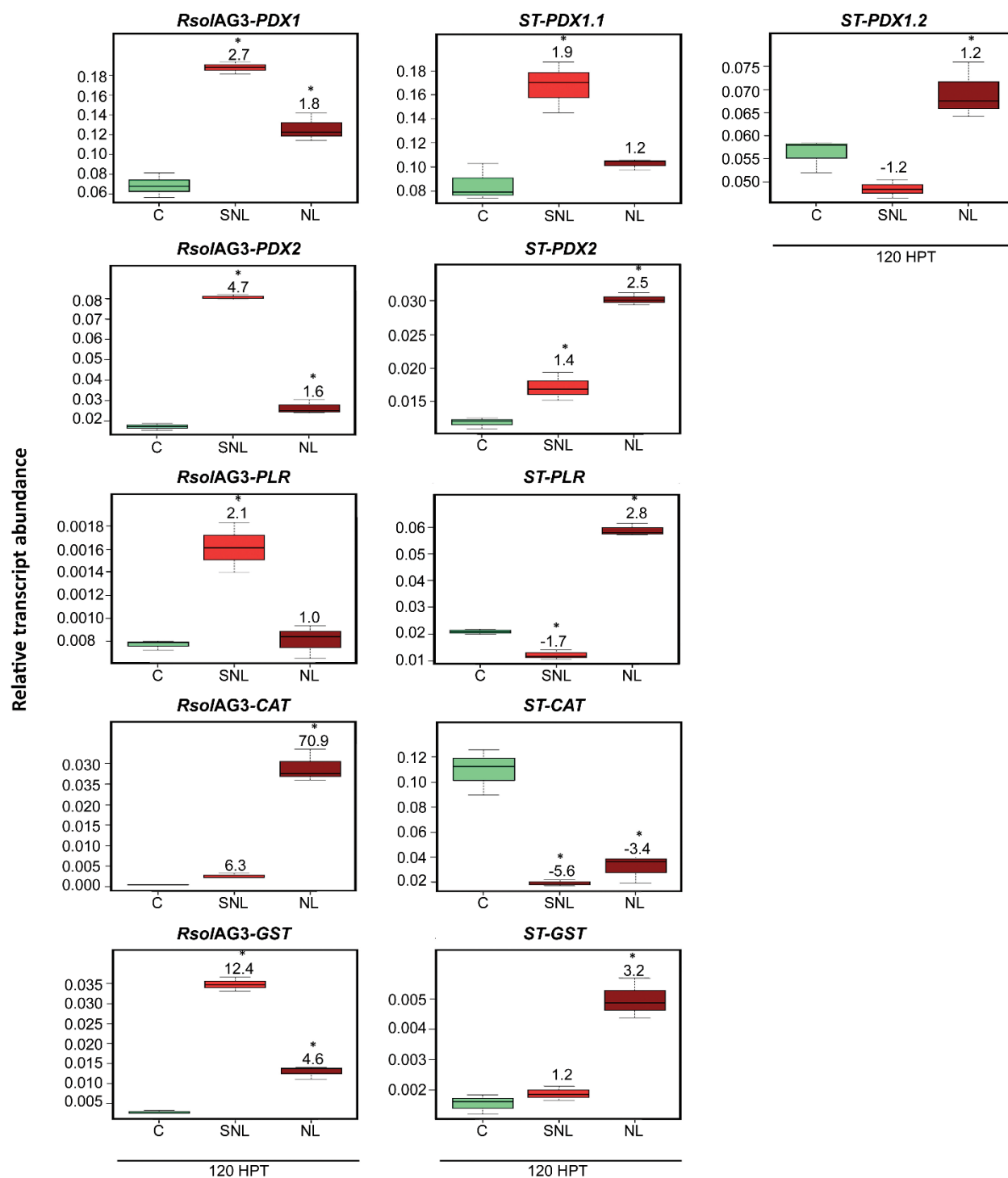


Figure 4.4. Relative transcript abundance of antioxidants in pathosystem I. Vitamin B6 (*PDX1*, *PDX2*, and *PLR*), *GST*, and *CAT* genes of the pathogen *R. solani* AG3 (*RsolAG3*) and

the host, *Solanum tuberosum* (ST) in control and in infected and surrounding tissues at 120 HPI during *R. solani* AG3 and potato sprouts interaction. C: control; *R. solani* AG3 grown alone or potato sprouts inoculated with sterile with PDA SNL: tissue surrounding necrotic lesion. NL: necrotic lesion. Asterisks indicate significant relative transcript abundance ratios between the control and interaction using least significant difference (LSD) test ($P < 0.05$). Fold change is calculated in relation to the control at 120 HPI. *PDX1* and *PDX2*: Pyridoxine biosynthesis genes, *PLR*: *PYRIDOXAL REDUCTASE*, *CAT*: *CATALASE*, *GST*: *GLUTATHIONE S-TRANSFERASE*

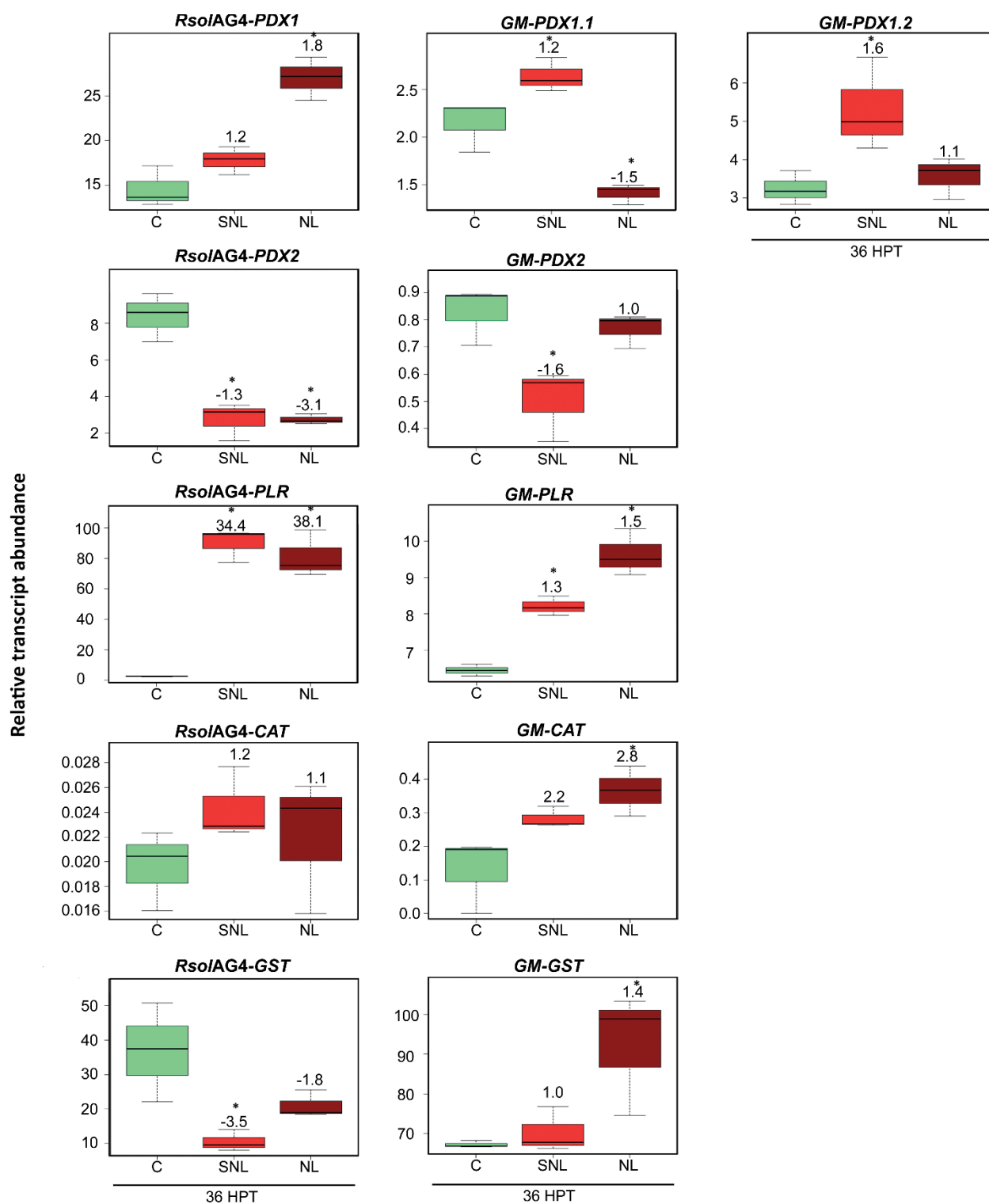


Figure 4.5. Relative transcript abundance of antioxidants in pathosystem II. Vitamin B6 (*PDX1*, *PDX2*, and *PLR*), *GST*, and *CAT* genes of both the pathogen *R. solani* AG4 (*RsolAG4*)

and the host, *Glycine max*, (*GM*) in control and infected and surrounding tissues at 36 HPI during *R. solani* AG4 and soybean hypocotyls interaction. C: control; *R. solani* AG4 grown alone or soybean hypocotyl inoculated with sterile PDA. SNL: tissue surrounding necrotic lesion. NL: necrotic lesion. Asterisks indicate significant relative transcript abundance ratios between the control and the interaction using least significant difference (LSD) test ($P < 0.05$). Fold change is calculated in relation to the control at 36 HPI. *PDX1* and *PDX2*: Pyridoxine biosynthesis genes, *PLR*: *PYRIDOXAL REDUCTASE*, *CAT*: *CATALASE*, *GST*: *GLUTATHIONE S-TRANSFERASE*

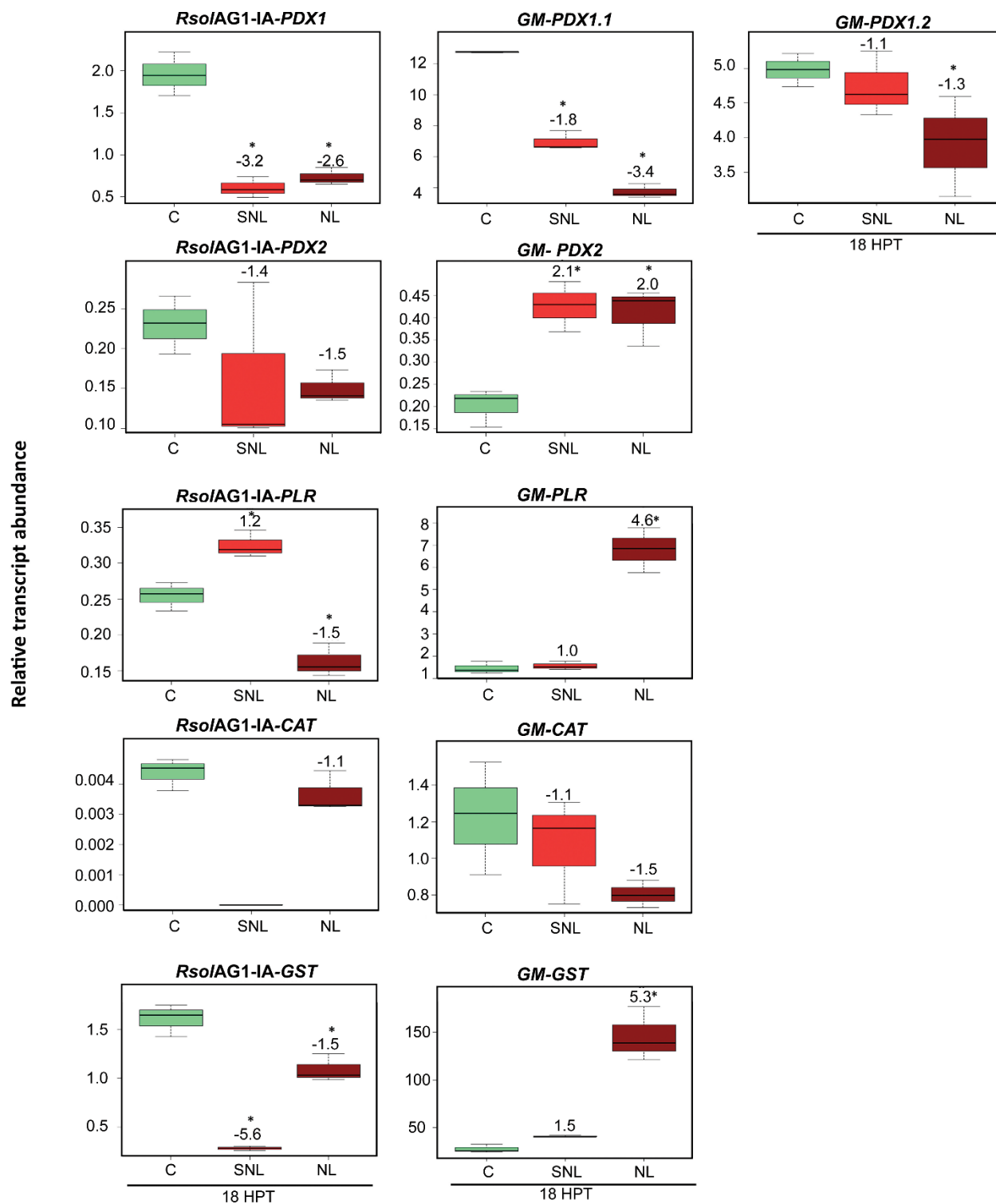


Figure 4.6. Relative transcript abundance of antioxidants in pathosystem III. Vitamin B6 (*PDX1*, *PDX2*, and *PLR*), *GST*, and *CAT* genes of both pathogen *R. solani* AG1-IA

(*RsolAG1-IA*) and the host, *Glycine max* (*GM*) in control and in infected and surrounding tissues at 18 HPI during *R. solani* AG1-IA and soybean leaves interaction. C: control; *R. solani* AG1-IA grown alone or soybean leaf confronted with sterile PDA. SNL: tissue surrounding necrotic lesion. NL: necrotic lesion. Asterisk indicates significant relative transcript abundance ratios between the control and interaction using least significant difference (LSD) test ($P < 0.05$). Fold change is calculated in relation to the control at 18 HPI. *PDX1* and *PDX2*: Pyridoxine biosynthesis genes, *PLR*: *PYRIDOXAL REDUCTASE*, *CAT*: *CATALASE*, *GST*: *GLUTATHIONE S-TRANSFERASE*.

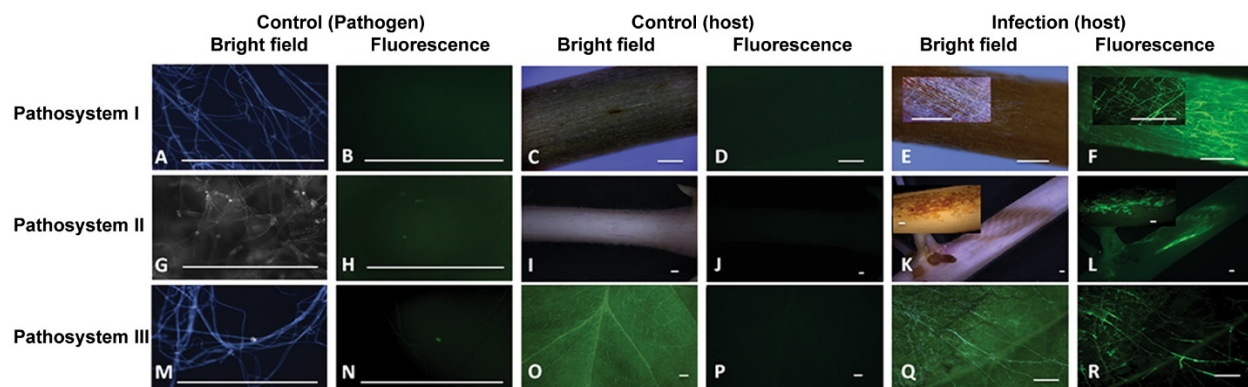


Figure 4.7. Intracellular production of ROS during disease development in pathosystems I-III. ROS production as visualized using H₂DCF-DA in control and infected potato sprouts, soybean hypocotyls and leaves along with mycelia of control *R. solani* AG-3, AG-4, AG1-IA, grown on half-strength PDA overlaid with cellophane membranes. (A-B) Control mycelia with no evidence of endogenous ROS at 120 h, (G-H) 36 h, and (M-N) 18 h upon growth on PDA membrane overlaid with cellophane membranes. (C-D) Control *S. tuberosum* sprout, (I-J) *G. max* hypocotyl and (O-P) *G. max* leaf with no evidence of endogenous ROS. Mycelia of *R. solani* during disease development in pathosystems I-III (Fig E-F, Fig K-L, and Fig Q-R, respectively). Bar = 500 μ m.

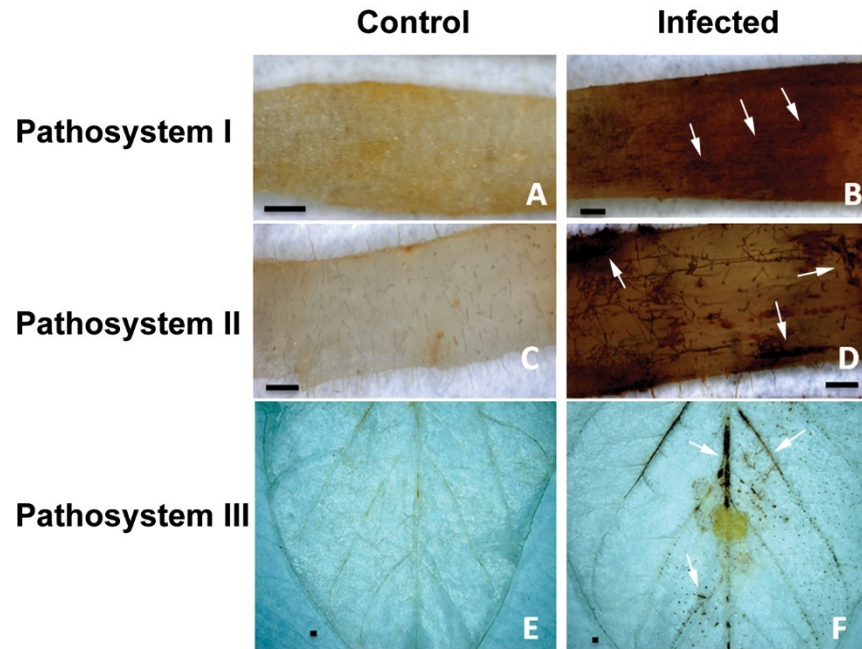


Figure 4.8. Microscopic detection of H₂O₂ accumulation using DAB staining during disease development in pathosystems I-III. H₂O₂ accumulation was visualized by DAB staining in control and infected potato sprouts and soybean hypocotyls and leaves. White arrows indicate dark brown precipitate developed at necrotic and surrounding areas. Control potato sprout (A), soybean hypocotyl (C), and soybean leaf (E) with no evidence of endogenous H₂O₂ at 120 HPT, 36h HPT, and 18h HPT, respectively. *R. solani* mycelia and *S. tuberosum* sprout (B), *G. max* hypocotyl (D), and *G. max* leaf (F) in pathosystems I-III with H₂O₂ accumulation. Bar = 500 μ m.

4.10 CONNECTING TEXT

In Chapter 4, we provided evidence that genes of vitamin B6 biosynthetic machinery are implicated as antioxidants in response to oxidative stress formed during potato-*R. solani* interaction and they are differentially and spatially co-regulated in different potato tissues in response to *R. solani* infection. In this chapter, we tested whether potato genotypes containing variable amounts of VB6 would exhibit distinct degrees of tolerance to *R. solani* AG3 and that this tolerance is tightly linked with the differential expression of genes of VB6 *de novo* and salvage biosynthetic pathways. We also examined phenotypes of Arabidopsis mutants with defects in the *de novo* pathway of VB6 biosynthesis against infection by *R. solani*, and compared their VB6 content, providing evidence that mutations in PDX1.3 led to increased disease, and suggesting the role of VB6 in plant resistance.

J. Samsatly and Dr. S. Jabaji conceived and designed the experiments. J. Samsatly performed the experiments. Data acquisition and analysis was done by J. Samsatly and Dr. S. Bayen. J. Samsatly and Dr. S. Jabaji interpreted the data. Dr. S. Jabaji contributed reagents and materials and analysis tools. The manuscript was written by J. Samsatly and revised by all co-authors and will be submitted to frontiers in plant science.

CHAPTER 5

Vitamin B6 is under a tight balance during disease development of *Rhizoctonia solani* with different cultivars of potato

Jamil Samsatly¹, Stéphane Bayen² Suha H. Jabaji¹

¹Department of Plant Science, and ²Department of Food Science and Agricultural Chemistry
McGill University, Ste-Anne-de-Bellevue, QC, Canada

Corresponding Author:

S. Jabaji; Department of Plant Science, McGill University, 21,111 Lakeshore Rd., Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9.

Telephone: +1 (514)-398-7561

E-mail: suha.jabaji@mcgill.ca

5.1 Abstract

Vitamin B6 is well recognized as an important antioxidant and plays a role in stress responses. Co-expression of plant and pathogen-derived vitamin B6 genes has been previously shown to be critical during disease development of *R. solani*. However, little is known about the functionality of vitamin B6 during plant-*R. solani* interactions and its involvement in disease tolerance. Here, we explored the possible involvement of vitamin B6 during disease progression of potato cultivars of different susceptibility levels to *R. solani*. A distinct pattern of expression, PN (Pyridoxin) concentration, and fungal biomass was found in the susceptible cv. Russet Burbank and tolerant cv. Chieftain. Accumulation of ROS in *R. solani* mycelia or plant tissues as determined by non-fluorescence or fluorescence methods was related to up-regulation in the vitamin B6 pathway and indicative of oxidative stress. Russet Burbank showed more susceptibility to *R. solani* which was linked to reduced amounts of VB6 content. Prior to infection, constitutive PN levels were significantly higher in Russet Burbank by 1.6 folds compared to Chieftain. Upon infection with *R. solani*, PN levels in infected tissues increased more in Chieftain (1.7 folds) compared to Russet Burbank (1.4 folds). *R. solani* AG3 infection of potato sprouts in both cultivars significantly activates the fungal and plant vitamin B6 and GST genes in a tissue specific response. Significant fold increases of transcript abundances of the fungal genes ranged from a minimum of 3.60 (*RsolSG3GST*) to a maximum of 13.91 (*RsolAG3PDX2*) in the surrounding necrotic lesion tissues (zone 1). On the other hand, PCA showed that the top plant genes *STGST* and *STPDX1.1* were linked to both tissues of necrotic lesions (zone 2) and their surrounding areas of necrotic lesions. Functional characterization of Arabidopsis *pdx1.3* mutants challenged with *R. solani* provided evidence into the role of the vitamin B6 pathway in the maintenance of plant tolerance during disease progression. Overall, we demonstrate that the regulation and production of vitamin VB6

is under tight control and is an important determinant of disease development during the interaction of *R. solani* with potato cultivars.

5.2 Introduction

Rhizoctonia disease of potato or black scurf, caused by *Rhizoctonia solani* Kuhn (telomorph *Thanatephorus cucumeris* Frank Donk), is an important fungal disease attacking potato (*Solanum tuberosum* L.). The disease occurs in most potato producing areas world-wide. Isolates of *R. solani* anastomosis group AG3 are identified as the etiological agents for this disease. Disease symptoms manifest on potato sprouts, stolons and roots causing sprout nipping and cankers which are reddish to brown lesions on stolons and roots (Gutierrez, Shew et al. 1997). Severe damage at this stage leads to poor, uneven crop stand and reduced yield (Hide and Horrocks 1994, Hide, Welham et al. 1996). Tubers are also affected with the appearance of black sclerotia (resting vegetative bodies of the fungus), thus the name black scurf, that appear on the surface of the tuber and in severe cases deformed tubers form in a tight cluster next to the stem. To date, no variety has been found with immunity to the sprout nipping and stem lesion phase, and the pathogen is not controlled by breeding for resistance. However, some varieties show varying degrees of resistance to formation of sclerotia on tubers.

Like many necrotrophic pathogens, *R. solani* is a destructive pathogen causing extensive necrosis, tissue maceration and plant rots (Laluk and Mengiste 2010). The shared elements in early disease development of *R. solani* isolates are the intimate association of fungal hyphae with the epidermis of the host forming infection cushion or aggregates, tissue penetration, dismantling and maceration by cell wall degrading enzymes and other enzymes which eventually leads to tissue browning and necrosis associated with high levels of reactive oxygen species (ROS) and tissue

death (Keijer 1996, Sneh, Jabaji-Hare et al. 2013). ROS molecules are highly reactive and toxic, and are not only important regulators of plant growth, but are involved in limiting pathogen spread, induction of cell death and signal transduction in host-plant interactions (Torres 2010, Barna, Fodor et al. 2012). There is increasing evidence that pathogenic fungi also produce ROS during pathogenic interactions (Daub and Ehrenshaft 2000, Samsatly, Copley et al. 2018). Therefore, the regulation of ROS in fungal cells and tolerance to external ROS produced by the host plant represent a balanced control and detoxification by both partners which can govern the fate of disease progression of necrotrophic pathogens (Heller and Tudzynski 2011). To fine-tune and maintain this balance, plant and fungal cells possess a complex battery of protective mechanisms such as oxalic acid or the NADPH oxidase RBohD (Torres, Jones et al. 2005, Kadota, Shirasu et al. 2015), or ROS-quenching molecules including vitamin B6 and several antioxidation enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), Glutathione-S-transferase (GST) and glutathione reductase (Das and Roychoudhury 2014, Samsatly, Chamoun et al. 2015, Zhang, Jin et al. 2015, Girard, Tong et al. 2017). In the case of *R. solani*, the initiation of basal resistance in plants to *R. solani* in different pathosystems is closely linked to ROS-scavenging mechanisms, oxylipins production, cell-wall bound phenolic compounds, and accumulation of metabolites related to vitamin B6 biosynthetic pathway (Taheri and Tarighi 2011, Nikraftar, Taheri et al. 2013, Aliferis, Faubert et al. 2014, Foley, Kidd et al. 2016, Samsatly, Copley et al. 2018).

Vitamin B6 (VB6) plays a critical role in all living organisms and is recognized as an important cofactor required for many enzymatic reactions (Hellmann and Mooney 2010). Vitamin B6 is a collection of six vitamers: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives (Fitzpatrick, Basset et al. 2012, Vanderschuren, Boycheva et al. 2013).

In plants and fungi, the *de novo* biosynthesis of VB6 vitamers (the so called DXP-independent pathway) requires two pyridoxal protein families (PDX1 and PDX2) that are highly conserved. PDX1 and PDX2 proteins contain the glutamine amidotransferase where PDX1 functions as the synthase and PDX2 constitutes the glutaminase domain (Raschle, Amrhein et al. 2005, Fitzpatrick, Amrhein et al. 2007). Another important component of the VB6 biosynthetic machinery is the salvage pathway, which is responsible for the interconversion between the different forms of VB6 vitamers (González-Guerrero, Cano et al. 2007, Herrero, González et al. 2011, Rueschhoff, Gillikin et al. 2012). It includes pyridoxal reductase (PLR), a downstream enzyme in the VB6 biosynthesis pathway, which converts PL into PN (Morita, Takegawa et al. 2004, Herrero, González et al. 2011).

In the last decade, the regulatory function of key genes involved in the VB6 biosynthetic pathways have been characterized in the model plant *Arabidopsis*, uncovering the importance of VB6 in plant development and response to stress (Hellmann and Mooney 2010). There are three *PDX1* homologs in *Arabidopsis*, two (*PDX1.1* and *PDX1.3*) of which are catalytically active and they encode PDX (Tambasco-Studart, Titiz et al. 2005, Titiz, Tambasco-Studart et al. 2006). *PDX1.3* is required for disease resistance to bacterial and fungal diseases in *Arabidopsis* (Moccand, Boycheva et al. 2014, Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). In contrast to *Arabidopsis*, potato (*Solanum tuberosum* L.) has only two *PDX1* homologs (*PDX1.1* and *PDX1.2*) and only *PDX1.1* is catalytically active (Mooney, Chen et al. 2013). Several studies had shown that VB6 is a potent antioxidant with ROS quenching ability that matches that of tocopherols or ascorbic acid. This novel role of VB6 as ROS scavenger and its ability to increase resistance to biotic and abiotic types of stress has been demonstrated in microbe-microbe (Chamoun and Jabaji 2011, Gkarmiri, Finlay et al. 2015) and in plant-microbe interactions (Ehrenshaft, Bilski et al.

1999, Benabdellah, Azcon-Aguilar et al. 2009, Mooney, Leuendorf et al. 2009, Vanderschuren, Boycheva et al. 2013). During plant-microbe interactions, plant-derived VB6 genes, involved in the *de novo* biosynthetic pathway, were upregulated in response to various stresses (Denslow, Walls et al. 2005, Bagri, Upadhyaya et al. 2018, Chandrasekaran and Chun 2018, Samsatly, Copley et al. 2018). These results suggest that VB6 vitamers might contribute to disease resistance in modulating plant defence responses against different types of pathogens. Zhang and co-workers (2014) provided direct evidence supporting this notion by examining Arabidopsis mutant ecotypes that are defective in the *de novo* and the salvage pathways of VB6 biosynthesis. Mutations in the Arabidopsis *PDX1.2* and *PDX1.3* genes involved in the *de novo* pathway ended up in increased levels of diseases caused by *B. cinerea* and decreased content level of VB6 (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015) Despite these findings, studies on the regulation and co-expression of VB6 genes in the host and the pathogen of the *de novo* and salvage biosynthetic pathways during host–pathogen interactions remain limited to the best of our knowledge to only one study (Samsatly et al., 2018).

In a previous research, we provided evidence that genes of the vitamin B6 biosynthetic machinery are implicated as antioxidants in response to oxidative stress formed during potato-*R. solani* interaction and that VB6 genes are co-expressed in both the potato and *R. solani* during their interaction. We also showed evidence that the differential transcription of the genes in infected necrotic tissues and their surrounding areas is spatially regulated. These results indicate that from the host view-point, VB6 antioxidant genes are critical during disease development, and from the fungal view-point, are crucial for detoxification and pathogenicity. Therefore, developing new strategies to combat yield losses in potato requires a thorough understanding of the antioxidant

genes and gene regulatory networks underlying the plant defense response, especially in existing tolerant breeding potato cultivars.

In this study we extended the research to first test whether potato genotypes containing variable amounts of VB6 (Mooney, Chen et al. 2013) would exhibit distinct degrees of tolerance to *R. solani* AG3 and that this tolerance is tightly linked with the differential expression of genes of VB6 *de novo* and salvage biosynthetic pathways. Second, we used LC-MS based-method to report on the content of the VB6 vitamer, pyridoxine (PN) in infected potato cultivars to assess *R. solani* induced expression of VB6 biosynthetic genes, and third, we examined phenotypes of Arabidopsis mutants with defects in the *de novo* pathway of VB6 biosynthesis against infection by *R. solani*, compared their VB6 content, and providing evidence that mutations in *PDX1.3* led to increased disease, suggesting the role of VB6 in plant resistance.

5.3 Materials and Methods

5.3.1 Biological material and inoculation

5.3.1.1 *Rhizoctonia solani* inoculum preparation

Starter cultures of *R. solani* AG3 isolate Rs114 (ATCC 10183), and AG4 isolate A76 provided by M. Cubeta, North Carolina University, USA, were recovered from cryogenic storage at -80°C on potato dextrose agar (PDA; Difco Laboratories) and grown for 3 days at 24°C in the dark, and used as a source of inoculum for plant infection. Inoculum was made of strips of PDA ($2\text{ cm} \times 8\text{ cm}$) containing mycelia growth *R. solani*. For RNA and DNA extraction, cultures of *R. solani* strains of AG3 and AG4 were grown on PDA overlaid with cellophane membrane following the method of Samsatly et al (2018).

5.3.1.2 *Solanum tuberosum* L. growth conditions

Certified potato tubers with different susceptibility levels to *R. solani* were used in this study. *Solanum tuberosum* cv. Russet Burbank, susceptible to *R. solani* was kindly provided by D.J. Donnelly (McGill University), and *S. tuberosum* cv. Chieftain, a tolerant to *R. solani* (Dowley 1972) was obtained from the Northern Horticultural Research/SPUD Unit, New Liskeard Agricultural Research Station, University of Guelph. For sprout induction, uniform-sized tubers, previously kept at 4°C in the dark, were surface sterilised and sprouted for 12 days under controlled conditions following the methods of (Samsatly, Copley et al. 2018). Sprout tips of 3 cm in length were sprayed with 1.5 mL solution of CaNO₃ (0.5M) to prevent browning. Inoculation of sprouts with *R. solani* was performed when sprouts were 8 cm in length.

5.3.2 Sprout inoculation and tissue collection

To induce *R. solani* infections on potato sprouts, excised sprouts (8 cm in length) were sandwiched with two PDA strips (2 cm × 8 cm) of a 3-day-old *R. solani* AG3 Rs114 culture and placed in sterile Pyrex trays (40 cm × 26 cm) lined with wet sterile Whatman No. 1 paper as fully described in Samsatly et al. (2018). Mock-inoculated control potato sprouts were sandwiched with sterile PDA strips. There were 3 biological replicates per treatment and each treatment replicate consisted of 4 excised potato sprouts.

Pyrex trays were covered with transparent plastic film and placed in a growth chamber under photosynthetically inactive black light (365 nm) at 24°C. PDA strips were removed 120h post-inoculation (HPI) of potato sprouts to reveal the infection areas with visible necrotic lesions on the sprouts. This time point was optimal to secure the beginning of infection cushions and mycelial aggregates that were stereoscopically examined (**Figure 5.1**). Two distinct zones were

sampled from potato stolons. Zone 1 consists of a one-cm area bordering the lesions and zone 2 contains the necrotic lesions with infection structures of *R. solani* (**Figure 5.1**). Similar areas and amounts of tissues were harvested from mock-inoculated controls (**Figure 5.1**). Harvested tissues were flash-frozen with liquid nitrogen and stored at -80°C for downstream applications.

5.3.3 Optical and fluorescence microscopy

The accumulation of cellular and extracellular ROS accumulation during disease progression was visualized by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) staining methods in mock inoculated control and potato by 3,3'-diaminobenzidine (DAB) staining methods in control and infected potatoes. For ROS detection and H₂O₂ accumulation *in situ* we followed the protocol of (Samsatly, Copley et al. 2018). All samples were examined under Zeiss SteREO Discovery.V20 microscope (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada). Fluorescence detection from plant tissues was read at an excitation wavelength of 470 nm using a GFP filter. Light microscopy was performed to monitor disease development and capture fungal infection cushions, and images were digitally documented with the Moticam 2300 digital camera (GENEQ Inc. Montreal, Quebec) for light microscopy.

5.3.4 Detection and quantification of fungal biomass on infected sprouts

The quantification of fungal biomass of *R. solani* AG3 was used to estimate disease severity in infected tissues by amplifying the gDNA of Glyceraldehyde-3-phosphatedehydrogenase in AG3 isolate 114 using the primer pair set G3PDH-F/R (**Table 5.1**). The putative amplified products were purified using the QIAquick PCR Purification Kit (Qiagen), and sub-cloned in pDrive cloning vector (Qiagen). Absolute quantification of genomic gene copies was established from a standard curve using six consecutive 10-fold dilutions of genomic DNA for *R. solani* AG3. Absolute quantification

of genomic gene copies was established from a standard curve using six consecutive 10-fold dilutions of genomic DNA for *R. solani* AG3. Initial copies were calculated using the genomic size presented by (King, Urban et al. 2015). Total genomic DNA was isolated from 100 mg of mock-inoculated control plant tissues and infected tissues (necrotic lesions) of potato using the DNeasy Plant Mini Kit™ (Qiagen, Toronto, ON, Canada). The concentration and purity of DNA were assessed by spectrophotometry with ND1000 (NanoDrop, Wilmington, Delaware). All Q-PCR reactions were carried on using Stratagene Mx3000 (Stratagene, Cedar Creek, USA).

5.3.5 Vitamin B6 analysis by LC-MS

To determine whether the level of vitamin B6 was affected in infected tissues as compared to mock-inoculated controls, the vitamer pyridoxine (PN) was extracted from lyophilized inoculated and mock inoculated control tissues of potato cultivars following the modified method of (Denslow, Walls et al. 2005). Briefly, 0.1 g of tissues were ground in liquid nitrogen, mixed with 30 ml 0.44 N HCl, and autoclaved at 121°C for 2 hours. The pH was brought to 4.8 using sodium acetate and each sample was de-glycosylated using fresh B-glucosidase (5mg in 1ml H₂O) and de-phosphorylated with fresh acid phosphatase (75 mg in 2 ml H₂O). Samples were added to flasks (50 ml capacity) and incubated overnight in a 37°C incubator shaker at 70 rpm. The mixture in each flask was brought to 50 ml with deionized H₂O and filtered through 0.22µm filters (Millex-FG, Millipore, MA, USA). Samples were stored at -20°C and all later steps of vitamin B6 analysis were done in dark conditions. Surrogate analog, 400 ng of Pyridoxine-(methyl-d3) (Sigma-Aldrich, Oakville, Canada) (**Table 5.2**), was spiked prior to extraction in all samples. Procedural blanks were prepared in the same manner. Four samples were spiked with 400 ng of Pyridoxine (Sigma-Aldrich) to estimate the recovery rates of the method. Matrix effects were assessed by comparing the signal obtained from spiked extracts with PN standard and with the equivalent

signal obtained from pure solvent standards. Unless otherwise stated, VB6 concentrations refer to the amounts of the vitamin PN.

High-performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (HPLC-QTOF-MS) analysis was performed using an Agilent 1290 Infinity LC System coupled to a 6540 Accurate-Mass [QTOF](#) System operated in the positive ionization mode (ESI+) (Agilent Technologies, Santa Clara, CA, USA). The separation was performed with a Poroshell EC-C18 Column (3.0 x 100 mm, 2.7 μ m, Agilent Technologies) fitted with an Infinity Lab Poroshell 120 pre-column (3.0 x 5 mm, 2.7 μ m) in a thermostated column compartment set at 20°C. HPLC grade solvents (water, methanol), as well as LC/MS grade formic acid were all purchased from Fisher Chemical (Pittsburgh, PA, USA) (**Appendix II**). The mobile phase consisted of water (A) and methanol (B), both containing 0.1% of formic acid. The HPLC gradient was as follows: 1–6 min (100% A), 6–10 min (75% A and 25% B), 10–12 (100 % B) min, 12–12.01 min (100 % B), 12.01–14 min (100 % B). The flow rate was 0.5 mL.min⁻¹ with an injection volume of 2.00 μ L. MS scans (m/z 50–500) were completed at a scan rate of 4 spectra/s. Prior to analysis, the QTOF was tuned (mass accuracy <1ppm), and mass accuracy was maintained throughout the batch using the continuous infusion of a reference mass mix. Compound identification was based on mass spectra and retention times of pure VB6 analytical standards (**Table 5.2**). Target analytes were quantified using a 6-points calibration range (10–400 ng.mL⁻¹) based on the extracted chromatogram for their specific protonated molecular ion [M+H]⁺. Concentrations were calculated from the relative response versus their respective mass-labeled analog.

5.3.6 RNA extraction, and quantitative RT-PCR

Total RNA was extracted from flash frozen pulverized tissues (100 mg) using TRIZOL (Generay Biotech, Shanghai, China) following the manufacturer's instructions. RNA (500 ng) was reverse transcribed with the Quantitect Reverse transcriptase kit™ (Qiagen) according to the manufacturer's protocols. QRT-PCR analyses were conducted on fungal and plant target antioxidant genes (**Table 5.1**). Primers for fungal VB6 and *GST* encoding genes were designed as previously described (Samsatly et al., 2018). Internal reference genes and potato VB6 and *GST* encoding genes were synthesized based on published sequenced primers (**Table 5.1**). QRT-PCR conditions were optimized for each primer set (**Table 5.1**), and putative products were confirmed by sequencing. Reverse transcription PCR assays were performed on three biological replicates and two technical replicates. QRT-PCR assays were conducted on fungal and plant target antioxidant genes encoding *GST*, the *de novo* VB6 biosynthesis genes, a synthase (*PDX1*) and a glutaminase, (*PDX2*), and the VB6 salvage pathway encoding gene, *Pyridoxal Reductase (PLR)* and appropriate internal reference genes (**Table 5.1**) using Stratagene Mx3000 (Stratagene, Cedar Creek, USA). PCR assay conditions were performed using suitable annealing temperature for each primer pair (**Table 5.1**) with the following conditions: Each 20 µL reaction contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd.), 0.16 µM each primer, and 500 ng cDNA. The thermocycling profile used an initial denaturation at 98°C for 2 min, followed by 35 or 40 cycles of denaturation at 98°C for 15 s, annealing for 30 s at the appropriate primer temperature and extension at 72°C for 30 s, followed by a dissociation curve analysis. In all QRT-PCR assays, routine negative and positive controls were performed at every run. The relative transcript abundance levels of the plant and fungal-derived genes were estimated

and normalized against their respective reference genes according to the method Zhao and Fernald (2005).

5.3.7 Arabidopsis pathogenicity experiments

Seeds for *Arabidopsis thaliana* wild type, ecotype Col-0 and *pdx1.3* mutants (At5g01410, SALK 086418C) were obtained from Arabidopsis Biological Resource Center (Ohio State University, Columbus; (Alonso, Stepanova et al. 2003). PCR-based genotyping was carried out for SALK 086418C lines to screen for homozygous plants. Gene-specific primers SALK 086418-LP/RP and T-DNA primers SALK LB-1.3 (**Table 5.1**) were included following the T-DNA Express Web site (<http://signal.salk.edu/tdnaprimers.2.html>). Plants homozygous for PDX1.3 mutation were used for downstream applications.

Seeds were surface sterilized by immersing them in 70% ethanol for 45 seconds, followed by 1.3% bleach for 3 min and rinsed five times with sterile water. Sterile seeds were stratified at 4°C for 5 days in the dark prior to planting. Seeds were sown in Square Pots 10 x 10 x 11 cm 0.69L containing AgroMix® G10 (Fafard Ltd.) and sand (1:1, v/v) with one seed per pot and placed in a growth cabinet with a 16/8 h light/dark photoperiod and 100 µmol/ms of light at 22°C/19°C, respectively, with 60% relative humidity. Eight weeks-old seedlings were used for disease assays.

Arabidopsis seedlings of wild type and mutant ecotypes were carefully removed from the potting mix and the roots were rinsed with distilled water to remove soil particles. Ten seedlings of Arabidopsis wild type ecotype Col-0 and *pdx1.3* mutants (At5g01410, SALK 086418C) were placed in sterile Pyrex trays as described above and sandwiched with two strips of PDA strips (1cm × 8 cm) of a 3-day-old *R. solani* AG4 isolate A76 culture covering the lower stem, crown, and part of the roots. Mock-inoculated controls were sandwiched with sterile PDA strips. There

were 3 biological replicates per treatment and each treatment replicate consisted of 10 Arabidopsis seedlings. Progression of infection was determined at 72 HPI. One zone was sampled which consists of necrotic lesions developing at the crown area and lower stem parts. Similar areas and amounts of tissues were harvested from mock-inoculated controls.

RNA isolation, cDNA synthesis, and qPCR reactions were carried out as described above for potato. Primer sets were designed for Arabidopsis based on sequences from NCBI, and were checked for specificity to amplify only their target gene (**Table 5.1**). Specific primers used for the amplification of VB6, *GST* encoding genes of Arabidopsis and of *R. solani* AG4 were designed as previously described (Samsatly et al., 2018). Internal reference genes for Arabidopsis and *R. solani* AG4 (**Table 5.1**) were used for normalization of relative transcript abundance levels.

R. solani AG4 fungal biomass was used to estimate disease severity in infected tissues of Arabidopsis wild type and pdx1.3 mutant line, by amplifying the gDNA using the primer pair set RsolAG4-PDX1-F/R in Q-PCR assays (**Table 5.1**). PCR assays were carried out as described above for potato. Optical and fluorescence microscopy to detect ROS accumulation during disease progression in the wild type and mutant lines of Arabidopsis infected or not with *R. solani* AG4 was visualized by H₂DCF-DA staining methods following the protocol described above.

5.3.8 Statistical analyses

Two-way analysis of variance (ANOVA) and the least significant differences (LSD) at $P < 0.05$ were used to compare the values of the relative transcript abundance, VB6 concentrations and fungal biomass between the treatments and the controls using the SPSS statistical package v. 22.0, (IBM Corp., Armonk, NY, USA). Transcript changes were deemed statistically and biologically significant if $P < 0.05$ and fold changes were $> +1.5$ or > -1.5 .

In order to understand the simultaneous relationships among the relative abundance of host and fungal-derived antioxidant genes, VB6 concentrations and fungal biomass, and to predict the effect and impact of a change in one variable will have on other variables, a multivariate analysis of the data was performed on potato-*R. solani* and Arabidopsis-*R. solani* interactions (**Figure 5.1**) using the SIMAC-P+v12.0 (Umetrics, MKS Instruments Inc., Andover, MA, USA). The data matrix for potato-*R. solani* consisted of biological replicates of control fungal tissues, mock-inoculated control plant tissues, plant tissues of zone 1, and plant tissue of zone 2 (columns; X variables), and the variables for relative transcript abundance of antioxidant genes, VB6 concentration, fungal biomass (rows; Y variables). Data were obtained from the analysis of relative transcript abundance, PN concentrations, and fungal biomass for potato sprouts-*R. solani* AG3 (10 antioxidant genes) [21 rows x 12 columns]. The data matrix for Arabidopsis-*R. solani* consisted of biological replicates of control fungal tissues, mock-inoculated control plant tissues, plant tissues with necrotic lesions (columns; X variables), and the variables for relative transcript abundance of antioxidant genes, VB6 concentrations, fungal biomass (rows; Y variables). Data were obtained from the analysis of 11 antioxidant genes (11 antioxidant genes) [15 rows x 13 columns].

The principal component analysis (PCA) was performed to determine which of the genes were most affected and check if they were correlated with VB6 concentration and fungal biomass in each interaction. Therefore, PCA loading coefficient plots (coupled with p values) and loading biplots for the effect of VB6 concentration, fungal biomass, and the relative transcript abundance of genes in each tissue type were built for the visualization of correlations between the X and Y variables using the pc (corr) (i.e., correlation scaled loadings of the correlation between Y variables

and X scores based on the variable importance of X as scores and loadings) setting in SIMCA-P+. Mean-centering and UV scaling were used for data normalization.

5.4 Results

5.4.1 Distinct patterns of VB6 gene expression, differential PN concentration, and fungal biomass among potato cultivars and *Arabidopsis* lines

To explore sources of variance within transcript levels, VB6 concentrations (measured as vitamer PN) and fungal biomass, we used principal component analysis (PCA). The analysis revealed tight groups with no outliers ($P < 0.05$) (**Appendix IV**). Briefly, the principal components (PCs) which are the PCA axes are identified (**Figure 2 A, C, D, E**), and they describe sources of variation that are uncorrelated to each other. These uncorrelated axes are linear combinations of the original variables and can be used to reveal sources of variance in the data set. The extent of correlation between the expression levels of a gene, VB6 concentration and fungal biomass and a PC is indicated by the magnitude of their loadings as shown by the value on the PC axes (**Figure 5.2, Appendix VI, and Appendix VII**). This approach identifies genes, VB6 concentration and fungal biomass having expression levels that are highly positively or negatively correlated with a given PC.

The strength of the correlations is pathosystem-dependent (**Figure 5.2**). In potato-*R. solani* AG3, PC1 and PC2 explained the majority of the variance in the dataset with tight grouping that led to the separation between the treatments (i.e., infected and control). Also, selected antioxidant genes were highly associated with the interaction zone as revealed by the PCA-loading biplot of the potato-*R. solani* AG3 interaction (**Figure 5.2A**). In *Arabidopsis* –*R. solani* interaction, only PC2 and PC3 but not PC1 explained most of the variance in the dataset (**Figure 5.2C-E**).

Interestingly, in both plant-fungus interactions, the PCA analysis showed that both fungal biomass and VB6 concentration contributed significantly to disease development (**Figure 5.2B, F, Appendix VI, and Appendix VII**).

5.4.2 Accumulation of ROS in *R. solani* mycelia and potato plant tissues is indicative of oxidative stress.

The presence of ROS in the fungal hyphae and plant tissues was detected by fluorescent H₂DCF-DA and non-fluorescent DAB methods in Potato-*R. solani*. Tissues of mock-inoculated control cv. Russet Burbank and Chieftain sprouts (**Figure 5.3B-C, and H-I**), did not display any green fluorescence. An intense green fluorescence was detected in the tissues of infected Russet Burbank and Chieftain sprouts (**Figure 5.3E-F, and K-L**). To demonstrate whether the generation of ROS was induced by *R. solani* infection, potato tissues were assayed *in situ* for the production of H₂O₂ in response to infection, using DAB staining. Tissues of infected potato cv. Russet Burbank sprouts displayed stronger brown precipitate pattern when compared to potato cv. Chieftain perhaps at necrotic and surrounding areas (**Figure 5.3N, and P**, respectively). However, control uninfected tissues did not show any browning (**Figure 5.3M, and O**, respectively).

5.4.3 Potato cv. Russet Burbank showed more susceptibility to *R. solani*

Analysis of the transcript for the *R. solani* G3PDH gene as an indicative of fungal growth rate *in planta* confirmed that potato cv. Russet Burbank plants were more susceptible to *R. solani* infections than the moderately tolerant potato cv. Chieftain. Potato cv. Russet Burbank displayed a statistically higher copy number of fungal gDNA compared to the moderately tolerant cv. Chieftain by 2.924 folds (**Figure 5.4A**), indicating that fungal biomass of the virulent strain 114

was closely correlated with stolon interaction, high loading coefficient values of fungal mass were observed (**Appendix VI, Appendix VII, Figure 5.2B, and F**)

5.4.4 Enhanced VB6 content has only limited impact on stress performance in potato cultivars

VB6 analysis revealed strong recovery rates of 98.5 ± 1.5 % for pyridoxine (PN). The method of detection limit was $0.124 \mu\text{g/g}$ with a matrix effect of $102.41 \pm 7.4\%$. Vitamin B6 (PN) content in plant tissues before and after inoculation with *R. solani* AG3 were linked with potato tissues of both cultivars based on loading coefficient values (**Appendix VI, Figure 5.2A, E**). Without pathogen inoculation, the constitutive levels of VB6 were significantly ($P < 0.05$) higher in the susceptible potato cv. Russet Burbank (1.6-fold) compared to the tolerant potato cv. Chieftain. After infection, VB6 content in infected potato tissues increased by 1.7 fold ($P < 0.05$) in potato cv. Chieftain, whereas in potato cv. Russet Burbank, the VB6 content slightly increased by 1.4-fold change (**Figure 5.4B**).

5.4.5 The potato-*R. solani* interaction zones show significant shifts of potato-derived VB6 and GST antioxidant genes and may have an impact on disease resistance of potato against *R. solani*

The transcript abundance of potato antioxidant genes, *STGST* (p, 65%), and the VB6 *de novo* *STPDX1.1* (p, 63%) were positively correlated with potato cultivar and with the interaction zone sampled (i.e. zone 1 vs zone2). In the susceptible potato cv. Russet Burbank, a significant fold increase in relative transcript abundance of *STPDX1.1* (1.97 fold) and *STGST* (4.68 fold) was observed in zone 2. In the tolerant potato cv. Chieftain, an opposite trend was observed. The

surrounding tissues in zone 1 significantly expressed fold increase of *STPDX1.1* (1.62 fold) and *STGST* (7.86 fold) (**Figure 5.5**). Gene expression of *STPDX2* was tightly linked to potato cultivar. A strong activation of *STPDX2* in tissues of zone 2 of potato cv. Russet Burbank with a 3.34-fold increase, and a downregulation in zone 1 and zone 2 in potato cv. Chieftain (-2.94 and -3.35-fold decrease, respectively). Despite the low loading value (p, 27%), the VB6 salvage pathways *STPLR* showed a significant upregulation of the relative transcript abundance in zones 1 and 2 in both potato cultivars, but with higher levels in potato cv. Chieftain (**Figure 5.5**). *STPDX1.2*, the non-catalytic homolog, showed a biologically significant down-regulation in both cultivars (up to a maximum of -3.01-fold decrease in cv. Russet Burbank) except in potato cv. Chieftain, the relative transcript abundance in zone 1 remained similar to the control tissues (1.14 fold) (**Figure 5.5**).

5.4.6 Expression of fungal –derived VB6 biosynthetic and *GST* antioxidant genes are not correlated with basal resistance of potato cultivars but are tissue zone-driven

A notable upregulation of the fungal-derived genes encoding the vitamin B6 *de novo* and salvage pathway genes, and *GST* was observed during disease development of *R. solani* AG3 on sprouts of cv. Russet Burkank and Chieftain potato cultivars. Fungal genes with high loadings for PC1 were noted for *RsolAG3PDX1*, *RsolAG3PDX2*, *RsolAG3PLR* and *RsolAG3GST* (**Appendix VI**) and were abundantly expressed and tightly linked with interaction zone 1 (i.e., tissues of surrounding areas of necrotic lesions of both potato cultivars). (**Figure 5.2A, B**). Significant fold increase of transcript abundance of these genes ranged from a minimum of 3.60 for *RsolSG3GST*) to a maximum of 13.91 for *RsolAG3PDX2*) (**Figure 5.5**).

5.4.7 Confirmation of Arabidopsis null mutants and presence of ROS during the interaction with *R. solani*

To explore the possibility of the role of VB6 in plant disease resistance, we have compared the expression of fungal-derived and plant-derived genes of Arabidopsis wild type Col-0 and *pdx1.3* mutants. Homozygous mutant plants were identified by genotyping with gene-specific primers flanking the insertion sites (**Appendix III**). RT-PCR analysis revealed that no *ATPDX1.3* transcripts were detected in the *pdx1.3* mutant plants (**Figure 5.8**). These data indicate that the *pdx1.3* are null mutants of the *ATPDX1.3* genes. These homozygous mutant lines were used for the further experiments as described below.

Tissues of mock-inoculated control Arabidopsis wild type Col-0 and *pdx1.3* mutant lower stems (**Figure 5.6 B-C, and H-I**) did not display any green fluorescence. An intense green fluorescence was detected in the tissues of Arabidopsis wild type and *pdx1.3* mutant lower stems, (**Figure 5.6 E-F, and K-L**) which supports the presence of oxidative stress during the interaction.

5.4.8 The *pdx1.3* Arabidopsis mutants showed more susceptibility to *R. solani* coincide with lower amounts of VB6

Arabidopsis *pdx1.3* plants were more susceptible to *R. solani* infections than the Arabidopsis wild type Col-0. The stems of the *pdx1.3* mutant Arabidopsis lines had significantly higher gDNA copy number of strain A76 than that in the wild type plants by 1.80 folds (**Figure 5.7A**). Prior to infection, the constitutive VB6 content in Arabidopsis wild type was significantly higher ($P < 0.05$) by 3.31fold compared to *pdx1.3* mutant line. After infection a drop in the VB6 levels in infected tissues of Arabidopsis wild type (1.6-fold decrease) was observed and no change occurred in the *pdx1.3* mutant line (**Figure 5.7B**).

5.4.9 Arabidopsis vitamin B6 genes and *ATGST* mark a potentially adequate defence response during disease development

The VB6 biosynthesis gene *ATPDX1.3* is tightly linked with disease development based on coefficient loading values (**Appendix VII, and 5.2F**). After infection, levels of *PDX1.3* transcripts were similar to those in the control of the wild type. No transcripts were detected in the *pdx1.3* mutant lines. As expected, higher transcript levels of *ATPDX1.1* and *ATPDX1.2* were found in both control and infected tissues of the *pdx1.3* mutant lines compared to the wild type with 5.68-fold and 6.52-fold increases, respectively (**Figure 5.8**). Interestingly, the relative transcript abundance for *ATPDX1.2* in the wild type infected tissues showed a significant 4.33-fold decrease in comparison to the control (**Figure 5.8**). The plant-derived VB6 salvage pathway gene *ATPLR* and the antioxidant *ATGST* gene had a significant role in Arabidopsis –*R. solani* AG4 interaction as estimated by the strong loading coefficient value (**Appendix VII**). Expression profile patterns of *ATPLR* were down regulated while expression of *ATGST* was induced in both the wild type and the *pdx1.3* mutants. Fungal-derived vitamin B6 genes and *GST* were suppressed during the disease development of *R. solani* AG4 on Arabidopsis. Only *RsolAG4PDX1* was detected in the plant-infected tissues of both the wild type and the *pdx1.3* mutant (**Figure 5.8**).

5.5 Discussion

During disease development in plants, both the host and pathogen deploy a tight control and regulation of ROS production where the latter can function as a signaling factor leading to more fungal spread or suppression by the host defense machinery (Barna, Fodor et al. 2012, Foley,

Kidd et al. 2016). This is reflected by unique differential expression of the antioxidant genes including VB6 for different pathosystems. In this study, we compared the differential co-expression patterns of fungal and host VB6 and *GST* genes across the two potato cultivars.

This research further supports our previous findings (Samsatly et al. 2018) that both the pathogen and the host employ unique expression strategies in the necrotic lesion and in the surrounding tissue. The co-expression and accumulation of plant and pathogen vitamin B6 and other antioxidant genes in potato cultivars might be critical for ROS management and disease development and pathogenicity. To further study the factors governing the tight balance of ROS and the role of VB6, we investigated in this current work whether different potato cultivars containing variable amounts of vitamin B6 (Mooney, Chen et al. 2013) exhibit differential degrees of susceptibility to *R. solani* AG3. The experimental set-up described in this study provides further support to our previous findings (Samsatly et al. 2018) that both the pathogen and the host employ unique expression strategies of VB6 and *GST* antioxidant genes in the bordering tissue (zone 1) and in the necrotic tissue (zone 2). The use of multivariate analysis enabled us to draw meaningful biological associations of gene expression of VB6 biosynthetic pathways, *GST* antioxidant genes, VB6 content, and fungal biomass with potato genotypes exhibiting different degrees of susceptibility to *R. solani*. Our assumption that potato genotypes having different susceptibility background to *R. solani* would express unique VB6 and *GST* antioxidant genes profiles is established.

In the susceptible potato cv. Russett Butbank, the *de novo* VB6 biosynthetic genes *STPD1.1*, *STPD2*, *STGST* and the VB6 salvage gene *STPLR* are upregulated in necrotic tissues (zone 2) in a manner consistent with increasing levels of VB6 mounting a pathogen response, but maintaining

low expression levels in tissues bordering defence (zone 1). In sprouts of the tolerant potato cv. Chieftain infected with *R. solani*, an opposite transcript profile was established. A sharp increase in transcript abundance of these genes except for *STPD_X2* in the bordering tissue (zone1) was detected with increasing levels of VB6 content. This fine-tuning expression strategy of VB6 and *GST* genes, is most likely that the tolerant potato plants must sustain ROS levels at the site of infection to defend against pathogens and to stimulate the defense response, yet keep ROS levels lower in regions (zone 1) not affected by the pathogen to maintain tissue integrity and viability. In potato, all *STPD_X* genes are expressed with the expression of *STPD_X1.2* highest in sink areas such as tubers, roots and stolons (Mooney, Chen et al. 2013). The role of the catalytic homolog *STPD_X1.1* in response to biotic and biotic stress and its involvement during disease development is established (Moccand, Boycheva et al. 2014, Samsatly, Copley et al. 2018). It is noteworthy that the non-catalytic *STPD_X1.2* is the most sensitive of *STPD_X*s to biotic stress in this study. We show that the expression of the *STPD_X1.2* is strongly downregulated in sprouts under biotic stress in both infected potato genotypes compared to the controls (this study; *Samsatly et al. 2018*), whereas its catalytic homologs are not. *Our results do not support the positive regulatory role of STPD_X 1.2 with STPD_X 2 (Dell'Aglio, Boycheva et al. 2017) as established in certain crops (Zhang, Liu et al. 2014), but likely supports the negative regulatory role of STPD_X 1.2 with STPD_X 2 in the tolerant potato genotype to R. solani.* The differential regulation of VB6 could also involve cross-talk between *de novo* and the salvage gene pathway. This may imply that the salvage pathway may compensate for the absence of *STPD_X2* with corresponding high expression levels of the *STPLR* in the tolerant potato cv. Chieftain. It is probable that the requirement of the salvage pathway for disease resistance in potato is essential. At this point, we cannot fully account for the observed downregulation of *STPD_X1.2* and *STPD_X2*. An interesting finding that may be worth

pursuing in the future is to understand how this diminution affects the regulation of VB6 biosynthesis.

In an attempt to understand how *R. solani* can regulate the levels of ROS for developmental and virulence purposes, we have also compared the regulation of the fungal-derived VB6 genes of the biosynthetic pathways (*de novo* and salvage) and the antioxidant *GST* in cv. Russet Burbank and Chieftain potato cultivars. Contrary to their counterpart in the plant, the activation of all VB6 encoding genes and *GST* was similar and comparable between both cultivars indicating they are essential for fungal pathogenicity. It is noteworthy that the escalated expression of all the genes were detected exclusively in areas surrounding the necrotic lesions (zone 1). This corroborates previous results of another study on gene regulation of *R. solani* antioxidants such as *CAT*, *GST* and VB6 (Samsatly, Copley et al. 2018) indicating that transcript abundance of these genes are tissue-specific. The results of tissue specificity mesh well with the limited data published on the VB6 expression in different infected tobacco tissues ((Denslow, Walls et al. 2005). Upon infection of tobacco leaves with *P. syringae* pv. *phaseolicola*, levels of tobacco *PDX1* expression were higher in the area surrounding the infiltration area. This increase most likely indicates that pathogens invest more on turning their antioxidant arsenal outside the killing zone in the necrotic lesion in order to expand further.

To provide direct evidence supporting the role of potato *PDX* genes in plant biotic defense against *R. solani*, we examined the phenotypes of Arabidopsis mutants with defect in the *de novo* VB6 biosynthesis pathway against infections by *R. solani* AG4. We compared the changes of VB6 biosynthetic gene, *ATPDX1.3* and VB6 (PN) contents in the wild type and *pdx1.3* plants before and after inoculation with *R. solani* AG4 strain A76. We selected Arabidopsis mutant defective in

PDX1.3 instead of *PDX1.2* because it is more requisite than *PDX1.1* for VB6 synthesis (Tambasco-Studart, Titiz et al. 2005, Titiz, Tambasco-Studart et al. 2006), and has a role in disease resistance against *Pseudomonas syringae* pv. tomato in Arabidopsis compared to *ATPDX1.1* (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). In this study, we examined the levels of disease caused by *R. solani* and compared the *in-planta* fungal growth on the wild Col-0 and the mutant plants *pdx1.3*. Analysis of *R. solani* AG4 *PDX1* gene, indicative of *R. solani* fungal growth *in planta*, confirmed that *pdx1.3* mutant plants were more susceptible to *R. solani* infection than the wild type plants. Coined with this significant increase of *R. solani* gDNA copies in the *pdx1.3* mutant was the reduction of the VB6 vitamer PN compared to the infected wild-type. These results indicate that mutations in *PDX1.3* gene resulted in increased susceptibility to *R. solani*. Similar trends were reported in studies dealing with interaction of the necrotrophic *Botrytis* with Arabidopsis *pdx1.3* mutant lines or tomato *slpdx1.3* silenced lines that led to increased disease severity (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). As expected, silencing of *PDX1.3* but not *PDX1.2* resulted in a decreased level of VB6 in the mutant plant. This is in agreement with what has been previously published (Tambasco-Studart, Titiz et al. 2005, Wagner, Bernhardt et al. 2006). Recent studies support the antioxidant role of VB6 and that it can modulate plant defence by regulation of the antioxidant status in plants that are under biotic stress. Such regulation is distinctively expressed and depends on the crop, tissue infected and the type of the pathogen such as in the case of tobacco-*P. syringae* pv. *Phaseolicola*, tomato-*Botrytis cinerea*, and tomato-*Pseudomonas syringae* pv (pst) or Arabidopsis-*Pseudomonas syringae* pv-Arabidopsis (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). Taken together, these findings indicate that data from the molecular and biochemical analysis signify that VB6 and its *de novo* biosynthetic pathway are essential for the regulation of defence response through the modulation of cellular antioxidant capacity.

In this study, we also evaluated the expression of VB6 de novo (*PDX2*, *PDX1.2*, *PDX 1.1*) and the salvage pathway genes (*ATPLR*) and *GST* antioxidant gene in the *pdx1.3* mutant line and in the wild type before and after infection. Expression analysis of the VB6 *de novo* biosynthetic pathway genes was similar in the control and infected wild and mutant lines. It has been reported that *Arabidopsis* (*ATPDX1.2*) is a pseudoenzyme that acts as a positive regulator of vitamin B6 abundance during abiotic stress (Moccand, Boycheva et al. 2014). The drop of VB6 vitamer PN content is most likely due to the drastic down regulation of *ATPDX1.2* in the infected wild type. On the other hand, the expression of *ATPLR*, an important component in the VB6 salvage pathway, was down-regulated in both infected lines which can also explain the drop in the VB6 content. This might also mean less ability of the *Arabidopsis* lines to resist the pathogen given the role of *PLR* in detoxification of ROS during biotic and abiotic stress (Morissette, Dauch et al. 2008, Chamoun and Jabaji 2011, Gkarmiri, Finlay et al. 2015, Samsatly, Chamoun et al. 2015). It is noteworthy that *ATGST* gene was substantially overexpressed in the wild and mutant lines of *Arabidopsis* during disease development as compared to the mock-inoculated controls of both lines. The high induction of *ATGST* is likely required to scavenge excess ROS in response to *R. solani* infection and bring the generated toxic oxygen metabolites to balanced level between production and quenching (Torres 2010, Sharma, Jha et al. 2012, Das and Roychoudhury 2014).

We also evaluated the expression *R. solani* VB6 biosynthetic de novo genes (*RsolPDX1*, *RsolPDX2*), the salvage gene (*RsolPLR*) and *RsolGST*. Interestingly, most of the fungal VB6 and *GST* genes were not detected and *RsolPDX1* was significantly suppressed. This pattern of downregulation can result in an overflow of ROS in infected areas similar to what is found in other plant-necrotroph systems which appear to play an important role during infection and initiation of the oxidative burst to. For example, in response to infection with *Botrytis cinerea*, leaves of ivy

pelargonium (*Pelargonium peltatum*), showed a strong nitric oxide (NO) burst and H₂O₂ accumulation to arrest disease progression through NO-dependent reversible inhibition of catalase (Floryszak-Wieczorek, Arasimowicz et al. 2007).

PCA analysis identified vitamer PN as important contributor during disease development in the potato-*R. solani* and Arabidopsis-*R. solani* interactions. Analysis of PN in potato genotypes before and after inoculation with *R. solani* by LC-MS showed that without the pathogen, the VB6 contents in potato cv. Russet Burbank and cv. Chieftain were approximately 21.6 µg/g and 13.7 µg/g, respectively which are comparable to VB6 contents generally found in different potato genotypes (Mooney, Chen et al. 2013). On the other hand, the VB6 content in *pdx1.3* mutant line of Arabidopsis was reduced by approx. 70% lower as compared to that of the wild-type plants. This observation is in agreement that *pdx1.3* plants grown in sterile culture medium contain reduced VB6 content (Zhang, Liu et al. 2014). After infection of potato genotypes by *R. solani* AG3, the content of PN in the susceptible cv. Russet Burbank and in the moderately tolerant cv. Chieftain significantly increased by 1.4 and 1.7 folds, respectively. The highest boost of PN in cv. Chieftain as compared to cv. Russet Burbank might be a factor leading to increased resistance to *R. solani*. This and the expression of VB6 *PDX1.1*, *STPLR* and *GST* in tissues bordering the necrotic lesion is another factor that might determine the degree of susceptibility to *R. solani*. We put forward the assumption that most important factor during disease development is not dependent only how much PN can be produced, but it is dependent on the ability of each plant to maintain or boost the production of PN at the right time the right place.

After infection by *R. solani* AG4, the VB6 content in the Arabidopsis wild type was significantly reduced by 37 %. However, the VB6 content in the infected *pdx1.3* mutant line was not affected and was similar to *pdx1.3* mutant lines before infection. Our results indicate that

reduced amounts of VB6 content leads to more *Rhizoctonia* infections in *pdx1.3* mutant lines. These results along with the downregulation of PDX1 after *R. solani* infections indicate that reduced biosynthesis and reduced VB6 content most likely represents a natural defence response to certain plants such as Arabidopsis, tomato, and tobacco to pathogenic infection (Denslow, Walls et al. 2005, Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). It is not yet clear why increased active content of VB6 in potato genotypes led to more disease in the susceptible cultivar, in contrast to what has been reported for Arabidopsis and other crops merits further research. Nevertheless, the data in this study provide direct evidence on the important role of VB6 content in plant disease resistance.

It is common knowledge that ROS is generated at the site of attempted invasion through an oxidative burst during disease development (Alvarez, Pennell et al. 1998, Lehtonen, Somervuo et al. 2008, Torres 2010, Foley, Kidd et al. 2016). ROS production was also reported in different anastomosis groups of *R. solani* that are pathogens of potato, soybean, sugar beet and wheat. The induced ROS were also linked to the regulation of VB6 antioxidant genes and their collective possible role in oxidative stress alleviation (Taheri and Tarighi 2011, Foley, Kidd et al. 2016, Samsatly, Copley et al. 2018). Oxidative damage can be prevented in both the host and the pathogen when their antioxidant machinery is efficiently used (Apel and Hirt 2004, Sharma, Jha et al. 2012). Microscopic analysis of *Rhizoctonia solani* AG3 and AG4 infections on stolons and stems confirmed that *Rhizoctonia* disease development in potato sprouts and Arabidopsis provokes an oxidative stress in fungal hyphae and plant tissues and could be related to the oxidative status of the fungus as well as the plant. Our data clearly showed that ROS formation was induced in the tissues of infected cv. Russet Burbank and Chieftain sprouts, and Arabidopsis wild type and *pdx1.3* mutant lower stems. Tissues of infected potato cv. Russet Burbank sprouts displayed stronger

brown precipitate pattern when compared to Chieftain specifically in zones 1 and 2. This indicates that cv. Russet Burbank is experiencing a higher oxidative state and is less successful in quenching the H₂O₂ produced in the infected tissues.

In conclusion, this work portrays the importance of VB6 during disease development of *R. solani* over the different potato cultivars or different lines of Arabidopsis. Our study showed a distinct pattern of expression, PN concentration, and fungal biomass between the cv. Russet Burbank and Chieftain. The characterization of Arabidopsis pdx1.3 mutant line challenged with *R. solani* offers convincing evidence into the role of VB6 in maintenance and enhancement of plant tolerance against *R. solani* AG4. The results obtained suggests the VB6 role in resistance to oxidative stress resulting from biotic interaction of *R. solani* with its natural hosts. Last, the regulation and production of VB6 is under tight control and is an important determinant of disease development of *R. solani* with potato or Arabidopsis

5.6 Acknowledgements

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5.7 Author Contributions

Conception and design of the study: JS and SJ. Acquisition of data for the study: JS and SB. Analysis of data for the work: JS and SB. Interpretation of data for the work: JS and SJ. Manuscript revision and approval: JS, SB and SJ

5.8 SUPPORTING INFORMATION

Appendix II. Working conditions of Agilent 6540 Accurate-Mass QTOF

Appendix III. Genotyping for homozygous pdx1.3 mutants (SALK086418) and wild (Col-0) Arabidopsis plants using SALK gene-specific primer pairs and T-DNA primer LB1.3 along with SALK086418-RP.

Appendix IV. Principle component analysis score plots (PC1/PC2) for the effect of transcriptional abundance of antioxidant genes, PN concentrations, and fungal biomass on control, infected tissues in (A) potato-*R. solani* AG3 pathosystem (PC1/PC2), and (B, C, D) Arabidopsis-*R. solani* AG4 pathosystem (PC1/PC2, PC1/PC3, and PC2/PC3) ($P < 0.05$).

Appendix V. Diagram showing transcript abundance, vitamin B6 concentration, and fungal biomass analysis during the interaction of Arabidopsis lines or potato cultivars with *R. solani*.

Appendix VI. p values of principal component analysis loading coefficient plots for the potato-*R. solani* AG3 interaction.

Appendix VII. p values of principal component analysis loading coefficient plots for the Arabidopsis-*R. solani* AG4 interaction.

Table 5.1. List of primers used in this study. Primer names were preceded by the plant or fungus abbreviation: *ST*, *Solanum tuberosum*, *AT*, *Arabidopsis thaliana*, or *Rsol*, *R. solani*. QRT-PCR conditions were optimized for each primer set, and products were confirmed by sequencing. Internal reference genes for each plant type and fungal strain were used for normalization of relative transcript abundance levels.

Target organism	Primer	Sequence (5' → 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Arabidopsis	PDX1 (Pyridoxine biosynthesis gene)				
	At ^a -PDX1.1-F	CGAAGGAGCCATGACGGAGA	58	196	At2G38230
	At-PDX1.1-R	AGCAACACCGCCTTGAGCTCGA			
	At-PDX1.2-F	TGTTACTCTCTACAGCGGCAC	54	130	At3G16050
	At-PDX1.2-R	CTTAGCTTGGTTAACCGAGGAA			
	At-PDX1.3-F	TAACGGTGCGATAACGGAGG	53	231	AT5G01410
	At-PDX1.3-R	TGTTTGATTCTTTAATCATTTG			
Potato	ST ^b -PDX1.1-qF	CTGTGACTATTCCTGTAAT	55	83	(Mooney, Chen et al. 2013)
	ST-PDX1.1-qR	GTAATCTACTCCGATAGC			
	ST-PDX1.2-qF	TGCTCTAATCCTTACAAG	55	162	
	ST-PDX1.2-qR	GTAGGTCTCATCACTAAC			

<i>R. solani</i> AG3	5'GSP1-(PDX1)	TGACGAACAGCCTCGACAACATTTCC	55	219	(Samsatly, Chamoun et al. 2015)
	3'GSP2-(PDX1)	TCCGTTTGTCTGTGGGGCTACATCTCTC			
<i>R. solani</i> AG4	<i>Rsol^εAG4-PDX1- F</i>	ATGATCAAAGAGATCGTGGAC	53	243	AVOZ01001318.1
	<i>RsolAG4-PDX1-R</i>	GATCATTGCTGCACCTTCGGA			
<i>PDX2</i> (Pyridoxine biosynthesis gene)					
Arabidopsis	<i>At-PDX2-F</i>	ACTAACATCTCAAGAAGGTGG	53	145	AT5G60540
	<i>At-PDX2-R</i>	TTGAATTTGTACGGTGGAGCTT			
Potato	<i>ST-PDX2-qF</i>	ATTCCAATCCTGCTATTC	55	185	(Mooney, Chen et al. 2013)
	<i>ST-PDX2-qR</i>	CACAATATCAGAAGTTCCT			
<i>R. solani</i> AG3	3'GSP1-(PDX2)	AATCTTGCTTGCCTCTGGTGGTGTG	55	225	(Samsatly, Chamoun et al. 2015)
	5'GSP1-(PDX2)	ATCCCATTAATGGTCGGTCCTCATCA			
<i>R. solani</i> AG4	<i>RsolAG8-PDX2-F</i>	ATTATATCACGCGTGACACC	53	207	JATN01000314.1

RsolAG8-PDX2-R

GGCGCCTTCAACACCACCAGA

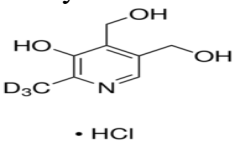
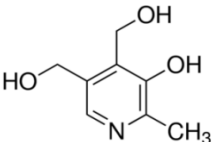
PLR (PYRIDOXAL REDUCTASE)

Arabidopsis	At -PLR-F	TGGGTCCTTTAAGTGTTTCT	53	170	AT5G53580
	At -PLR-R	TTTGGCCATTAAGCCTACCAGTG			
Potato	ST-PLR-F	GCCGCCCAAACCCGAACCCGA	58	118	XM_006358747.2
	ST-PLR-R	AAGGAGGATCTCGTTAGTGT			
R. solani AG3	PLR AKR8 F	GAAAGCCTCCTCTTGGAATCT	58	300	(Chamoun and Jabaji 2011)
	PLR AKR8 R	GGGTAAGATTGGATCGATTGGG			
R. solani AG4	RsolAG1-IA-PLR-F	AAGGGGCACTAACCGGAAAGC	58	194	AFRT01000983.1
	RsolAG1-IA-PLR-R	ATGTTGCCCGAGAGAGGAGAC			
GLUTATHIONE S-TRANSFERASES					
Arabidopsis	At -GST ^d -F	TGGCTCTCAAGCTTAAAGGCG	54	213	AT2G29450
	At -GST-R	TGTCATATGGACTTTGCGGTA			

Potato	ST-GST-F	TCCTTTTAGCCATAGAGTTGA	51	200	POTPR1A
	ST-GST-R	CTTCAAATGCCTCATCAATGC			
R. solani AG3	RsolAG3-GST-F	AGAAGACGAGGCAAATGCGA	57	256	(Samsatly, Chamoun et al. 2015)
	RsolAG3-GST-R	ATCTCTTCAACCGCCTTCCAGT			
R. solani AG4	RsolAG1-IA-GST-F	TACAAACGCATACCTTATCGC	53	217	ELU40094.1
	RsolAG1-IA-GST -R	TTGGAGCCGGGTAAGTTTGT			
Reference genes					
Arabidopsis	PP2AA3- ^e _F	TAACGTGGCCAAAATGATGC	53	60	(Libault, Thibivilliers et al. 2008)
	PP2AA3_R	GTTCTCCACAACCGCTTGGT			
	At-TIP4I ^f -F	GTGAAAACGTGTTGGAGAGAAGCAA	55	60	Sarah Muniz Nardeli 2017
	At-TIP4I-R	TCAACTGGATACCCTTTCGCA			
Potato	ST-Actin7-qF	GGCTATGTATGTTGCTAT	55	186	(Mooney, Chen et al. 2013)
	ST-Actin7-qR	ATCTTCATCAGGTTATCAG			
R. solani AG3	G3PDH [®] -F	GGTATTATTGGATACACTGA	55	129	(Chamoun and Jabaji 2011)

	<i>G3PDH -R</i>	TTAAGCCTCAGCGTCTTTCT			
<i>R. solani</i> AG4	<i>GM-RS-4^h</i>	CGGTTCATCTGCATTTACCTT	55	88	(Johanson, Turner et al. 1998)
	<i>GM-RS3-R</i>	AGTGTTATGCTTGGTTCCACT			
	<i>Genotyping primers</i>				
Arabidopsis	<i>SALK 086418-LP</i>	AGCGAACCTCTCAACCTTCTC	60	534-834	(http://signal.salk.edu/tdnaprimers.2.html)
	<i>SALK 086418-RP</i>	TGTTTTAATCTTGACCGTCCG			
	SALK LB-1.3	ATTTTGCCGATTTCGGAAC	60		
<i>a Arabidopsis thaliana</i>					
<i>b Solanum tuberosum</i>					
<i>c Rhizoctonia solani</i>					
<i>d Glutathione S-Transferase</i>					
<i>e Protein phosphatase 2A subunit A3</i>					
<i>f TIP41-like family protein</i>					
<i>g G3PDH: Glyceraldehyde-3-phosphatedehydrogenasegen</i>					
<i>h ITS1 of 5.8S rRNA</i>					

Table 5.2. Vitamin B6 vitamers used in the study and their description

Vitamer	Mw g/mol	MRM m/z	Empirical Formula	Retention time (minute)
Pyridoxine-(methyl-d3) hydrochloride  • HCl	208.66	173.10	C8D3H8NO3·HCl	2.33
Pyridoxine (PN) 	169.18	170.08	C8H11NO3	2.33

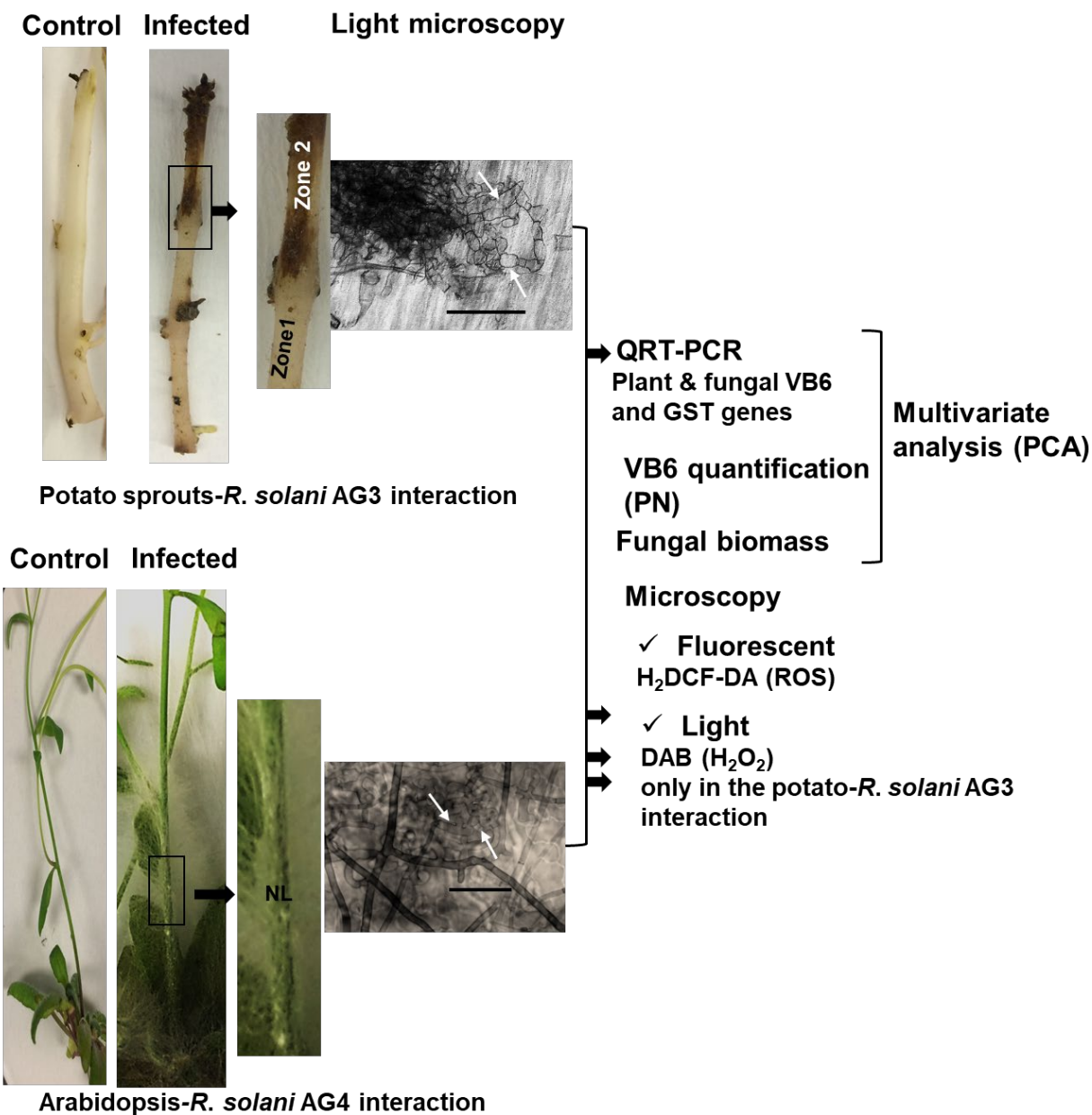


Figure 5.1. Flow chart of the experimental set up for disease development in potato sprouts-*R. solani* AG3 and Arabidopsis-*R. solani* AG4. Sprouts and Arabidopsis lower stems were sandwiched between two PDA strips of a 3-day-old *R. solani* AG3 or AG4 cultures, respectively. Sprouts or Arabidopsis lower stems sandwiched with sterile PDA strips served as controls. Samples were taken at 120 HPI (Hour post-inoculation) (Potato-*R. solani* AG3

interaction) from adjacent surrounding areas (Zone 1) and necrotic lesions (Zone 2) , and 72 HPI (Arabidopsis-*R.solani* AG4 interaction) from infected lower stems of Arabidopsis. Corresponding segments were taken from controls. Typical initials of infection cushions with mycelial mass aggregates are clearly visible under the light microscope (white arrows). Transcriptional abundance of antioxidant genes, PN concentrations, and fungal biomass from both the plant and pathogen was analyzed in zones1 and 2 (Potato-*R.solani* AG3 interaction) and lower stem areas (Arabidopsis-*R.solani* AG4 interaction) followed by multivariate analysis (PCA). The two interactions were examined for ROS accumulation using fluorescent (H₂DCF-DA) and DAB (only the potato interaction) staining methods in control and infected sprouts or Arabidopsis lower stems. Zone1: tissue surrounding necrotic lesion. Zone 2: necrotic lesion. NL: necrotic lesion. Bar = 50 μ m.

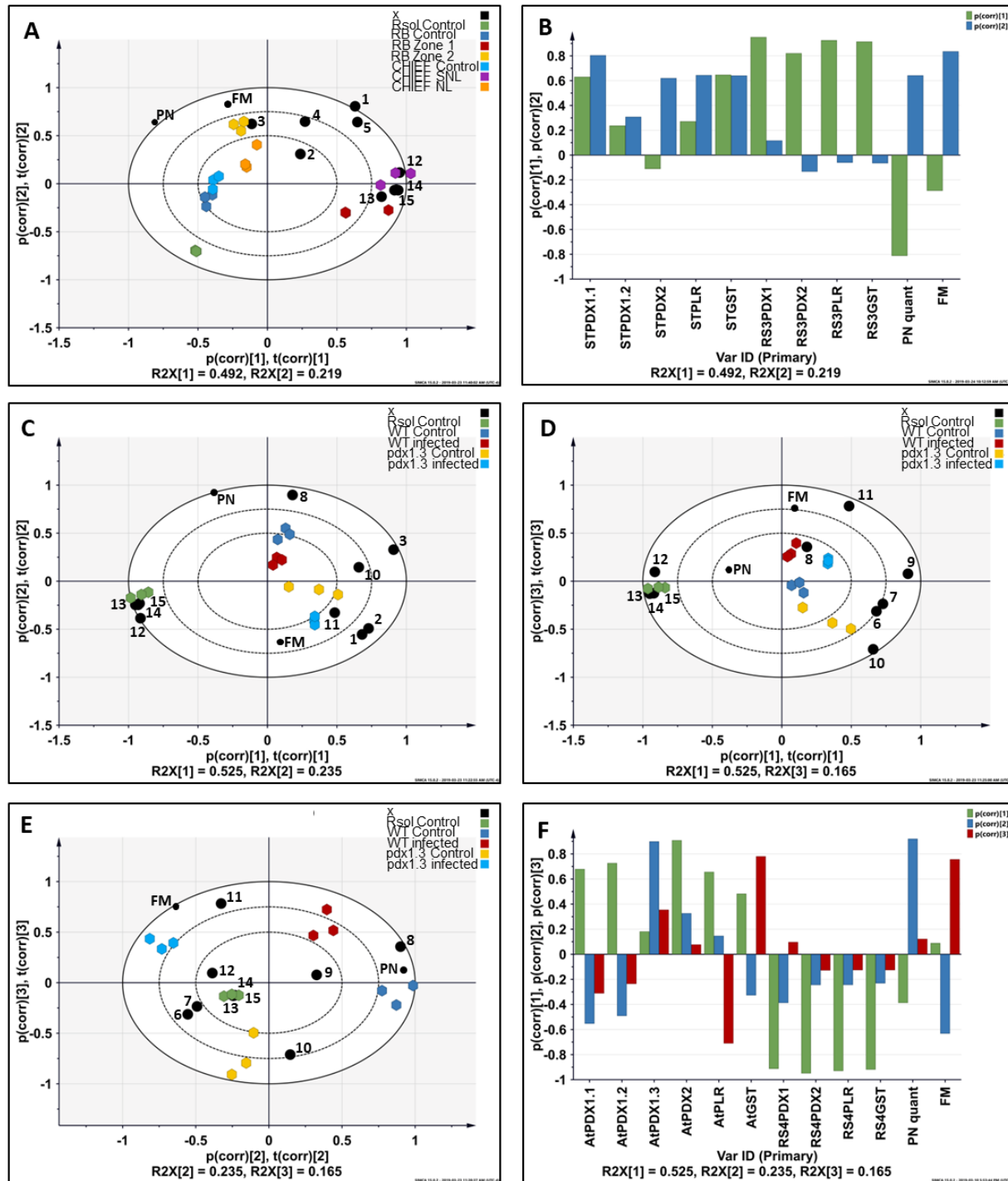
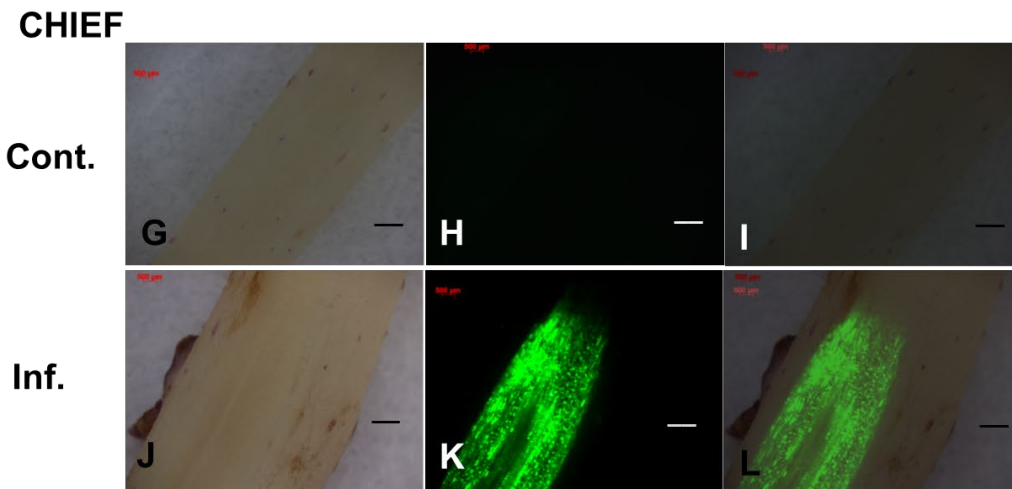
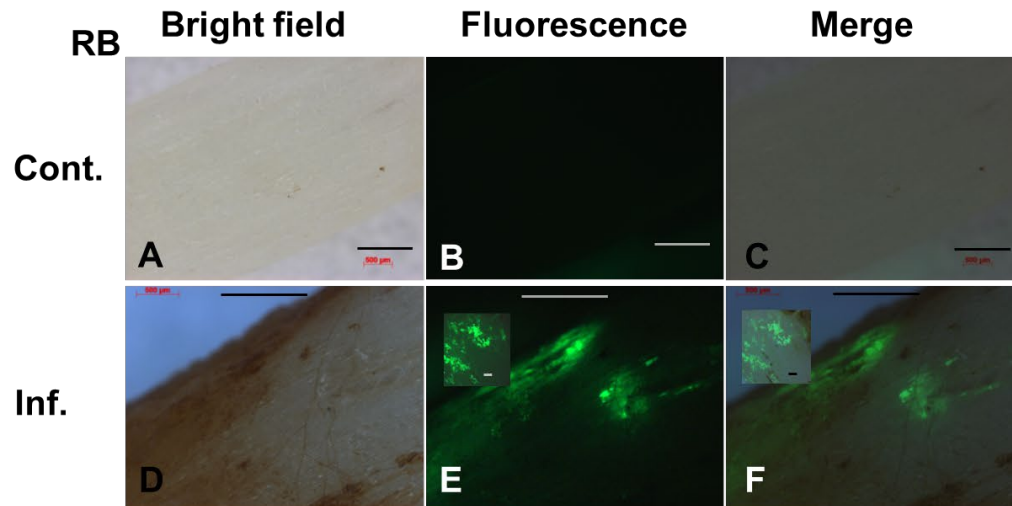


Figure 5.2. Principal component analysis (PCA) loading biplots for the effect of transcriptional abundance of antioxidant genes, PN concentrations, and fungal biomass on control, infected tissues in (A) potato sprouts-*R. solani* AG3 interaction, and (C, D, E) and Arabidopsis-*R.*

solani AG4 interaction ($P < 0.05$). Principal component analysis (PCA) loading coefficient plots for the effect of transcriptional abundance of antioxidant genes, PN concentrations, and fungal biomass on control, infected tissues in **(B)** potato sprouts-*R. solani* AG3, and **(F)** Arabidopsis-*R. solani* AG4. The scaled loading vectors pc (corr) and t (corr) are displayed for the first and the second component for potato- *R. solani* AG3 interaction and the first, the second, and the third component for Arabidopsis-*R. solani* AG4 interaction. Outer ellipses represent the Hotelling's T^2 at a 95% confidence interval. R^2X represents the fraction of the sum of squares of the two principal components. *ST*: *Solanum tuberosum*, *AT*: *Arabidopsis thaliana*, RS3: *R. Solani* AG3, RS4: *R. Solani* AG4. Black circle represents genes. 1 = *STPDX1.1*, 2 = *STPDX1.2*, 3 = *STPDX2*, 4 = *STPLR*, 5 = *STGST*, 6 = *ATPDX1.1*, 7 = *ATPDX1.2*, 8 = *ATPDX1.3*, 9 = *ATPDX2*, 10 = *ATPLR*, 11 = *ATGST*, 12 = *RsolPDX1*, 13 = *RsolPDX2*, 14 = *RsolPLR*, 15 = *RsolGST*. FM: fungal biomass. PN: vitamin B6 (pyridoxine) concentration. RB: Russet Burbank, CHIEF: Chieftain, Zone1: Tissue surrounding necrotic lesion. Zone 2: Necrotic lesion. Rsol: *R. solani* AG3 in the potato interaction and *R. solani* AG4 in the Arabidopsis interaction. WT: wild type.

Fluorescence microscopy



DAB microscopy

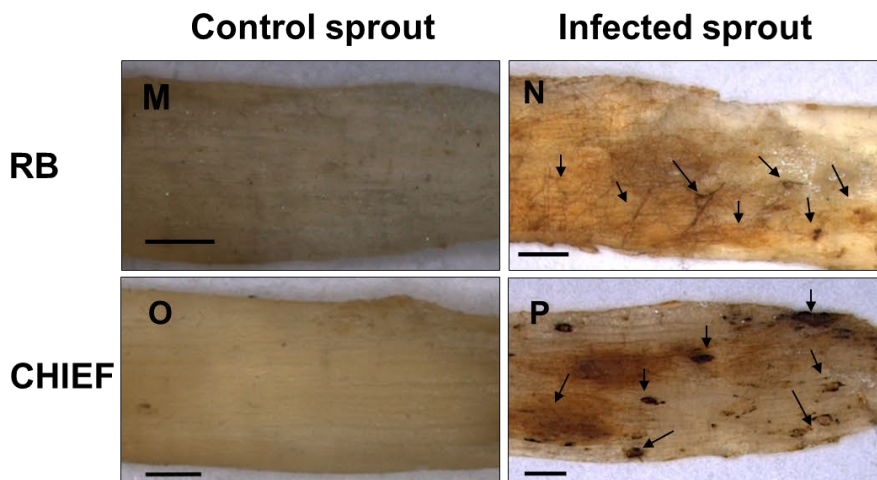


Figure 5.3. Intracellular production of ROS (A-L) and microscopic detection of H₂O₂ accumulation using DAB staining (M-P) during disease development in the potato-*R. solani* AG3 interaction. ROS production was visualized using H₂DCF-DA in control and infected potato sprouts, and Arabidopsis lower stems. (A-C, G-I) Control *S. tuberosum* sprouts, with no evidence of endogenous ROS. Infected tissues during disease development (D-F, and J-L). H₂O₂ accumulation was visualized by DAB staining in control and infected potato sprouts. Arrows indicate dark brown precipitate developed at necrotic and surrounding areas. Control potato sprouts (M, O) with no evidence of endogenous H₂O₂ at 120 HPT. *R. solani* mycelia and *S. tuberosum* sprout (N, P) with H₂O₂ accumulation at 120 HPT. RB: Russet Burbank, CHIEF: Chieftain, Cont.: control, Inf.: infected. Bar =1mm.

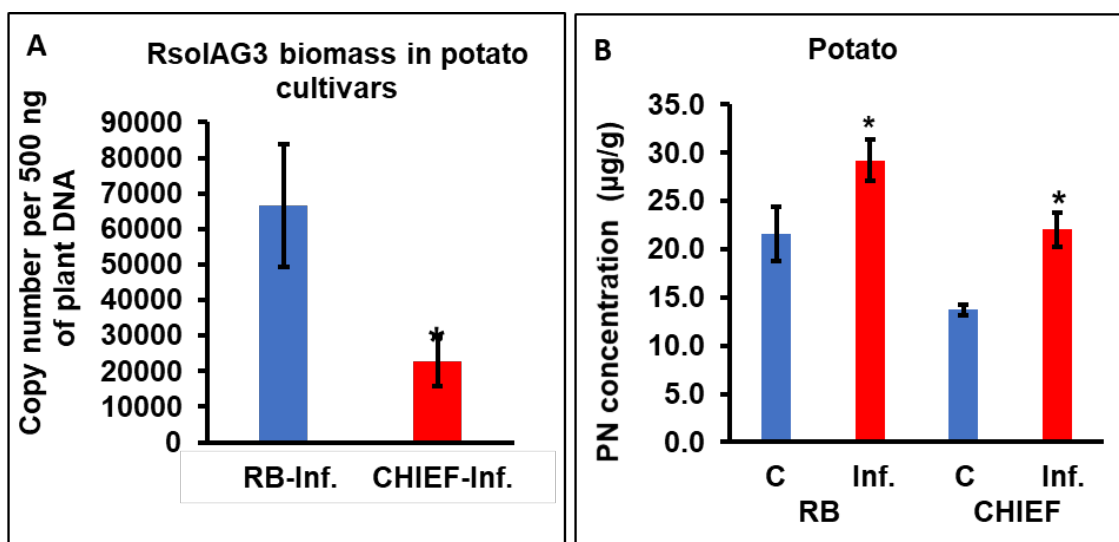


Figure 5.4. Quantification of fungal gDNA (A) and concentration (µg/g) of Pyridoxine (PN) (B) in two cultivars of infected potato sprouts, RB and CHIEF. Fungal gDNA was measured using real-time quantitative PCR from 500ng of plant samples. * indicate significant relative transcript abundance ratios between the control and the infected tissues using least significant difference (LSD) test ($P < 0.05$). RB: Russet Burbank, CHIEF, C: control, Inf.: infected.

Relative transcript abundance

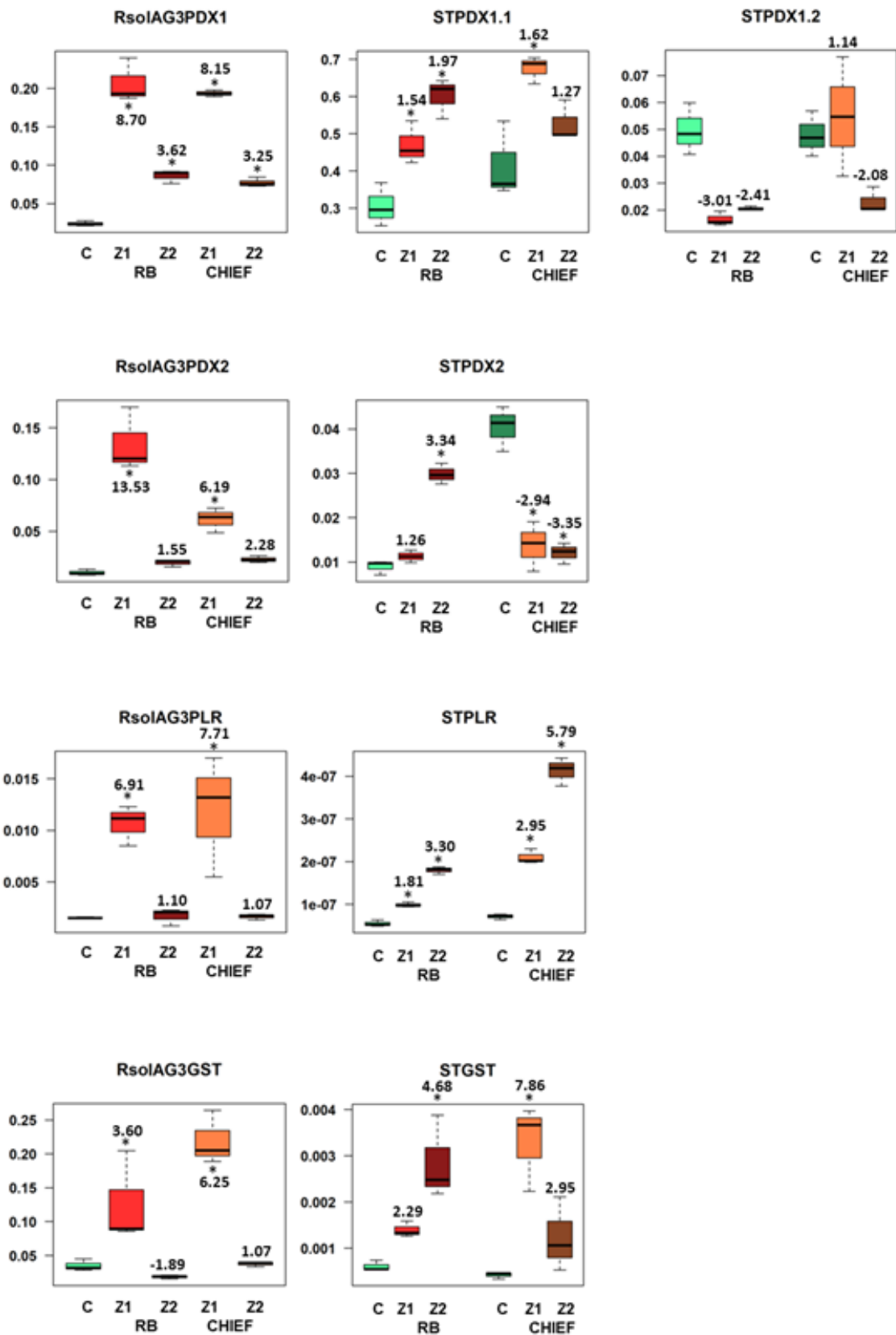


Figure 5.5. Relative transcript abundance of antioxidants expressed in potato-*R. solani* AG3 interaction. Vitamin B6 (*STPDX1*, *STPDX2*, and *STPLR*), and *STGST* genes of the pathogen *R. solani* AG3 (*RsolAG3*) and the host, *Solanum tuberosum* (ST) in control and in infected and surrounding tissues at 120 HPI during *R. solani* AG3 and potato sprouts interaction. C: control; *R. solani* AG3 grown alone or potato sprouts inoculated with sterile with PDA. Zone1: tissue surrounding necrotic lesion. Zone 2: necrotic lesion. RB: Russet Burbank. CHIEF: Chieftain. Asterisks indicate significant relative transcript abundance ratios between the control and interaction using least significant difference (LSD) test ($P < 0.05$). Fold change is calculated in relation to the control at 120 HPI. *PDX1* and *PDX2*: *Pyridoxine biosynthesis* genes, *PLR*: *Pyridoxal Reductase*, *GST*: *Glutathione S-Transferase*.

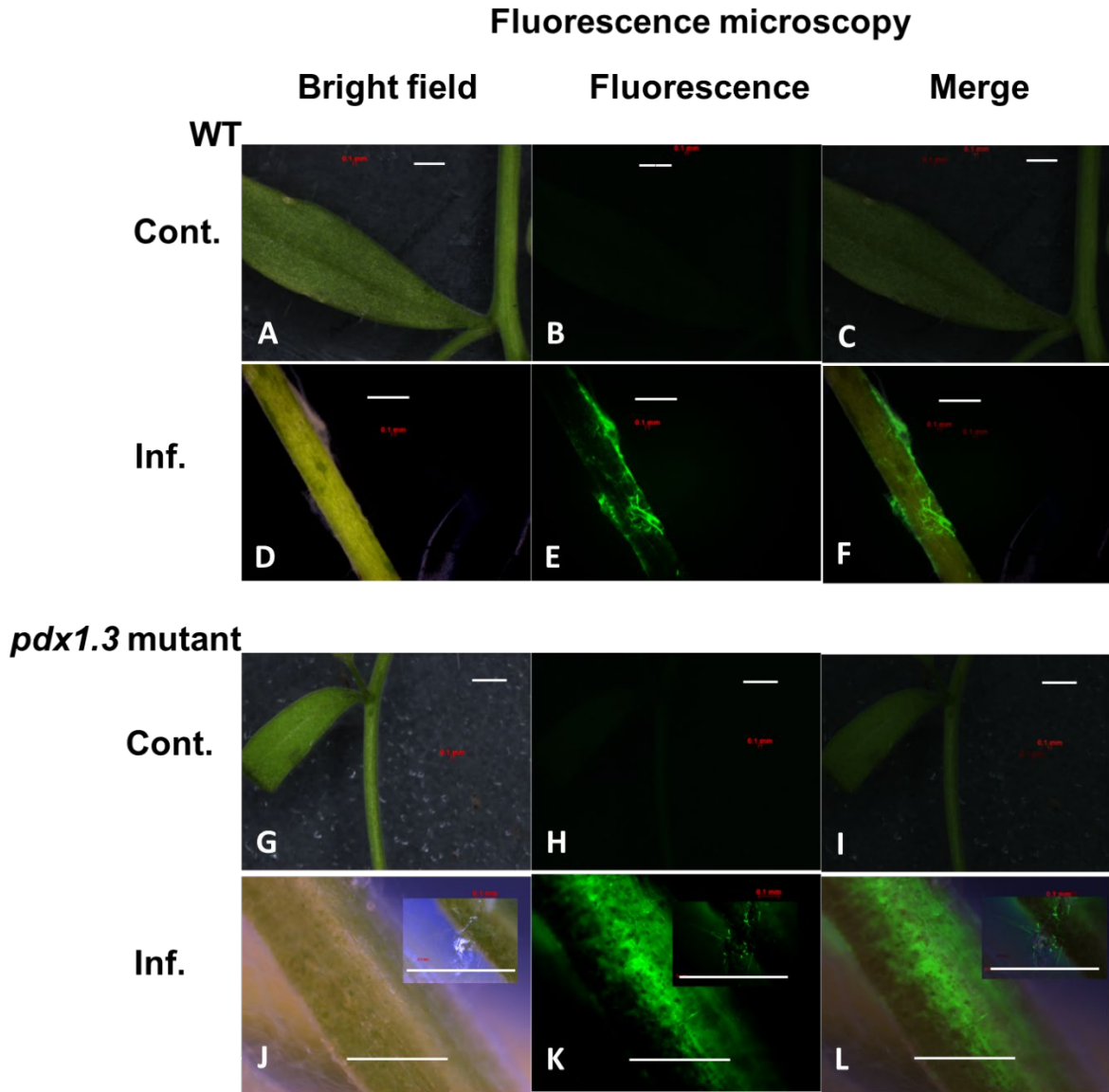


Figure 5.6. Intracellular production of ROS during disease development in the *Arabidopsis-R. solani* AG4 interaction. ROS production as visualized using H₂DCF-DA in control and infected *Arabidopsis* lower stems. *A. thaliana* lower stems with no evidence of endogenous ROS (A-C, G-I). Infected tissues during disease development in *Arabidopsis-R. solani* AG4 interaction (D-F, J-L). RB: Cont.: control, Inf.: infected, Col-o or WT: wild type. Bar = 1 mm.

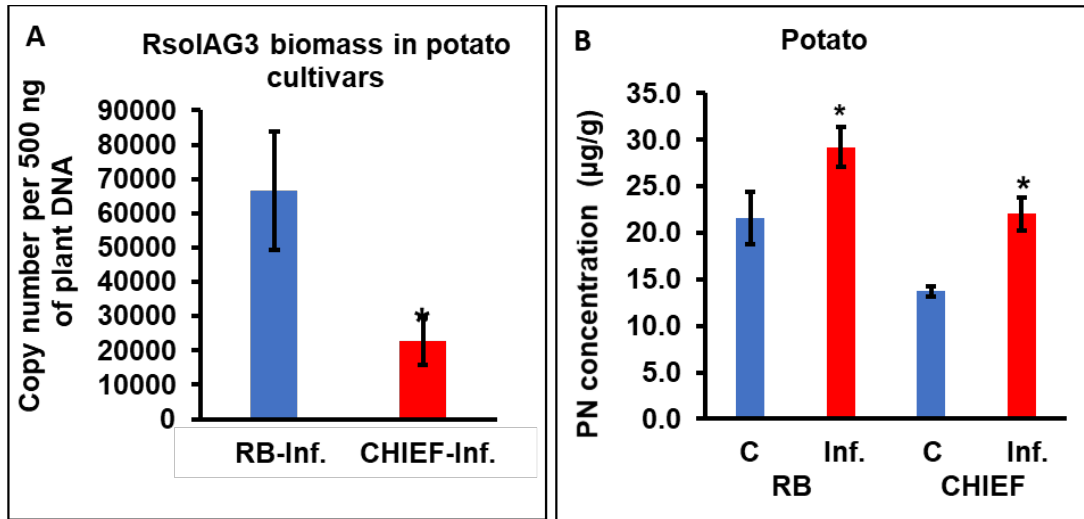


Figure 5.7. Quantification of fungal gDNA (A) and concentration (µg/g) of Pyridoxine (PN) (B) in two in tissues of two Arabidopsis lines (WT: wild type, pdx1.3 mutant) during disease development of *R. solani* AG4 on Arabidopsis, 72 Hour post infection (HPI). Fungal gDNA was measured using real-time quantitative PCR from 500ng of plant samples. * indicates significant difference between control and infected tissues at $P < 0.05$. WT: wild type, C: control, Inf.: infected.

Relative transcript abundance

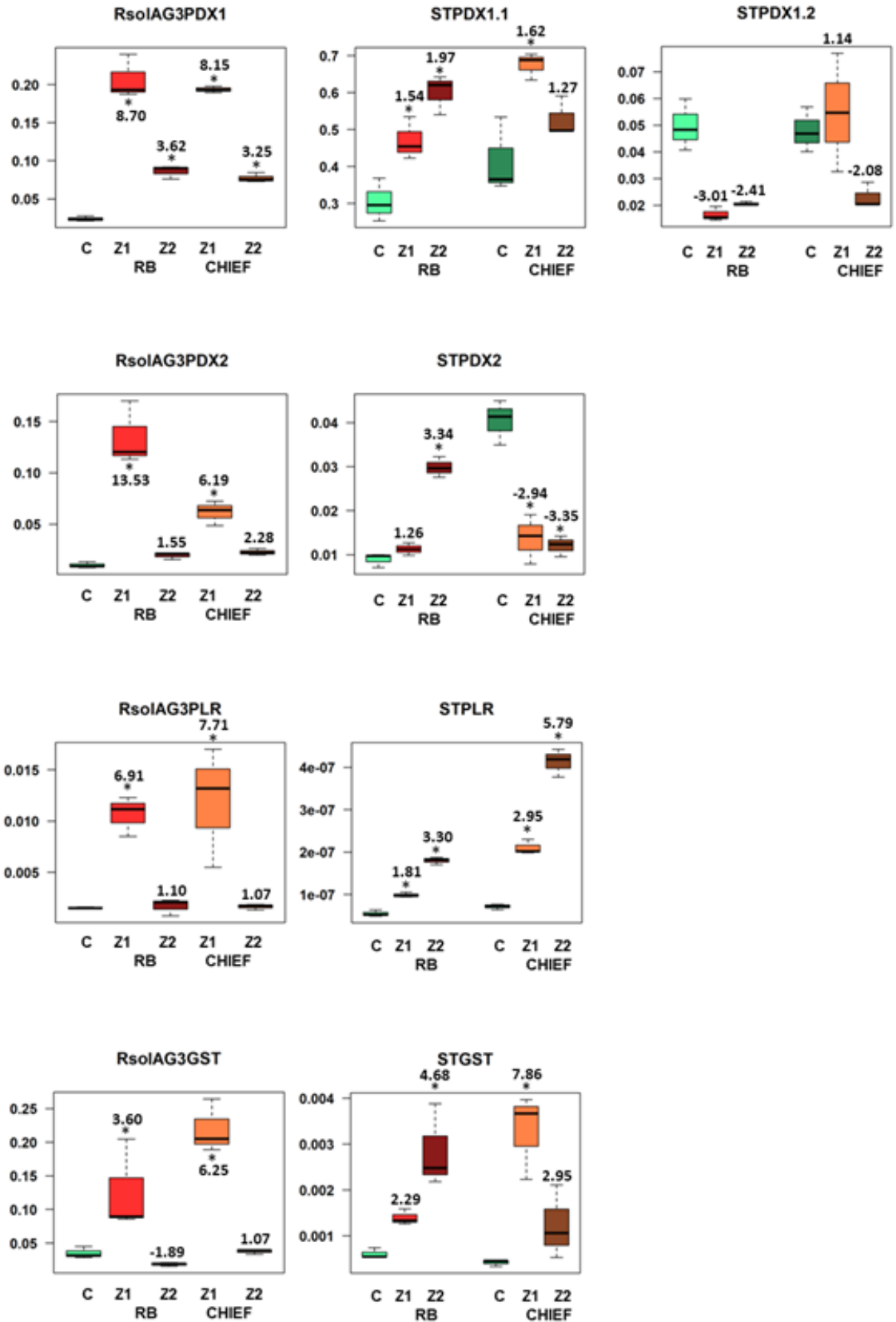


Figure 5.8. Relative transcript abundance of antioxidants in Arabidopsis-*R. solani* AG4 interaction. Vitamin B6 (*PDX1*, *PDX2*, and *PLR*), and *GST* genes of both the pathogen *R. solani* AG4 (*RsolAG4*) and the host, *Arabidopsis thaliana*, (*AT*) in control and infected tissues at 72 HPI during *R. solani* AG4 and Arabidopsis lower stems interaction. C: control; *R. solani* AG4 grown alone or *Arabidopsis* lower stems inoculated with sterile PDA. Inf.: infected. WT: wild type. Asterisks indicate significant relative transcript abundance ratios between the control and the interaction using least significant difference (LSD) test ($P < 0.05$). Fold change is calculated in relation to the control at 72 HPI. *PDX1* and *PDX2*: Pyridoxine biosynthesis genes, *PLR*: *Pyridoxal Reductase*, *GST*: *Glutathione S-Transferase*.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

This dissertation was written with the following broad objective in mind; to investigate the role of vitamin B6 in pathogenic fungi and in plants as an antioxidant in response to stress. Early in this study, we concentrated our efforts to understand the antioxidant role of VB6 in the plant pathogen *Rhizoctonia solani* as little is known about the possible function of the vitamin B6 pathways in this necrotrophic plant pathogen. Using molecular biology approaches, the *de-novo* biosynthetic pathway genes; *RsolPDX1* and *RsolPDX2* and the salvage biosynthetic pathway gene, *RsolPLR* were sequenced and characterized. This represents the first report on the presence of Vitamin B6 in *R. solani* and is the second report about the active role as an ROS quencher of VB6 in a plant pathogen. The characterization of VB6 biosynthetic genes allowed us to study their regulation when *R. solani* is under different types of reactive oxygen species (ROS) stressors such as paraquat and H₂O₂. Depending on the VB6 gene and the type of the stressor, transcript levels of the VB6 genes substantially increased with some as high as 33-fold. These results implied that accumulation of ROS in *R. solani* mycelia was linked to transcriptional regulation of the three genes and implicated the vitamin B6 biosynthesis machinery in *R. solani*, similar to well-known *catalases* and *GST*, as an antioxidant stress protector against oxidative stress. However, the direct participation of these genes in oxidative stress response in *R. solani* was established by yeast complementation assays.

Gene expression studies focusing on oxidative stress response in necrotrophic fungi and their hosts are limited to few organisms like *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Segal and Wilson 2018), and since the ROS produced from oxidative burst is a fine-tuned process and is

critical to necrotrophic pathogens, we compared co-expression analysis of genes encoding vitamin B6, *CAT* and *GST* in plant and fungal tissues of three pathosystems during disease development by different AG groups of *R. solani* on their respective hosts. Our findings presented new evidence on the co-expression of VB6 genes along with other well-known antioxidant genes (i.e., *CAT* and *GST*) in both the plant and pathogen side during disease development in each of the three *R. solani* pathosystems: potato sprout-*R. solani* AG3; soybean hypocotyl-*R. solani* AG4 and soybean leaves-*R. solani* AG1-IA interactions. A unique transcriptional pattern of the genes was expressed depending on the plant host and pathogen. Additionally, a coordinated shift in gene expression in necrotic tissue and in their surrounding tissues was observed. These results most likely imply that the host and the pathogen modulate the spatial expression of these genes for their own advantage leading to either disease control or pathogenicity.

Finally, we profiled VB6 gene expression and VB6 vitamer content at early stages of infection after exposure in the susceptible potato cv Russet Burbank and the tolerant potato cv Chieftain sprouts. Our study showed a distinct pattern of expression, PN concentration, and fungal biomass between the susceptible and tolerant potato genotype. *R. solani* AG3 infection of potato sprouts in both genotypes significantly activates the fungal and plant VB6 and *GST* genes in a tissue specific response. The susceptibility of potato cv Russet Burbank to *R. solani* was linked to reduced amounts of VB6 content. The functional characterization of VB6 *de novo* biosynthetic gene, PDX1.3 was examined by studying phenotypes of Arabidopsis mutant *pdx1.3* challenged with *R. solani*, and compared their VB6 content, provided evidence that mutations in PDX1.3 led to increased disease, providing compelling evidence into the role of VB6 *de novo* biosynthetic genes in the maintenance and enhancement of plant tolerance against fungal pathogens suggesting the role of VB6 in plant resistance.

Overall, this research offers novel insights into the biological correlation and identification of ROS antioxidant genes that can be used in soybean and potato breeding programs for resistance to *R. solani*.

6.2 FUTURE DIRECTIONS

6.2.1 GLOBAL OMICS STUDY OF THE TISSUE SPECIFIC RESPONSE IN POTATO

It is essential to perform transcriptomic and metabolomic analyses of the tissue specific response that was reported in the different varieties of potato at the expression level. Quantification of all VB6 vitamers (PN, PL, PM, and PLP) concentrations in the necrotic lesions and the surrounding tissues would allow to further understand the actual role of each vitamer in the dynamics of disease development and plant defense mechanisms. Global omics studies of these two types of tissues would provide us with in-depth insights into the molecular responses of potato during its interactions with *R. solani*.

6.2.2 TARGETING OF POTATO OR SOYBEAN VITAMIN B6 PATHWAY FOR ENHANCED RESISTANCE

Our studies have shown the importance of vitamin B6 as antioxidant during disease development of *R. solani* on potato and soybean and its implication in resistance. Mutations in the Arabidopsis PDX1.2 and PDX1.3 genes involved in the de novo pathway or Virus-induced gene silencing mediated knockdown of the tomato equivalents ended up in increased levels of diseases caused by *B. cinerea* and decreased levels of VB6 (Zhang, Liu et al. 2014, Zhang, Jin et al. 2015). Similar approach should be adopted in potato and soybean using CRISPR (Cong et al., 2013) in order to develop knock-outs or over-expressing lines. These will be used to validate the role of

vitamin B6 genes in defense. Over-expressing lines are also a sound strategy to adopt. Over expression of the *PDX2* gene in potato led to enhanced resistance to abiotic stresses (Bagri, Upadhyaya et al. 2018). Similar strategy should be followed with soybean and studies should focus on the implications on disease resistance.

6.2.3 TARGETED CONTROL MEASURES AGAINST *R. SOLANI*

The use of CRISPR/Cas is now validated in filamentous fungi (Nodvig, Nielsen et al. 2015) and therefore this approach of targeted gene manipulation should be used to develop vitamin B6 knock-outs or over-expressing lines in *R. solani*. This will provide further insight into their role in pathogenicity and during disease development in different plant hosts.

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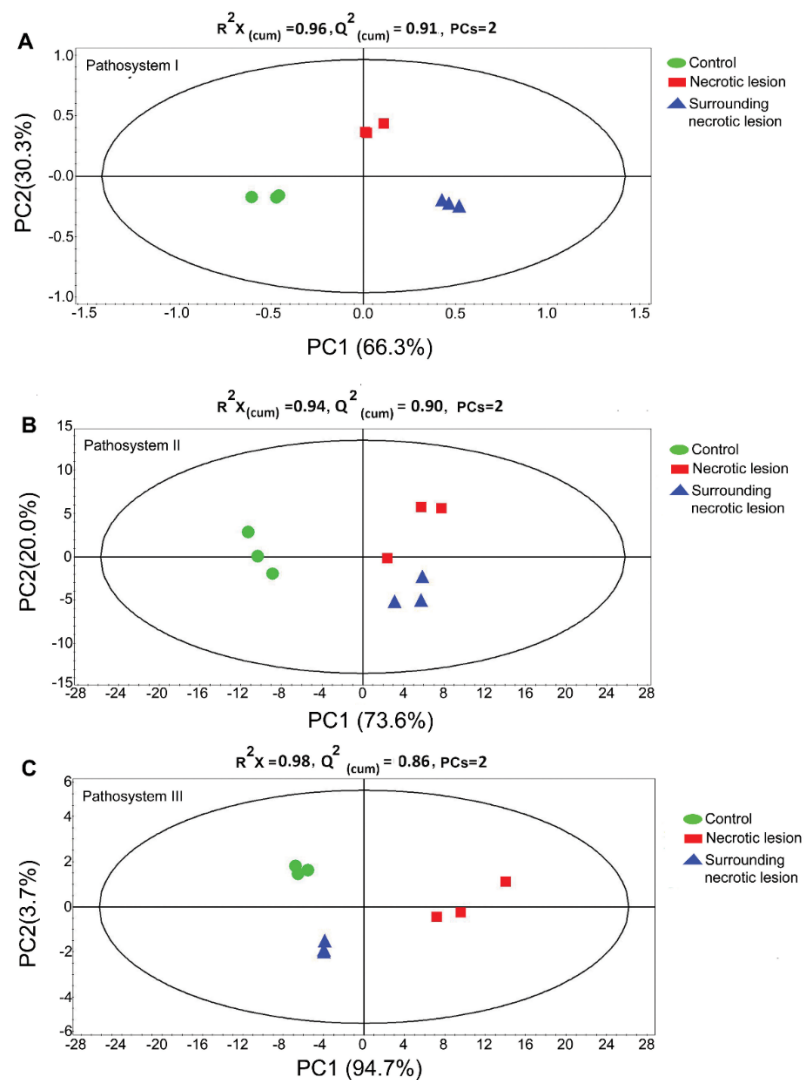
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APPENDICES

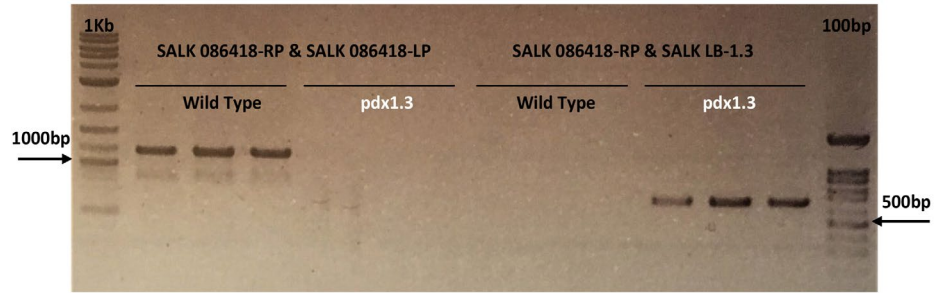
Appendix I. Principle component analysis score plots (PC1/PC2) for the effect of 11 antioxidant genes relative transcripts abundance on control, necrotic lesions and surrounding areas of necrotic lesions of Pathosystem I (A), II (B) and III (C). The ellipse represents the Hotelling T2 at a 95% confidence interval. Three biological replications were performed per treatment. Q^2 (cum); cumulative fraction of the total variation of the X's that can be predicted by the extracted components, R^2X ; the fraction of the sum of squares of the two principal components.



Appendix II. Working conditions of Agilent 6540 Accurate-Mass QTOF

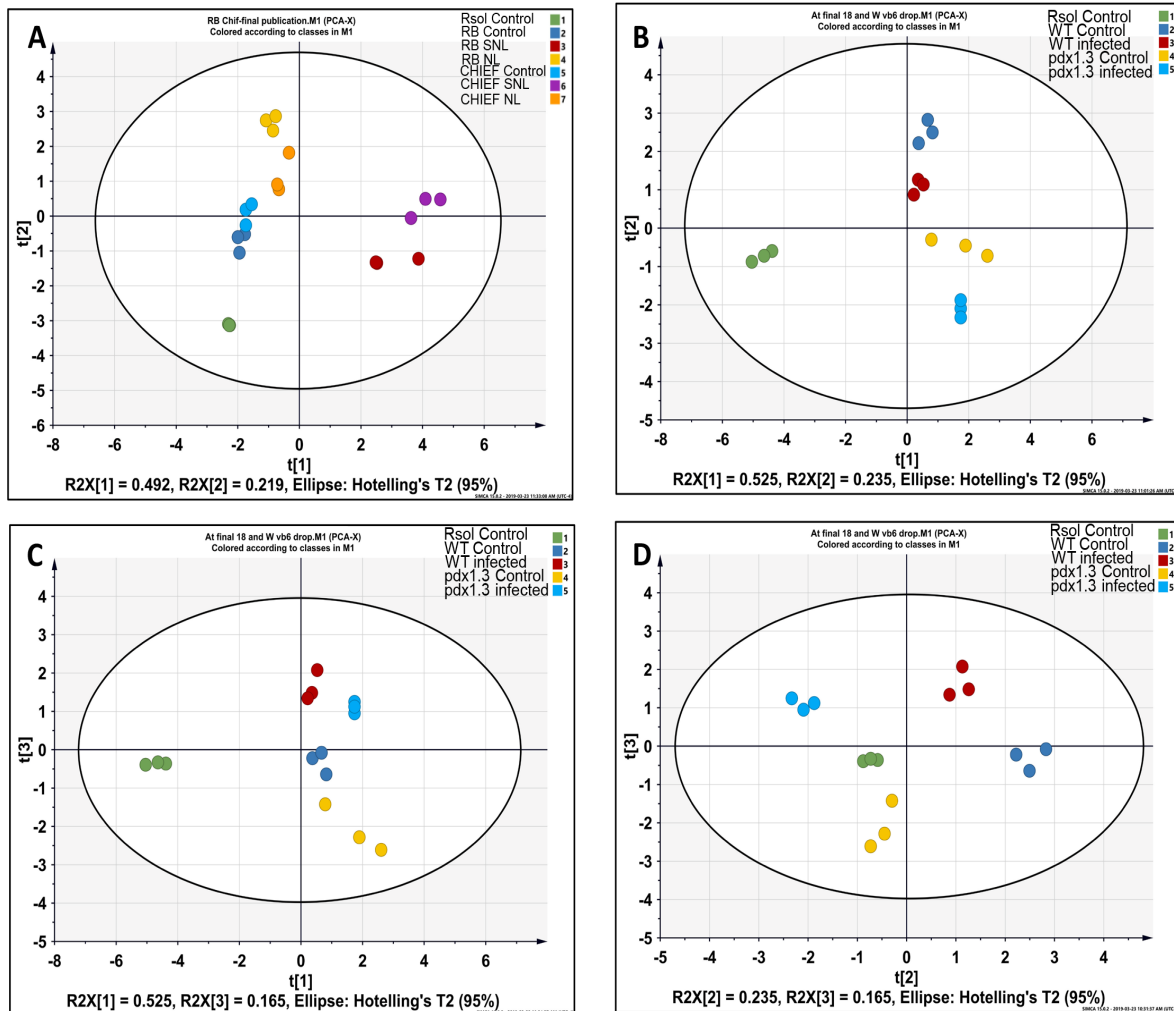
Ionization mode	Positive (ESI+)
Drying gas temperature	325°C
Drying gas flow	5 L.min ⁻¹ (nitrogen)
Sheath gas temperature	275°C
Sheath gas flow	12 L.min ⁻¹ (nitrogen)
Capillary voltage	3500 V
Nozzle voltage	2000 V
Fragmenter	175 V
Acquisition type	MS
Mass range	<i>m/z</i> 50–500
Acquisition rate	4 spectra/sec

Appendix III. Genotyping for homozygous *pdx1.3* mutants (SALK086418) and wild (Col-0) *Arabidopsis* plants using SALK gene-specific primer pairs and T-DNA primer LB1.3 along with SALK086418-RP.

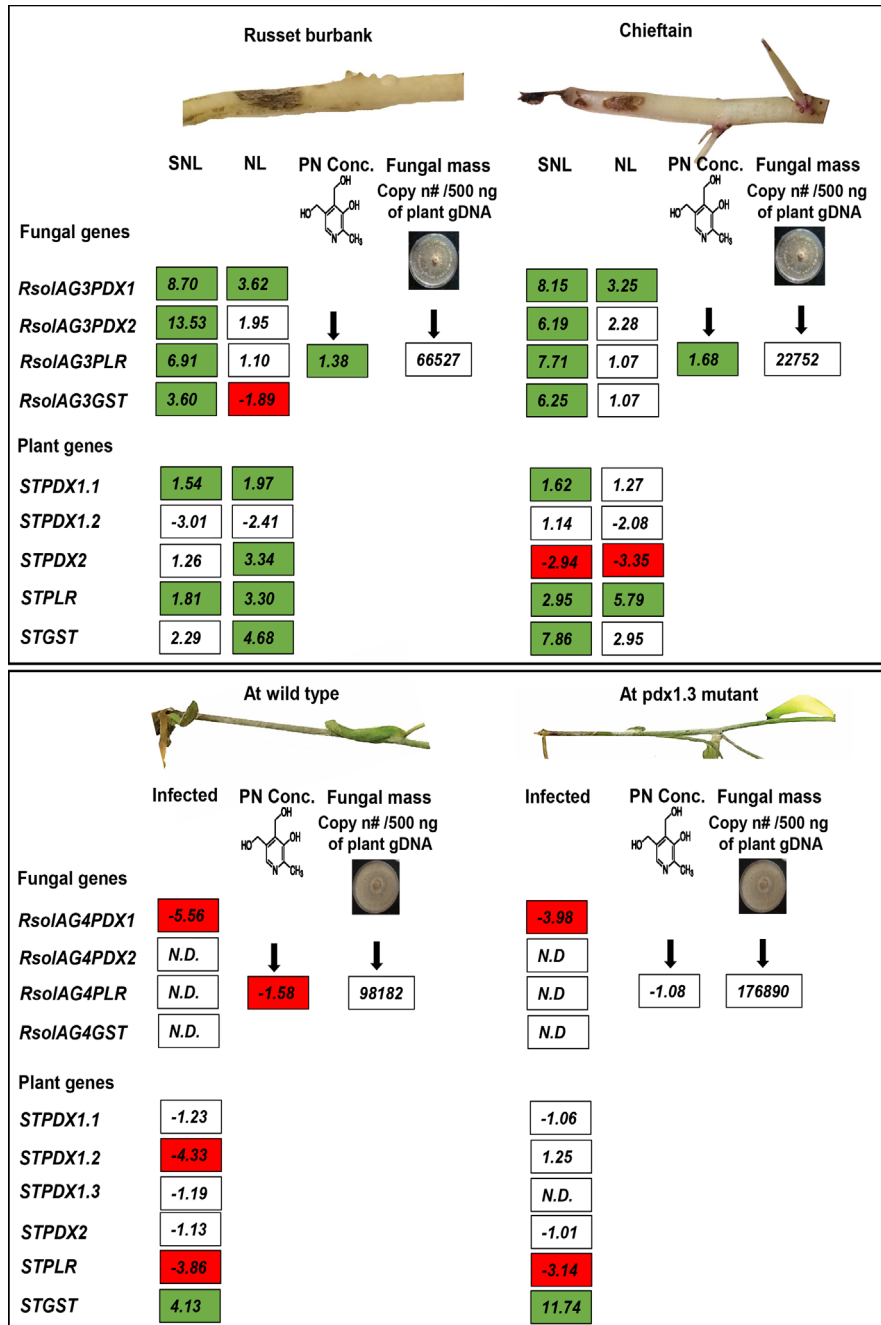


Appendix IV. Principle component analysis score plots (PC1/PC2) for the effect of transcriptional abundance of antioxidant genes, PN concentrations, and fungal biomass on control,

infected tissues in (A) pathosystem I (PC1/PC2), and (B, C, D) pathosystem II (PC1/PC2, PC1/PC3, and PC2/PC3) ($P < 0.05$). The ellipse represents the Hotelling T2 at a 95% confidence interval. Three biological replications were performed per treatment. Q2 (cum); cumulative fraction of the total variation of the X's that can be predicted by the extracted components, R2X; the fraction of the sum of squares of the two principal components.



Appendix V. Diagram showing transcript abundance, vitamin B6 concentration, and fungal biomass analysis during the interaction of Arabidopsis lines or potato cultivars with *R. solani*. Transcript fold changes of genes associated with vitamin B6 pathway (PDX1, PDX2, and PLR) and GST, PN concentrations, and fungal biomass shown in squares were quantified in infected Arabidopsis (Wild type and pdx1.3 mutant) or potato (Russet Burbank and Chieftain). Statistically significant ($P < 0.05$) and biologically significant (fold change ≥ 1.5 or ≤ -1.5) differences in transcript abundances are indicated in boxes where red represents down-regulation, green up-regulation, and white represents no significant differences ($n = 3$). Fold change is calculated in relation to the control at 72 HPI. *PDX1* and *PDX2*: Pyridoxine biosynthesis genes, *PLR*: Pyridoxal Reductase, *GST*: Glutathione S-Transferase.



Appendix VI. *p* values of principal component analysis loading coefficient plots for potato-*R. solani* AG3 interaction.

Variables	p(corr)[1]	p(corr)[2]
<i>STPDX1.1</i>	0.63	0.80
<i>STPDX1.2</i>	0.24	0.31
<i>STPDX2</i>	-0.11	0.62
<i>STPLR</i>	0.27	0.64
<i>STGST</i>	0.65	0.64
<i>RsolPDX1</i>	0.95	0.12
<i>RsolPDX2</i>	0.82	-0.14
<i>RsolPLR</i>	0.93	-0.06
<i>RsolGST</i>	-0.81	0.64
PN concentration	-0.81	0.64
Fungal biomass	-0.29	0.84

Appendix VII. *p* values of principal component analysis loading coefficient plots for the *Arabidopsis-R. solani* AG4 interaction.

Variables	p(corr)[1]	p(corr)[2]	p(corr)[2]
<i>ATPDX1.1</i>	0.70	-0.55	-0.31
<i>ATPDX1.2</i>	0.73	-0.49	-0.24
<i>ATPDX1.3</i>	0.18	0.90	0.36
<i>ATPDX2</i>	0.91	0.33	0.078
<i>ATPLR</i>	0.66	0.15	-0.71
<i>ATGST</i>	0.48	-0.33	0.78
<i>RsolPDX1</i>	-0.91	-0.39	0.10
<i>RsolPDX2</i>	-0.95	-0.25	-0.13
<i>RsolPLR</i>	-0.93	-0.24	-0.13
<i>RsolGST</i>	-0.92	-0.23	-0.13
PN concentration	-0.39	0.92	0.12
Fungal biomass	0.09	-0.63	0.76