SYSTEMIC ALTERATION OF DEFENSE-RELATED GENE TRANSCRIPT LEVELS IN MYCORRHIZAL BEAN PLANTS INFECTED WITH *RHIZOCTONIA SOLANI*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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ABSTRACT

A time course study was conducted to monitor disease development and expression of the defense-related genes phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and hydroxyproline-rich glycoprotein (HRGP) in bean (*Phaseolus vulgaris* L.) plants colonized by the arbuscular mycorrhizal (AM) fungus *Glomus intraradices*, and post-infected with the soil-borne pathogen *Rhizoctonia solani*. Pre-colonization of bean plants by the AM fungus did not significantly reduce the severity of rot symptoms. RNA blot analysis revealed a systemic increase in transcript levels of the four defense-related genes in response to *R. solani* infection. On the other hand, pre-colonization of bean plants with *G. intraradices* elicited no change in PAL, CHS and CHI transcripts, but an increase of HRGP transcripts in leaves was detected. A differential and systemic alteration in the expression of all four defense genes was observed in AM beans post-infected with *R. solani*. Depending on the time after infection with *R. solani* and the tissue examined, varying responses from stimulation, suppression, to no change in transcript levels were detected.

RÉSUMÉ

Une étude a été réalisée afin d'évaluer l'impact du champignon endomycorhizien arbusculaire *Glomus intraradices* sur la réduction de la pourriture racinaire causée par le champignon pathogène *Rhizoctonia solani* chez des plants de haricots (*Phaseolus vulgaris* L.). La pré-colonization des plants de haricot par le *G. intraradices* n'a pas significativement réduit la sévérité des symptômes de la maladie. L'étude de l'expression de gènes codant pour les enzymes de défense phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and hydroxyproline-rich glycoprotein (HRGP), effectuée à l'aide de l'analyse de transfert d'ARN sur membrane avec hybridization à des marqueurs moléculaires désignés (RNA blot analysis), a révélé une augmentation systémique de l'expression de ces quatre gènes dans les différentes parties de la plante en réponse à l'infection par le *R. solani* ainsi que chez les plantes mycorhizées et subséquemment infectées par le *R. solani*. La pré-colonization des plants d'haricot par le *G. intraradices* n'a pas modifié l'expression des gènes codant pour PAL, CHS et CHI, mais une augmentation de l'expression du gène codant pour HRGP a été détectée dans les parties foliaires de la plante. L'analyse détaillée de l'expression de ces gènes en fonctions des différents traitements expérimentaux, parties de la plante étudiées (racines, tiges, et feuilles), et temps de récolte (1, 3, et 5 jours après l'infection par le *R. solani*) a révélé des réponses variées (stimulation, suppression ou absence d'effet pour l'expression des différents gènes étudiés).

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor, Dr. Suha Jabaji-Hare, for her guidance and support. I would especially like to thank her for being a mentor to me during my entire time at Macdonald, beginning when I was a first year undergraduate student in her class.

I would also like to acknowledge my committee members, Dr. C. Hamel, Dr. T. Paultiz, Dr S. Sparace and Dr. M. St-Arnaud, for the technical expertise they provided in answering my numerous questions, and the Natural Science and Engineering Research Council of Canada for their financial support.

Many thanks to all the individuals in Dr. Hare's laboratory, which we affectionately call the Funlab, especially Martin, Greg, Carole and Diane. Their friendship and comradely made our laboratory more than a place of research, but also a home away from home.

Finally I would like to thank Corina Hay and my family for their unfailing support and encouragement throughout my Master's degree.

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LIST OF ABBREVIATIONS

AG	Anastomosis group
ANOVA	Analysis of variance
CHI	Chalcone isomerase
CHS	Chalcone synthase
CHT	Chitinase
DAI	Days after infection
GLU	Glucanase
HRGP	Hydroxyproline-rich glycoprotein
ISR	Induced systemic resistance
PAL	Phenylalanine ammonia-lyase
PAR	Photosynthetically active radiation
PGIP	Polygalacturonase inhibiting protein
PGPR	Plant growth promoting rhizobacteria
SA	Salicylic acid
SAR	Systemic acquired resistance
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulphate

CHAPTER I

Introduction

1.1 General introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with the roots of vascular plants, and based on fossil evidence, this type of symbiosis has existed for approximately 460 million years (Redecker *et al.*, 2000). As a result of the long evolutionary history of the AM symbiosis, it is estimated that 95% of vascular plants belong to families that can form AM symbioses (Trappe and Schenck, 1982). The association benefits plant health most notably by enhancing soil nutrient uptake (Hayman, 1983; Smith and Gianinazzi-Pearson, 1988), and by reducing the severity of diseases caused by soil-borne pathogens (Dehne, 1982; St-Arnaud *et al.*, 1995a).

1.2 Rationale

Although the reduction in disease severity caused by the AM symbiosis has been extensively studied in numerous plant-pathogen combinations (for reviews see: Dehne, 1982; Perrin, 1990; St-Arnaud *et al.*, 1994), the cellular and molecular mechanisms responsible for this phenomenon are not well understood partly due to the complexity of the biological system. Numerous mechanisms have been proposed to explain the bioprotection of plants by AM fungi, with the majority of research focusing on the improved nutrient status of the plant host, alteration of the soil microbial community and a stimulation or alteration of plant defense responses by AM fungi which allows the plant to more effectively respond to a subsequent pathogenic attack. Among these hypotheses,

the latter has received considerable attention (for reviews see: Linderman, 1994; St-Arnaud *et al.*, 1995a; Azcón-Aguilar and Barea, 1996; Blee and Anderson, 2000). In the past decade, the effect of the AM symbiosis on the plant's defense responses during a pathogenic infection has been investigated by measuring the amount of defense-related compounds, enzymes or transcripts encoding for plant defense-related processes in the root tissues of AM plants at one time-period after a pathogenic infection (Benhamou *et al.*, 1994; Pozo *et al.*, 1996; Cordier *et al.*, 1998; Dassi *et al.*, 1998; Mohr *et al.*, 1998; Pozo *et al.*, 1999). Only a few reports have assessed the plant's defense responses over several time points (Wyss *et al.*, 1991; Slezack *et al.*, 1999; Guenoune *et al.*, 2001), and none examined the defense responses in tissues other than the roots. Therefore, to determine if the AM symbiosis has an impact on the plant's defense responses during a pathogenic infection, it is crucial not only to assess the plant's defense responses at several time points during the initial stages of infection by the pathogen, but also to determine whether these responses are expressed in plant tissues distant from the site of AM or pathogen inoculation such as the stems or leaves.

Among all the mycorrhizal plant-pathogen interactions that have been investigated, to our knowledge no study has been undertaken to investigate whether the AM symbiosis can reduce root and hypocotyl rot of common bean (*Phaseolus vulgaris* L.) caused by *Rhizoctonia solani* Kühn. Bean is commonly used as a model plant to study plant defense responses during pathogenic infections because its defense responses to numerous biotic and abiotic factors have been well characterized (Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989), and several defense-related genes of bean have been cloned

and characterized for use as hybridization probes to measure transcript levels (Dixon and Harrison, 1990; Dixon *et al.*, 1991). Thus, *P. vulgaris* makes an excellent model plant to study AM-induced plant defense responses.

1.3 Hypotheses

It is hypothesized that the AM symbiosis reduces the severity of root and hypocotyl rot caused by *R. solani*, and that this protection is due to the systemic stimulation of the plant's defense responses as a result of root colonization by *G. intraradices*. These two hypotheses formed the basis for the undertaking of this thesis.

1.4 Objectives

Due to the limited amount of knowledge on the spatial and temporal effects of the AM symbiosis on plant defense responses during a pathogenic infection, and the lack of information on the effects of the AM symbiosis on disease caused by *R. solani,* the objectives of this thesis were to:

1. Determine how arbuscular mycorrhizal (AM) bean plants respond to the pathogen *R. solani*, and if the symbiotic association affords the plant any protection against the pathogen.
2. Determine whether levels of transcripts of selected defense-related genes in bean plants are altered due to the AM symbiosis.
3. Determine how AM bean plants respond to *R. solani* infection by assessing the spatial and temporal accumulation of transcripts of selected defense-related genes.

CHAPTER II

Literature review

2.1 Disease resistance

Disease resistance in plants results in the inability of pathogenic organisms to successfully infect the plant which results in reduced disease symptoms (Van Loon, 1997). In most plant-pathogen interactions, resistance is associated with a rapid deployment of a complex defense response that includes chemical compounds such as antimicrobial phytoalexins, hydrolytic enzymes and structural barriers such as lignin and hydroxyproline-rich glycoproteins. With few exceptions, activation of the plant's defense mechanisms due to infection by a pathogen involves the transcriptional activation of defense-related genes (Collinge and Slusarenko, 1987; Dixon and Harrison, 1990). Numerous plant models have been used to investigate the plant defense responses at the molecular and genetic level. Plant defense mechanisms in bean (*Phaseolus vulgaris*) have been especially well characterized, and a wealth of information on the activation of defense responses due to abiotic elicitors and pathogenic infection is available.

2.1.1 Phytoalexins

Plants possess a variety of mechanisms to defend themselves against pathogens. One method is the production of low-molecular-weight antimicrobial compounds known as phytoalexins (Kuc, 1995). Phaseolin, a major phytoalexin of *P. vulgaris* (Collinge and Slusarenko, 1987) is synthesized through the phenylpropanoid pathway. The first committed step of the phenylpropanoid pathway is the conversion of L-phenylalanine to

cinnamic acid which is catalyzed by phenylalanine ammonia-lyase (PAL) (Dixon, 1986). Aside from being utilized for the production of phytoalexins, cinnamic acid can be utilized in the production of lignin (Vance *et al.*, 1980; Hahlbrock and Scheel, 1989) and salicylic acid, a signal molecule involved in regulated plant defense responses (Reymond and Farmer, 1998). Other well-characterized enzymes in the phenylpropanoid pathway are chalcone synthase (CHS) and chalcone isomerase (CHI) (Lamb *et al.*, 1983; Dixon and Harrison, 1990). These enzymes catalyze the first and second committed steps of the flavonoid/isoflavonoid branch of the phenylpropanoid pathway, respectively (Dixon, 1986). Enzyme activity and transcript levels of PAL, CHS and CHI have been observed to dramatically increase during pathogenic infections and after elicitor treatment in bean (Ryder *et al.*, 1984; Edwards *et al.*, 1985; Lawton and Lamb, 1987; Mehdy and Lamb, 1987; Ryder *et al.*, 1987).

2.1.2 Hydrolytic enzymes

Hydrolytic enzymes, such as glucanases and chitinases are believed to inhibit colonization of plant tissue by the pathogen through the degradation of fungal cell walls (Mauch *et al.*, 1988; Bowles, 1990). Levels of chitinase and glucanase transcripts have been observed to rapidly increase in bean cell suspension cultures and whole plants due to treatment with an elicitor, infection by pathogens such as *Colletotrichum lindemuthianum* (Sacc. & Magnus) or wounding (Hedrick *et al.*, 1988; Edington *et al.*, 1991).

2.1.3 Hydroxyproline-rich glycoproteins

Plants can defend themselves against pathogens through the deposition of proteins, such as hydroxyproline-rich glycoproteins (HRGPs) within their cell walls (Collinge and Slusarenko, 1987; Dixon and Harrison, 1990). The amino acid sequence of HRGPs contains repeated hydroxyproline motifs and the proteins are believed to strengthen the cell wall through intermolecular cross linkages (Varner and Lin, 1989). HRGPs have also been implicated in immobilizing pathogens through electrostatic interactions or by providing sites for lignin deposition (Hammerschmidt *et al.*, 1984; Showalter, 1993). In bean hypocotyls, the levels of HRGP transcripts rapidly accumulated due to infection by *C. lindemuthianum*, wounding or treatment of a cell culture with an elicitor (Corbin *et al.*, 1987; Templeton *et al.*, 1990; Wycoff *et al.*, 1995).

2.1.4 Polygalacturonase inhibiting proteins

Plants may also respond to pathogens by producing proteins which directly inhibit the mechanisms that pathogens use to invade the plant. Necrotic pathogens such as *Rhizoctonia solani* produce polygalacturonases that are involved in pathogenesis due to their ability to degrade plant cell wall carbohydrates (Van Etten *et al.*, 1967). In response, plants such as *P. vulgaris* synthesize polygalacturonase inhibiting proteins (PGIPs) whose transcript levels have been observed to rapidly increase in tissues infected with a pathogen (Nuss *et al.*, 1996; Devoto *et al.*, 1997). PGIPs not only inhibit the activity of polygalacturonases (Cervone *et al.*, 1987), but cause an increase in the levels of partially hydrolyzed plant-cell well carbohydrates called oligalacturonides (De Lorenzo *et al.*,

1994). These oligalacturonides, produced through the action of PGIPs, may act as elicitors of additional defense mechanisms (De Lorenzo *et al.*, 1994).

2.1.5 Induced disease resistance

As mentioned in the preceding sections, infection by a pathogen can activate a wide variety of plant defense responses to limit the severity of disease caused by the pathogen. Induced resistance is the phenomenon in which a plant can exhibit an enhanced resistance response to pathogens as the result of prior stimulation of resistance mechanisms (Kuc, 1982; Van Loon, 1997; Van Loon *et al.*, 1998). There are two classes of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Hammerschmidt, 1999). SAR has been heavily studied and is the subject of numerous reviews (Ward *et al.*, 1991; Ryals *et al.*, 1994; Ryals *et al.*, 1996; Sticher *et al.*, 1997). SAR in plants develops in response to infection by necrotizing pathogens, is associated with the production of pathogenesis-related proteins (PR), and usually involves a faster and greater activation of defense-related genes after infection of induced plants (Van Loon and Van Strien, 1999). A large body of evidence suggests that salicylic acid (SA) plays a key role in both SAR and disease resistance (Klessig and Malamy, 1994; Reymond and Farmer, 1998) and it is believed that SA is likely the translocated signal that triggers SAR in distant plant organs (Ryals *et al.*, 1996).

Induced systemic resistance (ISR) has been much less studied than SAR and generally is the result of root colonization by beneficial plant growth promoting rhizobacteria (PGPR) such as Pseudomonads (van Peer *et al.*, 1991; Liu *et al.*, 1995a; Liu *et al.*, 1995b). ISR is

regulated not by salicylic acid (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997) but by jasmonates and ethylene (Van Loon *et al.*, 1998). In contrast to SAR, rapid and substantial accumulation of PR proteins does not generally occur upon challenge of a PGPR-protected plants with a pathogen (Hoffland *et al.*, 1995; van Wees *et al.*, 1999). However, levels of phytoalexins have been observed to be at higher levels in PGPR protected plants challenged with a pathogen as compared to challenged non-protected plants (van Peer *et al.*, 1991).

2.2 Effect of the AM symbiosis on disease severity

Like PGPRs, another type of beneficial non-pathogenic soil microbes are arbuscular mycorrhizal (AM) fungi, which form symbiotic associations with plant roots and can also modulate disease resistance (St-Arnaud *et al.*, 1995a). The benefits of the AM symbiosis are not limited to reduced disease severity, but include enhanced soil nutrient uptake most notably, phosphorus (Hayman, 1983; Smith and Gianinazzi-Pearson, 1988), increased drought tolerance (Nelsen and Safir, 1982), and improved soil structure (Sutton and Sheppard, 1976).

The ability of the AM symbiosis to affect the severity of disease caused by soil-borne pathogens has received considerable attention. The most recent comprehensive review of the literature reported that in approximately 65% of the 136 plant-soil-borne pathogen models studied, presence of the AM symbiosis reduced disease severity caused by soil-borne pathogens, but generally caused an increase in disease severity caused by foliar pathogens (St-Arnaud *et al.*, 1995a).

2.2.1 Effect of the AM symbiosis on *Rhizoctonia solani*

Rhizoctonia solani Kühn is a common soil-borne pathogen that causes damping-off of young seedlings, and hypocotyl and root rot in a variety of plant species (Howard *et al.*, 1994). Although several studies have investigated the role of the AM symbiosis in reducing the severity of diseases caused by *R. solani*, the results are not clear-cut. In pointsettia (Stewart and Pfleger, 1977) and *Brassica napus* L. (Iqbal *et al.*, 1977), the presence of the AM symbiosis decreased disease caused by *R. solani*. In contrast, the AM symbiosis did not decrease hypocotyl rot severity in soybeans (Zambolim and Schenck, 1983) nor did it affect the course and extent of root colonization by *R. solani* (Wyss *et al.*, 1991).

2.3 Mechanisms involved in disease reduction

Numerous mechanisms have been postulated to explain how the AM symbiosis can reduce disease severity caused by soil-borne pathogens, with the majority of research focusing on three proposed mechanisms: (i) increased nutritional status of AM plants, (ii) alteration of the soil's microbial community and (iii) alteration of the plant's defense mechanisms by the AM fungus (for reviews see: Perrin, 1990; Jalali and Jalali, 1991; Hooker *et al.*, 1994; Linderman, 1994; St-Arnaud *et al.*, 1995a; Azcón-Aguilar and Barea, 1996).

2.3.1 Mechanism I: Improved nutritional status

Increased nutritional status of AM plants was one of the first mechanisms proposed to explain the reduction in disease severity (Davis and Menge, 1980; Graham and Menge, 1982). The AM symbiosis would increase the plant's nutritional status resulting in a healthier and more vigorous plant better able to resist or tolerate an infection by a pathogen than a non-mycorrhizal plant (Linderman, 1994). Davis and Menge (1980) showed that the severity of Phytophthora root rot of citrus was equally reduced due to increased phosphorus fertilization and colonization of the roots by the mycorrhizal fungus *Glomus fasciculatus* (Thaxter) Gerd. and Trappe. However, recent studies have clearly demonstrated that the reduction in disease severity caused by various soil-borne pathogens could not always be attributed to the increase in phosphorus nutrition caused by the AM association (Caron *et al.*, 1986a; St-Arnaud *et al.*, 1994; St-Arnaud *et al.*, 1997).

2.3.2 Mechanism II: Alteration of the microbial community

The effect of the AM symbiosis on the soil's microbial community has received considerable attention. Meyer and Linderman (1986), and Secilia and Bagyaraj (1987) have shown that AM fungi caused quantitative changes in the levels of soil microbes as well as qualitative changes in the make-up of the types of organisms in the soil microbial community. In addition to the gross changes in the soil microbial community, the effects of the AM association on populations of specific soil-borne pathogens have also been illustrated on several occasions. Propagule levels of *Pythium ultimum* Trow (Kaye *et al.*, 1983), *Aphanomyces euteiches* Drechs (Rosendahl, 1985) and *Fusarium oxysporum* Schl.

(St-Arnaud *et al.*, 1997) decreased in the soil of AM plants versus non-AM plants. A study looking at the interaction between the AM fungus *G. intraradices* and the pathogen *F. oxysporum* was conducted using *in vitro* AM carrot root cultures (St-Arnaud *et al.*, 1995b). Conidial germination of *F. oxysporum* increased in the presence of *G. intraradices*, which led the authors to conclude that the substantial increase in conidial germination of the pathogen may result in an overall depletion of propagules in the soil. This proposed mechanism was recently investigated in more detail by Filion *et al.* (1999) who showed that compounds produced by the extraradical mycelium of the AM fungus *G. intraradices* directly influenced the growth of *F. oxysporum* and *Pseudomonas chlororaphis* (Guignard & Sauvageau).

2.3.3 Mechanism III: Alteration of plant defense mechanisms

Alteration of the plant's defense responses due to root colonization by AM fungi, which may allow the plant to more effectively respond to a pathogenic infection, is a third mechanism proposed to explain the bioprotective effect of the AM symbiosis (Rosendahl, 1985; Caron *et al.*, 1986b; St-Arnaud *et al.*, 1994; Niemira *et al.*, 1996). Much of the initial work has focused on how colonization of the roots by AM fungi effects the plant's defense responses by altering the levels of phytoalexins, activities of defense enzymes and defense gene transcript levels. More recent work has looked at how the AM symbiosis can affect the plant's defense responses during a pathogenic infection. The information provided below is a brief overview of some of the published studies. This subject has been described in more detail in several recent reviews (Dumas-Gaudot *et al.*,

1996; Gianinazzi-Pearson *et al.*, 1996; Morandi, 1996; Blee and Anderson, 2000; Lambais, 2000; Shaul *et al.*, 2000).

2.3.3.1 Defense responses of host roots to AM fungal colonization

Despite the intimate plant-fungal contact in the mycorrhizal symbiosis, strong induction of plant defense responses do not occur as they do during plant-pathogen interactions (Gianinazzi-Pearson *et al.*, 1996). Recent studies have shown that colonization of *P. vulgaris* by the AM fungi *Glomus mosseae* (Nicol and Gerd.) and *G. intraradices* generally do not cause an increase in the accumulation of defense-related gene transcripts such as chitinase, glucanase, PAL and HRGP (Blee and Anderson, 1996; Mohr *et al.*, 1998). However, several other examples demonstrated that mycorrhizal associations can elicit weak defense responses during the initial stages of colonization by the AM fungus, usually within the first two weeks after inoculation, but as colonization progresses the responses are suppressed. During the formation of the AM symbiosis in leek (*Allium porrum* L.) roots (Spanu *et al.*, 1989a), and *P. vulgaris* roots (Lambais and Mehdy, 1993), chitinase activities increased during the initial stages of colonization by the AM fungus, but as colonization progressed enzymatic activities were suppressed. In addition, activation followed by suppression of the components of the phenylpropanoid pathway such PAL and CHI transcripts levels and enzyme activities have been observed in alfalfa roots (*Medicago sativa* L.) colonized by *G. intraradices* (Volpin *et al.*, 1994; Volpin *et al.*, 1995). Equally, enzymes involved in cell wall strengthening such as peroxidase and catalase have been affected in a similar manner (Spanu and Bonfante-Fasolo, 1988; Blilou *et al.*, 2000). These findings indicate that despite the mutualistic character of AM

associations, the host plant recognizes the symbiotic fungi and may react at least initially by activating some defense mechanisms. However this response tends to be weak and transient (Gianinazzi-Pearson *et al.*, 1996).

2.3.3.2 Cellular localization of defense alterations due to the AM symbiosis

Aside from the overall changes in defense responses in the entire AM root system, studies have been conducted to observe the effects of the AM symbiosis at the cellular level. Lambais and Mehdy (1995) observed that expression of chitinase and glucanase genes were systemically suppressed in the roots of bean plants colonized by *G. intraradices*, but were locally enhanced in cortical cells containing arbuscules. Similarly, in mycorrhizal alfalfa and bean, levels of transcripts coding for genes in the phenylpropanoid pathway such as PAL and CHS have been shown to be localized only in cells containing arbuscules (Harrison and Dixon, 1994; Blee and Anderson, 1996). In corn roots colonized by the AM fungus *Glomus versiforme* (Karst) Berch., it was found that HRGP transcripts only accumulated in root cells colonized by the AM fungus (Balestrini *et al.*, 1997). As well as the effects on the entire root system, colonization of roots by AM fungi causes a localized activation of defense related genes specifically in cells containing arbuscules (Blee and Anderson, 2000).

2.3.3.3 Mycorrhizal specific hydrolytic enzymes

Several researchers have taken a different approach to studying the effects of the AM symbiosis on plant defense responses. Instead of measuring total activity of a specific enzyme or the transcript levels of a particular defense-related gene, they have looked at

the production of new isoforms of defense-related enzymes. Colonization of pea (*Pisum sativum* L.) roots by the mycorrhizal fungus *G. mosseae* has been shown to induce the production of a new acidic chitinase isoform that was not detected in the roots of non-AM peas (Dumas-Gaudot *et al.*, 1994; Dassi *et al.*, 1996). Similarly, colonization of tomato (*Lycopersicon esculentum* Mill.) roots by *G. mosseae* induced the production of new glucanase and chitinase isoforms (Pozo *et al.*, 1996; Pozo *et al.*, 1999). The implications of the AM specific hydrolases to bioprotection are discussed in a later section.

2.3.3.4 Defense responses of AM plants infected with fungal pathogens or elicitors

Only a limited number of studies have investigated the defense responses of AM plants infected with a pathogen. These works have shed light on whether or not the AM symbiosis causes an alteration of the plant's defense responses which would allow the plant to more effectively respond to a pathogenic infection.

Published reports have provided conflicting evidence indicating that the AM symbiosis can either cause an increase, decrease or no change in the plant's defense response to a pathogenic attack. For instance, Dehne and Schönbeck (1979), in an early study investigating the effects of the AM symbiosis on the plant's defense response during a pathogenic infection, found that AM tomato (*L. esculentum*) infected with *F. oxysporum* had higher PAL activity than non-AM plants infected with the pathogen. In a cytochemical study investigating holly (*Ilex crenata* Thunb.) colonized by *G. mosseae* and post-infected with the pathogen *Thielaviopsis basicola* (Berk. &Br.) Fr., it was found that AM plants produced wound structures at a faster rate and tended to compartmentalize

the pathogen more rapidly than non-AM plants infected with the pathogen (Wick and Moore, 1984). Similarly, mycorrhizal Ri T-DNA transformed carrot roots infected for one week with *F. oxysporum* had extensive defense-like host cell wall reactions and accumulation of phenolic compounds at the sites of fungal penetration and in intercellular spaces, which were not seen in non-mycorrhizal roots infected with *F. oxysporum* (Benhamou *et al.*, 1994). The authors suggested that colonization of the roots by the AM fungus may sensitize the plant to respond more rapidly to a microbial attack (Benhamou *et al.*, 1994). In a study investigating proteolytic activities in the root tissue of AM pea plants infected with *A. eutiches* it was found that total protease activity was generally higher in AM plants versus non-AM roots, 10 days after infection with the pathogen (Slezack *et al.*, 1999). As mentioned in section 2.3.3.2, colonization of tomato by an AM fungus induced the production of new isoforms of cell wall hydrolases. Interestingly, Pozo *et al.* (1999) demonstrated that 2 basic isoforms of glucanase were only present in tomato plants colonized by *G. mosseae* and subsequently infected with *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker and were absent in non-AM plants infected with the pathogen. The authors suggested that the AM induced isoforms of cell wall hydrolases may help plants respond to an invading pathogen either by their hydrolytic ability to degrade the pathogen's cell wall or by their ability to release elicitors that allow the plant's defense system to be quickly activated compared to non-AM plants (Pozo *et al.*, 1996; Pozo *et al.*, 1999).

In contrast, other studies have shown that defense responses in plants during a pathogenic infection are actively suppressed as a result of the AM symbiosis, and it has been

speculated that the suppression may be mediated by defense suppressor molecules or through the involvement of plant hormones. In tobacco, colonization by the AM fungus *G. intraradices* caused a suppression in the levels of a basic chitinase, and this suppression was maintained even after application of a chemical elicitor of defense responses (David *et al.*, 1998). It was proposed that the suppression was due to a plant-mediated factor potentially a plant hormone. A more detailed study by Guenoune *et al.* (2001) found that in alfalfa dual inoculated with the AM fungus *G. mosseae* and the pathogen *R. solani,* mRNA levels of chalcone isomerase and isoflavone reductase, as well as peroxidase enzyme activity in the root tissue were suppressed in comparison to non-AM alfalfa infected with the pathogen. The authors speculated that the absence of a defense response in AM plants infected with the pathogen may be the result of a suppressor molecule produced by the AM fungus.

Other researchers have found that the AM symbiosis causes no alteration of defense responses. Wyss *et al.* (1991) found that in soybean (*Glycine max* L.) plants pre-colonized with the AM fungus *G. mosseae* and post-inoculated with the pathogen *R. solani*, levels of the phytoalexin glyceollin and the pattern of accumulation in the root tissue, were similar to those observed for non-AM plants inoculated with the pathogen. Two recent studies have shown that levels of defense-related proteins or transcripts were unchanged in AM plants compared to non-AM plants during a pathogenic infection. Chitinase activity and transcript levels were the same in non-AM and AM bean colonized by *G.mosseae* and post-infected with the pathogen *Fusarium solani* (Mart.) Sacc. for one week (Mohr *et al.*, 1998). Also, levels of four pathogenesis-related proteins were

generally unchanged in tomato roots colonized by *G. mosseae* and post-infected with the pathogen *P. parasitica* for one week compared to non-AM plants infected with the pathogen (Dassi *et al.*, 1998).

Induced systemic resistance (ISR) by mycorrhizal fungi against pathogens has been demonstrated in only one study in which a split-root system technique was used (Cordier *et al.*, 1998). Formation of an AM symbiosis as the result of colonization of tomato roots by *G. mosseae* induced not only localized but also systemic resistance against *P. parasitica*. The authors also showed that the ISR was characterized by large reductions in root damage and in *P. parasitica* development in non-mycorrhizal root tissues of mycorrhizal root systems. This protection was correlated with the accumulation of PR-1a pathogenesis-related protein and induction of plant cell wall defense responses such as the deposition of callose and pectins (Cordier *et al.*, 1998). In contrast, to the systemic protection observed in the root system by Cordier *et al.* (1998), Shaul *et al.* (1999), utilizing an abiotic elicitor based system, observed an opposite phenomenon in the leaves of AM tobacco plants. Protein and gene transcript accumulation of two pathogenesis-related proteins was lower and the accumulation was delayed in AM leaf tissue in comparison to the leaves from non-AM plants after treatment with an elicitor. The authors suggested that the differences in defense responses may be caused by AM induced alteration of plant hormones (Shaul *et al.*, 1999).

In summary, there is continued debate on whether or not the AM symbiosis alters plant defense mechanisms that would result in some form of induced resistance to pathogens.

Some have suggested that the reduction in disease observed in AM plants is due to an alteration of plant defense mechanisms (Dehne and Schönbeck, 1979; Benhamou *et al.*, 1994; Cordier *et al.*, 1998). Others have found that the AM symbiosis does not result in a more pronounced defense response during a pathogenic interaction (Wyss *et al.*, 1991; Dassi *et al.*, 1998), while evidence from a third group of researchers have found that defense mechanisms are not stimulated but rather suppressed as a result of the AM symbiosis during a pathogenic infection (Guenoune *et al.*, 2001).

CHAPTER III

Materials and methods

3.1 Fungal material and inoculum preparation

A highly pathogenic *Rhizoctonia solani* Kühn isolate (A76 belonging to AG-4) was kindly provided by Marc Cubeta, Department of Plant Pathology, North Carolina State University, NC. The inoculum of *R. solani* was produced on oat kernels according to the method of Cardoso and Echandi (1987) and kept at 4 °C. The inoculum of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* Schenck & Smith (DAOM 181602) consisted of axenically-produced spores obtained from an *in-vitro* carrot root (*Daucus carota* L.) culture as described in St-Arnaud *et al.* (1996). Briefly, Ri T-DNA-transformed carrot roots were grown on a minimal (M) medium according to the method of Bécard and Fortin (1988), but solidified with 0.4 % (w/v) gellan gum (ICN Biochemical, Cleveland, Ohio, USA) instead of 1% (w/v) bacto-agar (modified M medium) and colonized by *G. intraradices*. The dual cultures were grown in the dark at 28°C for 2-5 months. Spores were extracted from the media by dissolving the media with 10 mM sodium citrate buffer (pH 6.0) at 30°C (Doner and Bécard, 1991). The spores were resuspended in sterile tap water and cold treated for 7-9 weeks at 4 °C prior to use.

3.2 Plant material and growth conditions

Bean seeds (*Phaseolus vulgaris* L.) cv. Contender UT15 (Stokes Seeds Ltd. St. Catherines, Ontario, Canada) were surface sterilized in 30% H_2O_2 for 5 minutes, rinsed

three times with sterile distilled water, and pregerminated for 48 hours at room temperature in the dark on filter papers soaked with 20 ml of sterile tap water.

Plants were grown in 164 ml Cone-tainers ™ (Stuewe and Sons, Corvallis, Oregon). Prior to use, the Cone-tainers were surface sterilized for 45 minutes in 2.5% (w/v) NaOCl and rinsed with distilled water. Cone-tainers received one glass marble to plug the drain hole, followed by 16ml of washed and autoclaved one-quarter inch gravel. A volume of 110 ml of soil mix which consisted of field soil (3mm sieved), Turface® (IMC Imcore, Mundelein, Illinois), peat (4 mm sieved), and perlite (1:1:1:1 vol) was placed on top of the gravel. The soil mix (70% sand, 19% silt, 11% clay, 8.0% organic matter) contained 170 mg /kg of P, 362 mg/kg of K, 2813 mg/kg of Ca, 330 mg/kg of Mg, pH of 5.29 and was autoclaved for 60 min at 121°C on three consecutive days before use. A small wooden stake was placed within the soil mix to a depth of 2.5 cm in order to facilitate the delivery of *R. solani* inoculum.

In order to reintroduce the natural microflora other than indigenous AM fungi found in the field soil, all Cone-tainers received 10 ml of a soil filtrate. The soil filtrate was prepared by mixing 50 g of fresh field soil in 1 litre of sterile physiological saline solution (0.85% NaCl) which was stirred for 10 minutes and then filtered twice through Whatman filter paper (#1). The microflora was reintroduced to the soil mix 6 days prior to sowing of the bean seeds to allow the soil's microflora to become established. The Cone-tainers were placed in a growth cabinet maintained at 23°C/20°C (day/night), with 80% relative humidity and watered once a day with 15-20 ml of double distilled water. Lighting was

maintained for 16 hours/day generating a photon flux of 210 μmol m^{-2} s^{-1} (PAR) at the surface of the Cone-tainer.

3.3 Plant inoculation

Two pregerminated bean seeds were placed within each Cone-tainer and inoculated with 4 ml of *G. intraradices* spore solution containing 2500 axenically-produced spores that was poured over the seeds. Cone-tainers not inoculated with *G. intraradices* received 4 ml of sterile tap water. The pregerminated seeds were then covered with 1.5-2.0 cm of the soil mix prepared as above, and the Cone-tainers were placed in a growth cabinet under the same conditions as above. Cone-tainers were watered as above with double distilled water and fertilization began 12 days after planting. Each Cone-tainer received 10 ml of a modified Long Ashton solution (St-Arnaud *et al.*, 1994) 2- 3 times a week up until inoculation with *R. solani*.

Prior to post-infection with *R. solani*, bean plants inoculated or not with *G. intraradices* were allowed to grow for 27 days at which time the percentage of total root length colonized with *G. intraradices* was approximately 25%. The small wooden stake was carefully removed from the soil and 4 oat kernels previously colonized with *R. solani* were dropped down the hole that was formed. Cone-tainers not inoculated with *R. solani* received 4 autoclaved oat kernels. The plants were then harvested 1, 3 and 5 days after infection (dai). In all, there were 4 treatment-combinations for each harvesting date: 1- not inoculated with *G. intraradices* and not infected with *R. solani* (-G-R), 2- inoculated with *G. intraradices* and not infected with *R. solani* (+G-R), 3- not inoculated with *G.*

intraradices and infected with *R. solani* (-G+R), and 4- inoculated with *G. intraradices* and infected with *R. solani* (+G+R).

3.4 Evaluation of disease and root colonization

At each harvesting date, the plants were carefully removed from the Cone-tainers and the root system was quickly washed in tap water to remove the adhering soil. The severity of hypocotyl rot caused by *R. solani* was visually evaluated for each plant using a disease severity scale ranging from 0 to 5 on the basis of necrotic lesion development (Cardoso and Echandi, 1987); where 0 = no lesions on hypocotyl, 1 = lesions ≤ 2.5 mm long, 2 = lesions 2.5-5.0 mm long, 3 = lesions ≥ 5.0 mm long, 4 = lesions girdling plant, 5 = plant is damped-off or dead. The plants were divided into three sections: roots, stems and leaves, and flash-frozen in liquid nitrogen and stored at -80°C for RNA analysis. For the determination of *G. intraradices* and *R. solani* colonization of the roots, a small sample of roots was randomly taken and cut into 1-cm sections, cleared in 10% KOH and stained with acid fuchsin (Kormanik and McGraw, 1982). The percentage of root length colonized by *G. intraradices* and *R. solani* was determined using the gridline intersect method (Giovannetti and Mosse, 1980). Hyphae of *R. solani* were differentiated from *G. intraradices* hyphae by the presence of distinctive septae, branching morphology (Sneh *et al.*, 1991) and staining colouration that was not observed in *G. intraradices* hyphae.

3.5 Experimental design and statistical analyses

The experimental design was a split-split plot consisting of three blocks. The harvesting dates (1, 3 and 5 dai with *R. solani*) were randomized among the main plots, while *G.*

intraradices inoculation (+G or -G) was randomized among the split plots and *R. solani* infection (+R or -R) was randomized among the split-split plots. There were three replicates of each treatment-combination for each harvesting date and the entire experiment was repeated twice. For the statistical analyses, the two experiments were considered temporal blocks so that the data from both experiments could be analyzed together. Statistical analyses were done with the General Linear Model procedures of the SAS statistical software (SAS Institute Inc., 1998) and log linear analysis of the STAT-GRAPHICS software (STSC Inc., 1988). The percentage of root length colonized by *G. intraradices* and by *R. solani* were analyzed using ANOVA, while disease severity caused by *R. solani* was analyzed with frequency table analysis using a log linear model (Lehmann, 1975). Where appropriate *a posteriori* comparisons between treatments were performed using Tukey's studentized range tests. For analysis of mRNA transcript levels, 10 μg of total RNA was prepared by pooling 3.33 μg from all three replicates of each treatment combination.

3.6 RNA extraction and blot analysis

The entire root, stem and leaf tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 0.1 g of ground tissue using the Rneasy Plant Mini Kit (Qiagen Inc, Mississuaga, Ontario, Canada). The RNA was quantified using a Beckman DU 640 spectrophotometer, assuming that an A_{260} of 1.0 approximates a solution of 40 μg/ml of RNA. RNA quality was verified by electrophoresis in formaldehyde agarose gels according to the instructions of the

manufacturer of the RNA extraction kit. Ten (10) μg of RNA/ sample was transferred onto Hybond N nylon membrane (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) using a Bio-Dot SF slot blot apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada) according to Sambrook *et al.* (1989). The RNA was fixed to the membrane by UV irradiation using a Strat-linker UV crosslinker (Stratagene, La Jolla, California).

The membranes containing the transferred RNA were incubated for 20 minutes in 3 X SSC (1 X SSC consists of 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7), and prehybdridized for 12-16 hours at 42 °C in a solution of 3 X SSC, 50% (v/v) formamide, 0.2% (w/v) SDS (sodium dodecyl sulphate), 60 mM sodium phosphate (pH 7.2), 5 X Denhardt's solution and 0.1 mg/ml denatured fragmented salmon sperm DNA. cDNA probes were labelled with [α-32P] dCTP using a random primer labelling kit (T7 QuickPrime Kit, Amersham Pharmacia Biotech) and subsequently purified on a Sephadex G50 column. The hybridization solution was identical to the prehybridization solution except that the former contained denatured labelled probe at a concentration of 0.5-2.0 X 10^6 cpm/ml. The membranes were hybridized with the probes for 12-16 hours at 42 °C and washed in 5 X SSC, 0.1% SDS for 1 hour at 55°C and in 0.2 X SSC, 0.1% SDS for 1 hour at 60°C. The membranes were exposed to phosphor screens (Kodak screen K) that were scanned using a Molecular Imager FX (Bio-Rad Laboratories, Mississauga, Ontario, Canada). All signal intensities were below the saturation threshold of the apparatus. The signals on the screens corresponding to either hydroxyproline-rich glycoprotein, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase or H1 mRNA were

quantified and analyzed using Quantity One software program (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Normalization of the signals involved arbitrarily setting equal the resultant accumulation of H1 transcripts with compensatory changes to the respective data obtained from the utilization of the other probes. For reprobing, the membranes were placed in boiling 0.1% SDS and allowed to cool for approximately 2 hours to strip the previous probe from the membrane.

3.7 cDNA probes

cDNA clones originating from common bean (*Phaseolus vulgaris* L.), and encoding for chalcone isomerase (pCHI1), chalcone synthase (pCHS1), phenylalanine ammonia-lyase (pPAL5), hydroxyproline-rich glycoprotein (pHYP4.1), chitinase (pCHT12.2), glucanase (pG101), polygalacturonase inhibiting protein (pAD16) and a constitutively expressed gene of unknown function (pH1) were used as probes for hybridization. Description of the gene product, clone, insert size and origin of the probes are listed in Table 1. Prior to performing a complete transcript analysis, all 8 probes were screened using RNA extracted from stem tissue in order to determine among which of the eight mRNA transcripts were expressed to a level that allowed accurate quantification (Table 1).

Table 1. cDNA clones used as hybridization probes for the analysis of transcript levels in bean tissue.

Gene product	Clone	Restriction site	Insert size (kb)	Size of RNA species (kb)	Eliminated during screening	Origin
Chalcone isomerase (CHI)	pCHI1	*Eco* R1	0.87	1.0		Mehdy and Lamb, 1987
Chalcone synthase (CHS)	pCHS1	*Eco* R1	1.30	1.5		Ryder *et al.*, 1984
Phenylalanine ammonia-lyase (PAL)	pPAL5	*Pst* 1	1.77	2.5		Edwards *et al.*, 1985
Hydroxyproline rich-glycoprotein (HRGP)	pHYP4.1	*Pst* 1	0.70	2.5 (may cross hybridize with 4.4, 3.3 and 1.8kb)		Corbin *et al.*, 1987
Chitinase (CHT)	pCHT12.2	*Bam* H1 and *Kpn* 1	0.65	1.2	X	Hedrick *et al.*, 1988
Class I β-1,3-Glucanase (GLU)	pG101	*Pst* 1	0.93	1.4	X	Edington *et al.*, 1991
Polygalacturonase inhibiting protein (PGIP)	pAD16	*Bam* H1	1.27	1.2	X	Toubart *et al.*, 1992
Unknown function	pH1	*Pst* 1	1.00	Several sizes		Lawton and Lamb, 1987

CHAPTER IV

Results

4.1 *G. intraradices and R. solani* colonization of bean roots

All structures of *G. intraradices* (intercellular hyphae, arbuscules and vesicles) were present in the root tissue of bean plants inoculated with *G. intraradices* and infected or not with the compatible fungal pathogen *R. solani*. The percentage of root length colonized by *G. intraradices* was not significantly different for all three time points after infection with *R. solani*, ranging from 23.5% to 35.0% (Table 2A). Infection with the compatible pathogen did not have a significant ($P > 0.05$) effect on mycorrhizal colonization, although mycorrhizal plants infected with *R. solani* had a slightly lower percentage of their roots colonized by *G. intraradices* (Table 2A). The presence of *G. intraradices* had no significant effect ($P > 0.05$) on the percentage of root length colonized by *R. solani* (Table 2B). Root colonization by *R. solani* significantly ($P < 0.05$) increased with time (Table 2B). Specifically, the plants harvested 5 dai had a significantly higher percentage of root length colonized with *R. solani* when compared to plants harvested 1 and 3 dai (Table 2B). Differential fertilization of AM and non-AM bean plants in order to compensate for phosphorus levels was not necessary since the results of a recent study (Filion *et al.*, 2001) using the same bean cultivar, AM fungus, potting substrate and fertilization regime found no significant differences ($P > 0.05$) in shoot and root dry weight between AM and non-AM bean plants over a six week period. In the current study, no obvious differences in the size of the root systems or aerial portions of non-AM and AM plants were observed throughout the duration of the study.

4.2 Severity of hypocotyl rot

To study the effect of *G. intraradices* on hypocotly rot caused by *R. solani*, the severity of lesions on the hypocotyls was estimated using a visual disease severity scale. Disease severity data were analyzed using frequency tables to allow proper analysis of the effect of time and *G. intraradices* inoculation on hypocotyl rot. Hypocotyl rot significantly increased with time, with more plants exhibiting a value of 4 on the disease severity scale at 5 dai, while significantly more plants exhibited no disease at 1 dai (Table 3). Severe hypocotyl rot appeared by 3 dai, and at 5 dai significantly fewer plants exhibited no disease compared with those harvested 1 and 3 dai. There was no significant difference ($P< 0.05$) in disease severity between -G+R and +G+R treatments, although a larger percentage of -G+R plants had higher rankings on the disease severity scale of hypocotyl rot (Table 4), which was most evident at 3 dai (data not shown).

Table 2. Effect of pre-colonization by *Glomus intraradices* and post-infection by *Rhizoctonia solani* on fungal root colonization.

A	Mycorrhizal colonization (%) #			
	Days after infection (dai)			
Treatment*	1	3	5	Mean $
+G-R	35.0	30.5	30.2	31.9a
+G+R	26.2	23.5	29.0	26.2a
Mean $	30.6a †	27.0a	29.6a	

B	*R. solani* colonization (%)			
	Days after infection (dai)			
Treatment	1	3	5	Mean
-G+R	0.0	1.5	5.5	2.3a
+G+R	0.0	2.5	5.7	2.7a
Mean	0.0a	2.0a	5.6b	

Percent root length bearing fungal structures of *G. intraradices* or *R. solani*, as evaluated using the gridline intersect method (Giovannetti and Mosse, 1980).

*+G-R = plants inoculated with *G. intraradices* and not infected with *R. solani*, +G+R = plants inoculated with *G. intraradices* and infected with *R. solani.* –G+R = plants not inoculated with *G. intraradices* and infected with *R. solani.*

$ Means were calculated using the values of six replicates.

† For part A, means with different letters between each column or row are significantly different ($P<0.05$) by analysis of variance. For part B, means with different letters between each column are significantly different ($P<0.05$) by Tukey's studentized range test; means with different letters between each row are significantly different ($P<0.05$) by analysis of variance. Data from +G treatments only were included in part A as no mycorrhizal colonization was observed in -G plants. Data form +R treatments only were included in part B as no *R. solani* colonization was observed in -R plants.

Table 3. Effect of post-infection by *Rhizoctonia solani* on the percentage of bean plants showing each ranking on the disease severity scale.

Disease severity scale #	Days after infection with *R. solani* 1	3	5
0	95.8+ *	62.5	4.2–
1	4.2	8.3	4.2
2	0	16.6	20.8
3	0	12.5	41.7
4	0	0	29.2+
Overall	a †	b	c

Disease severity scale for hypocotyl lesions ranged from 0 = no disease to 5 = damping-off or dead as evaluated with the method of Cardoso and Echandi (1987).

*A percentage with a – or + sign was significantly under or over represented, respectively, by comparison with the same ranking on the disease severity scale from the other infection periods.

† Columns with different letters are significantly different according to a frequency table analysis using a log linear model. Data for plants inoculated with *R. solani* only were included, as plants not infected with *R. solani* showed no signs of disease. As no plants exhibited a ranking of 5 on the disease severity scale, this rank was omitted from the analysis.

Table 4. Effect of pre-colonization by *Glomus intraradices* on the percentage of bean plants showing each scale of disease severity caused by *Rhizoctonia solani*.

Disease severity scale #	+G+R*	-G+R
0	61.1	47.2
1	8.3	2.8
2	8.3	16.7
3	11.1	25.0
4	11.1	8.3
Overall	a †	a

Disease severity scale for hypocotyl lesions ranged from 0 = no disease to 5 = damping-off or dead as evaluated with the method of Cardoso and Echandi (1987).

*+G+R = plants inoculated with *G. intraradices* and infected with *R. solani,* +G-R = plants inoculated with *G. intraradices* and not infected with *R. solani*.

† Columns with different letters are significantly different according to a frequency table analysis using a log linear model. Data for plants inoculated with *R. solani* only were included, as plants not infected with *R. solani* showed no signs of disease. As no plants exhibited a rank of 5 on the disease severity scale, this rank was omitted from the analysis.

4.3 Accumulation of defense-related gene transcripts

All cDNA probes listed in Table 1 were first tested to determine the transcript levels of the defense-related genes. For this, RNA extracted from stem tissue of mycorrhizal and non-mycorrhizal bean plants infected or not with *R. solani* were analyzed. Transcript levels of chitinase, β-1,3- glucanase and PGIP were at the threshold of the detection limit, which in turn prevented accurate quantification. Consequently, these cDNAs were not retained for further analysis (Table 1).

The slot blot technique (Figures 1-3), which allows the simultaneous analysis of many samples in a single hybridization step, was performed to determine quantitatively the temporal and spatial changes in transcript levels. The choice of this technique is justified by the well-established fact that Northern analysis of all the defense-related probes used in this study have been shown to hybridize to a single RNA species (Table 1). Although, the cDNA, pHYP4.1 in some cases may cross hybridize with other RNA species encoding HRGPs (Corbin *et al.*, 1987), several studies have shown that in pathogen-infected plants, pHYP4.1 hybridizes predominantly to a single RNA species 2.5 kb in size (Mahé *et al.*, 1992; Mahé *et al.*, 1993; Wycoff *et al.*, 1995). To determine whether the differences in transcript levels were due to treatment effects and not to unequal loading of the RNA on the slot blot, the transcript levels for HRGP, PAL, CHS and CHI were normalized with respect to the levels of transcripts of the H1 gene which has been shown to be constitutively expressed (Lawton and Lamb, 1987). The accumulation of H1 mRNA was similar in all tissues and in all four treatment-combinations (Figures 1-3).

Figure 1. Effect of pre-colonization by *Glomus intraradices* and post-infection by *Rhizoctonia solani* on HRGP, PAL, CHS, CHI and H1 transcript levels in the root tissue of -G-R (○), +G-R (□), -G+R (●) and +G+R (■) bean plants. Transcript levels were normalized with respect to H1 and are expressed as arbitrary units. HRGP, hydroxyproline-rich glycoprotein; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; H1, gene of unknown function.

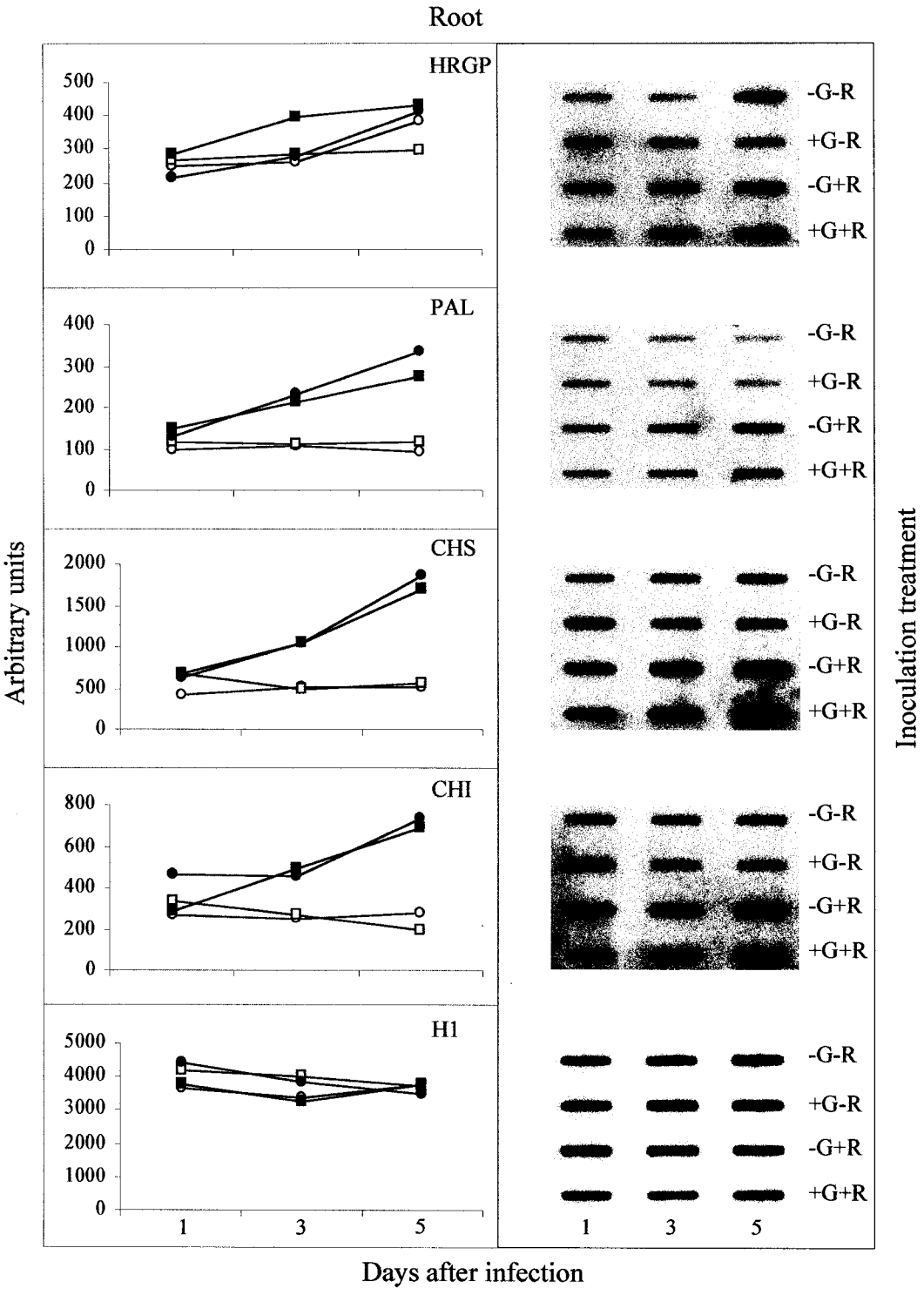

Root
HRGP
500
400
300
200
100
0
PAL
400
300
200
100
0
CHS
2000
1500
1000
500
0
CHI
800
600
400
200
0
H1
5000
4000
3000
2000
1000
0
1
3
5
-G-R
+G-R
-G+R
+G+R
Arbitrary units
Inoculation treatment
Days after infection

Figure 2. Effect of pre-colonization by *Glomus intraradices* and post-infection by *Rhizoctonia solani* on HRGP, PAL, CHS, CHI and H1 transcript levels in the stem tissue of -G-R (○), +G-R (□), -G+R (●) and +G+R (■) bean plants. Transcript levels were normalized with respect to H1 and are expressed as arbitrary units. HRGP, hydroxyproline-rich glycoprotein; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; H1, gene of unknown function.

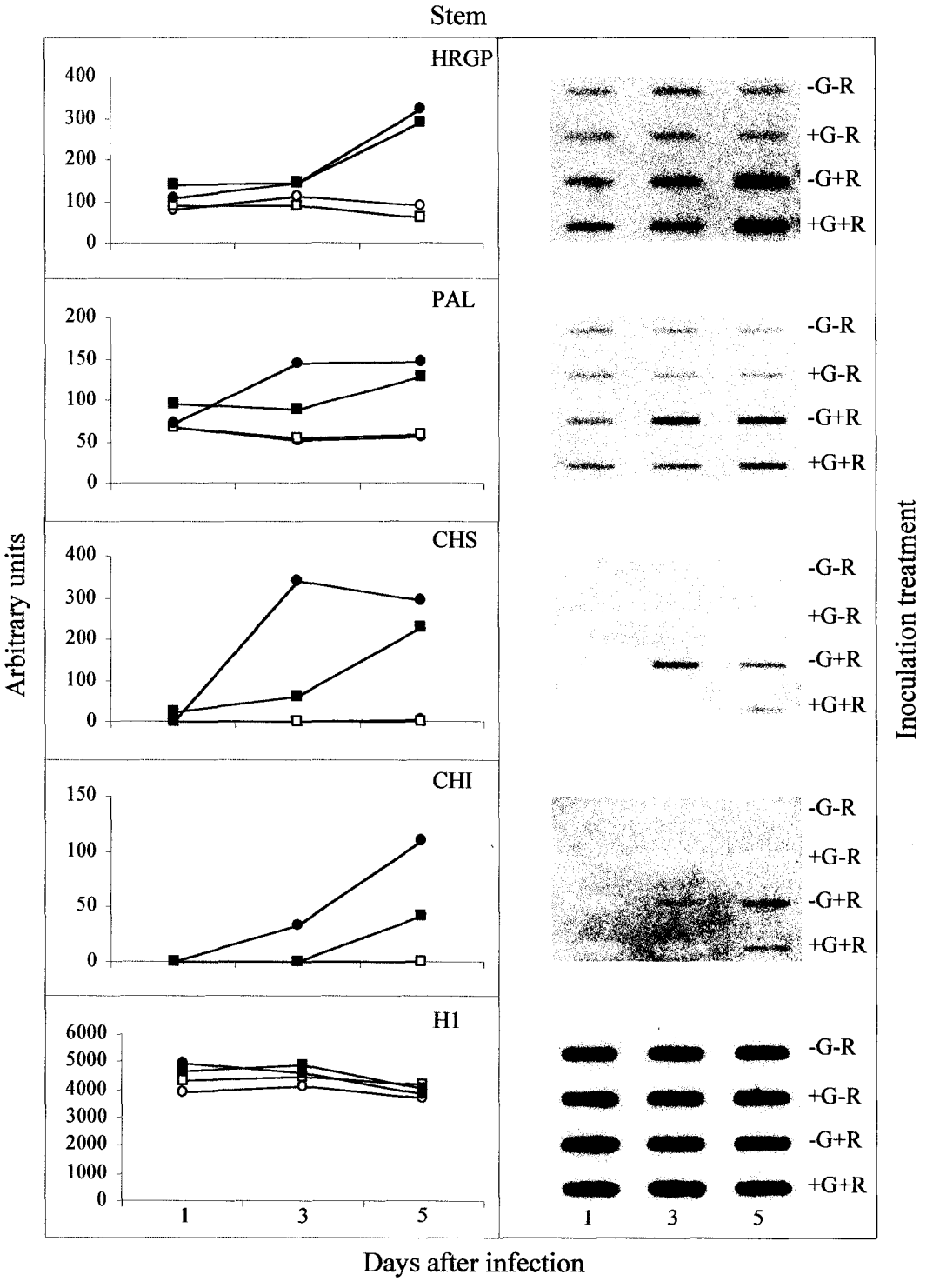
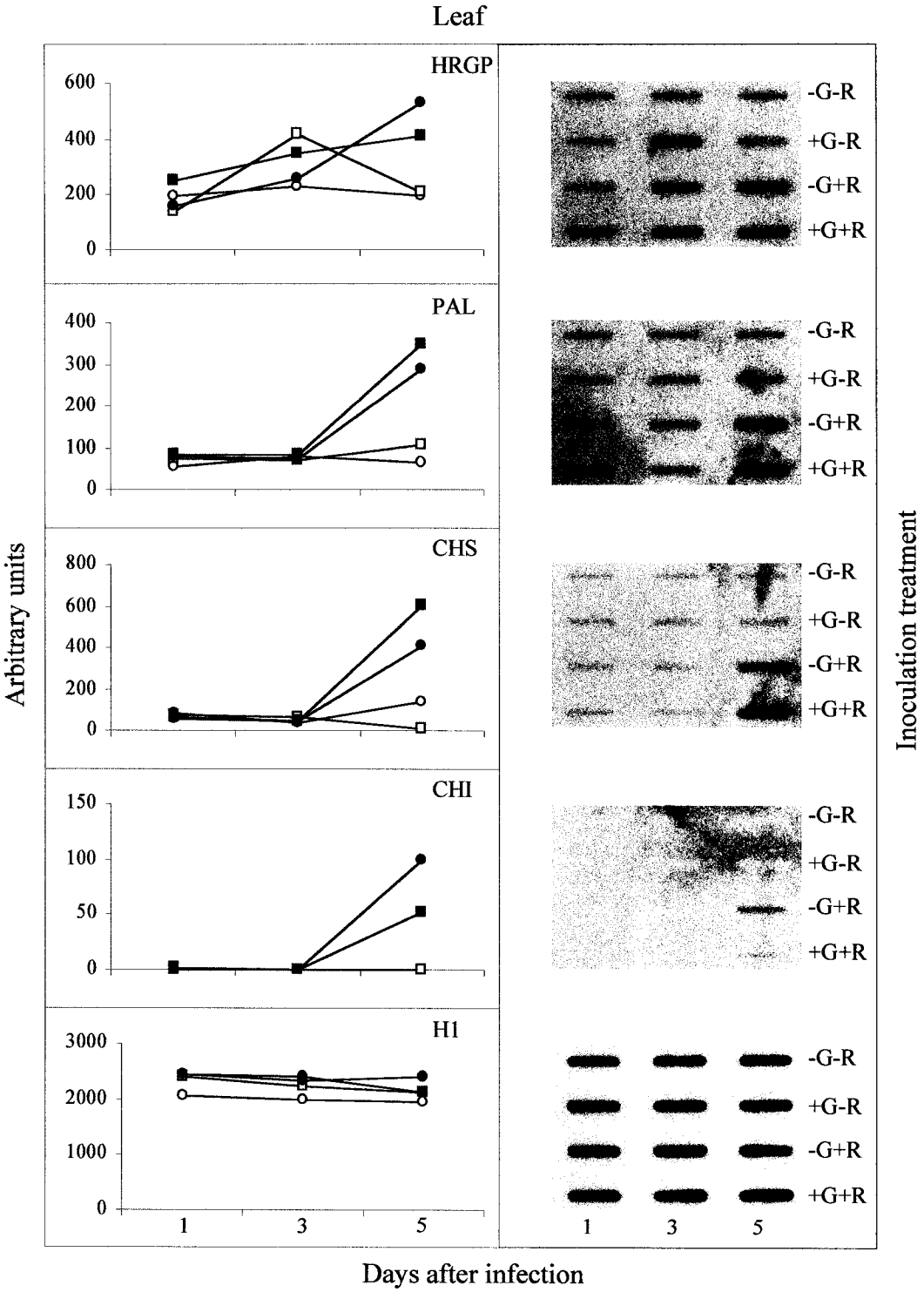

Stem
HRGP
PAL
CHS
CHI
H1
Arbitrary units
Inoculation treatment
-G-R
+G-R
-G+R
+G+R
Days after infection

Figure 3. Effect of pre-colonization by *Glomus intraradices* and post-infection by *Rhizoctonia solani* on HRGP, PAL, CHS, CHI and H1 transcript levels in the leaf tissue of -G-R (○), +G-R (□), -G+R (●) and +G+R (■) bean plants. Transcript levels were normalized with respect to H1 and are expressed as arbitrary units. HRGP, hydroxyproline-rich glycoprotein; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; H1, gene of unknown function.


Leaf
HRGP
PAL
CHS
CHI
H1
600
400
200
0
300
100
800
150
50
3000
2000
1000
1
3
5
-G-R
+G-R
-G+R
+G+R
Arbitrary units
Inoculation treatment
Days after infection

Interestingly, the H1 transcript levels in leaves (Figure 3) were slightly lower compared to those observed in the root and stem tissues (Figures 1-2).

4.3.1 Root tissue

Non-mycorrhizal (-G-R, ○) and mycorrhizal (+G-R, □) root tissue showed a similar trend in PAL, CHS, CHI and HRGP transcript levels over the three time points, with the exception of the first time point where +G-R (□) plants had slightly elevated levels of CHS transcripts and the last time point where -G-R (○) plants had slightly elevated levels of HRGP transcripts (Figure 1). Infection with *R. solani* caused an increase in transcript levels of all four defense-related genes, with the highest levels detected at 5 dai. Generally, transcripts of PAL, CHS and CHI, that code for three of the enzymes in the phenylpropanoid pathway, were first observed to accumulate at 3 dai in response to *R. solani* infection, and continued to increase for the duration of the experiment (Figure 1). Of interest, mRNA levels coding for CHI were slightly elevated at 1 dai in -G+R (●) plants only. Increased levels of HRGP transcripts were observed at all time points in the roots of +G+R (■) plants as compared to -G+R (●) plants, most notably at 3 dai, (Figure 1).

4.3.2 Stem tissue

No difference was detected in the trend of HRGP, PAL, CHS and CHI transcript accumulation between mycorrhizal (+G-R, □) and non-mycorrhizal (-G-R, ○) plants (Figure 2). Analysis of the expression of the four defense-related genes revealed that only mRNA coding for HRGP and PAL was detectable in the stem tissues of -G-R (○) and

+G-R (□) plants over the duration of the experiment (Figure 2). Similar to the root tissue (Figure 1), transcript levels of all four defense-related genes substantially increased in response to *R. solani* infection (Figure 2). Analysis of mRNA levels of the genes coding for enzymes in the phenylpropanoid pathway, revealed consistently higher levels of gene expression, with a 1.5 fold increase in PAL transcripts and a peak of a 7 fold increase in CHS transcripts in -G+R (●) plants at 3 dai compared to +G+R (■) plants. At 5 dai, the difference in transcript levels of these genes between -G+R (●) and +G+R (■) plants had decreased with the exception of CHI transcripts which were still 2.5 fold higher in -G+R (●) plants. Levels of mRNA coding for HRGP were similar in -G+R (●) and +G+R (■) plants over the duration of the experiment (Figure 2). At 5 dai, substantially elevated levels of HRGP transcripts (4 fold) were detected in *R. solani* infected plants versus plants not infected with *R. solani* (Figure 2).

4.3.3 Leaf tissue

Neither *G. intraradices* nor *R. solani* induced substantial alterations in PAL, CHI and CHS mRNA levels at 1 or 3 dai (Figure 3), while HRGP transcript levels peaked at 3 dai in response to *G. intraradices* and not to *R. solani* infection (Figure 3). Irrespective of the mycorrhizal treatment, *R. solani* caused a substantial increase in transcript levels of the four defense-related genes with the highest levels detected at 5 dai (Figure 3). As a result of the AM symbiosis, levels of HRGP and CHI transcripts were respectively 1.3 and 2.0 fold higher in -G+R (●) versus +G+R plants (■) (Figure 3), while PAL and CHS transcripts were respectively 1.2 and 1.5 fold higher in +G+R (■) versus -G+R (●) plants 5 dai (Figure 3).

Chapter V

Discussion

5.1 Fungal colonization and disease severity

We have examined the influence exerted by the AM symbiosis on root and hypocotyl rot caused by *R. solani* (AG-4) in beans. The results indicate that pre-inoculation with *G. intraradices* did not reduce Rhizoctonia root and hypocotyl rot, although mycorrhizal plants generally had less disease. Equally, post-infection of beans with a highly pathogenic isolate of *R. solani* did not alter mycorrhizal colonization. Thus, the symbiont and the pathogen mutually did not influence the extent of *R. solani* infection or *G. intraradices* colonization. These results are in agreement with previous studies conducted with mycorrhizal soybeans infected with *R. solani*, in which *G. mosseae* had no effect on the incidence of infection of soybeans by the pathogen (Zambolim and Schenck, 1983; Wyss *et al.*, 1991), and simultaneously the pathogen did not influence the course of mycorrhizal colonization (Wyss *et al.*, 1991).

5.2 Defense response of bean plants to *R. solani*

The present study with *R. solani*-infected bean plants demonstrates that, as in the case of many dicotyledenous plants, defense-related genes are induced in response to infection by fungal pathogens (Collinge and Slusarenko, 1987; Dixon and Harrison, 1990). This is further substantiated by the detection of constant levels of H1 transcript levels, confirming that the increases seen for the defense-related gene transcripts are therefore

part of a selective response to elicitation. The results demonstrate that expression of plant defense-related genes such as the cell wall structural HRGP and genes of the phenylpropanoid pathway are induced spatially and temporally in beans post-infected with the compatible pathogen *R. solani,* regardless whether or not the plants were mycorrhizal. Even though transcript levels of all defense genes increased concomitantly in roots, stems and leaves, there was a differential expression in which levels of all defense genes were generally higher in roots than in stems and leaves. The observation that defense reactions were expressed with a much higher magnitude in *R. solani*-infected mycorrhizal plants than in mycorrhizal plants not infected with the pathogen support the hypothesis that a signal produced by the pathogen is essential for triggering synthesis and accumulation of defense gene products. A similar conclusion was reached in the case of mycorrhizal Ri T-DNA carrot roots colonized by the AM fungus *G. intraradices* and post-infected with *Fusarium oxysporum* f. sp. *chrysanthemi* (Benhamou *et al.*, 1994).

Generally in this study, the highest levels of HRGP, PAL, CHS and CHI transcripts occurred late in the infection process when fully developed lesions had formed on the hypocotyls and *R. solani* root colonization was at the highest levels observed. These results reconfirm what others have reported on the accumulation of several plant defense compounds including phytoalexins (Stockwell and Hanchey, 1987), β-1,3-glucanases, chitinases and peroxidases (Wasfy *et al.*, 1984; Xue *et al.*, 1998), and the presence of cell wall structural phenolic compounds (Smith *et al.*, 1975; Jabaji-Hare *et al.*, 1999) at the sites of lesion development in response to infection of bean hypocotyls by highly virulent *R. solani* isolates.

It is well known that pathogens or elicitors cause activation of defense mechanisms not only at the site of infection or elicitor treatment, but also in distant uninfected tissues. (Lawton and Lamb, 1987; Lafitte *et al.*, 1993; Xue *et al.*, 1998). Consistent with these reports, *R. solani* infection of beans resulted in a systemic activation of all defense-related genes studied. Transcript levels of PAL, CHS and CHI showed a very similar spatial and temporal pattern of accumulation in response to *R. solani* infection. Similar results were found with PAL, CHS and CHI transcripts, using the same cDNA clones, in bean cell suspension cultures as well as in entire plants in response to fungal elicitors and infection by *C. lindemuthianium*, another highly virulent fungal pathogen of beans (Cramer *et al.*, 1985; Mehdy and Lamb, 1987; Bolwell *et al.*, 1988). These results together with the present study support the concept that the genes coding for enzymes of the phenylpropanoid and flavonoid/isoflavonoid pathways are coordinately regulated during pathogenic infections. This is also supported by the fact that several genes encoding enzymes of the phenylpropanoid pathway share similarity in the structure of their promoters (Leyva *et al.*, 1992; Arias *et al.*, 1993).

Phenolic compounds are complex polymers formed from a mixture of simple phenylpropanoids in which PAL is a key regulatory enzyme of the phenylpropanoid biosynthetic pathway (Dixon and Paiva, 1995). Histochemical tests, conducted by Jabaji-Hare *et al.* (1999), on hypocotyls of one-week-old bean seedlings infected with the same *R. solani* isolate used in the present study, showed that high amounts of phenolic compounds were located in the walls of epidermal cells and the outermost layers of the

cortex. In contrast, these compounds were absent in healthy bean seedlings. It is believed that the deposition of these compounds may provide an effective physical and/or chemical barriers to the invading hyphae of *R. solani* (Jabaji-Hare *et al.*, 1999). Our findings that PAL transcripts rapidly accumulated in the stem tissue in response to *R. solani* infection support this hypothesis.

In the case of HRGP transcripts, which code for a cell wall structural protein, the appearance and levels of HYP4.1 mRNA transcripts coding for a HRGP differed spatially and temporally, as compared to PAL, CHS and CHI transcripts. Notably, accumulation of HRGP mRNA was delayed in the stem tissue. Similarly, Lawton and Lamb (1987) studying the expression of HRGP, PAL and CHS also showed a delayed accumulation of HRGP gene transcripts in elicitor-treated cell cultures of bean. In contrast to the stem tissue, HRGP transcripts accumulated earlier than PAL, CHS and CHI transcripts in the leaf tissue in response to *R. solani* infection. These results differ from what has been reported in other studies where accumulation of HRGP transcripts in leaves of bean plants infected with *C. lindemuthianum* coincided with the accumulation of PAL and CHI transcripts (Mahé *et al.*, 1992; Mahé *et al.*, 1993). Why only HRGP transcripts accumulated faster in leaf tissue and not in other tissues is not clear. This discrepancy may be attributed to the age of the plants at inoculation, and to the different bean cultivar and pathogen used (cultivar $P_{12}S$ and *C. lindemuthianum*) used by Mahé *et al.* versus cultivar Contender UT15 and *R. solani* in our case.

The systemic induction of defense-related gene expression in this system also addresses the question of signal migration from the roots to the rest of the plant. Many studies have indicated that salicylic acids (SA), a natural phenolic compound, is an important signaling factor in the induction of systemic acquired resistance (Klessig and Malamy, 1994; Ryals *et al.*, 1994). In a previous study, it has been observed that the concentration of endogenous salicylic acid in beans increased during infection by *R. solani.* Compared to control tissues, the amount of SA was higher in roots and stems (Jabaji-Hare, personal communication). Additional experiments are needed to understand the role of SA in this interaction.

5.3 Accumulation of defense-related gene transcripts in response to the AM symbiosis

In the root tissue, we observed that mycorrhizal colonization neither stimulated nor suppressed the levels of defense-related gene transcripts to any appreciable amounts. Similar results were reported for HRGP, PAL, CHS, CHT transcripts in bean roots colonized by *G. intraradices* (Blee and Anderson, 1996) or *G. mosseae* (Mohr *et al.*, 1998). In other studies, both stimulation and suppression of plant defense responses have been observed in root tissue in response to colonization by AM fungi (Spanu *et al.*, 1989b; Lambais and Mehdy, 1993; Franken and Gnadinger, 1994; Volpin *et al.*, 1995; Ruiz-Lozano *et al.*, 1999). In general, these studies observed an early stimulation of plant defense-gene expression, generally one to two weeks after inoculation, followed by a weak suppression as the symbiosis matured. In our study, the plants were harvested approximately 4 weeks after inoculation with *G. intraradices.* Therefore, if some type of

alteration had occurred in the root tissue early in the development of the symbiosis it would not have been observed. Localized changes in mycorrhizal root cells in the levels of transcripts encoding defense-related genes have been investigated. Arbusculated cells of several plant species including *P. vulgaris* possessed elevated glucanase, chitinase (Lambais and Mehdy, 1995), PAL, CHS (Harrison and Dixon, 1994) and HRGP (Balestrini *et al.*, 1997) transcript levels. Since in this study we analyzed RNA from the whole root system, single cell responses might have been diluted.

As far as we are aware, this is the first report examining the effect of the AM symbiosis in absence of external biotic elicitors on defense gene transcript levels in distant, hitherto non-colonized plant parts such as stems and leaves. Although recently, Shaul *et al.* (1999) assessed PR protein defense responses in abiotic elicitor-treated AM and non-AM tobacco leaves, non-elicited AM and non-AM controls were not compared. Expression of genes that are involved in cell wall modification such as HRGP are influenced by a variety of factors such as wounding (Corbin *et al.*, 1987), elicitor treatment (Showalter *et al.*, 1985), infection by a pathogen (Templeton *et al.*, 1990) and mycorrhization (Franken and Gnadinger, 1994). In our study, the accumulation of HRGP mRNA in leaves of +G-R at 3 dai in response to *G. intraradices* indicates that the AM fungus is able to induce a systemic response. Similarly in other AM systems, elevated levels of HRGP encoding mRNA have been reported in the roots of parsley (Franken and Gnadinger, 1994) and in arbusculated *Zea mays* L. cells (Balestrini *et al.*, 1997). Since we are the first to investigate the expression of HRGP in leaves of mycorrhizal plants, direct comparison with results of other research is not possible. However, it is worthwhile to point out that

irrespective of the tissue examined, whether leaves (this study) or roots (Franken and Gnadinger, 1994), accumulation of HRGP was detected in cells or tissues that do not contain AM structures, indicating a systemic effect on HRGP expression.

5.4 AM induced alterations of plant defense responses during infection by *R. solani*

Contradictory evidence has shown that, in response to infection by a pathogen, the presence of the AM symbiosis can either stimulate (Benhamou *et al.*, 1994; Cordier *et al.*, 1998) suppress (Guenoune *et al.*, 2001) or have no effect (Mohr *et al.*, 1998) on plant defense mechanisms. The results of this study clearly demonstrate that a differential spatial and temporal accumulation of defense-related gene transcripts occurs in mycorrhizal pathogen-infected plants. Increased accumulation of the cell wall structural HRGP mRNAs in roots, and PAL and CHS in leaves in mycorrhizal pathogen-infected plants versus non-mycorrhizal pathogen-infected plants was observed. This observation supports the results of Benhamou *et al.* (1994) and Cordier *et al.* (1998) who showed that as a result of AM symbiosis, cell wall remodeling, such as the cell wall thickenings, was induced in mycorrhizal carrot roots infected with *F. oxysporum* and tomato roots infected with *P. parasitica*, respectively. Conversely, suppression of defense-related genes, most notably genes coding for the phenylpropanoid pathway, was detected in the stem tissue of mycorrhizal pathogen-infected bean plants. This co-ordinate suppression corresponds to the time when reduced disease severity in AM plants was most evident. Suppression of several defense-related responses such as CHI mRNA levels (Guenoune *et al.*, 2001), PR proteins levels (Shaul *et al.*, 1999) and peroxidase activity (Guenoune *et al.*, 2001) have been also observed in various mycorrhizal pathogen-infected or elicitor-treated systems.

In addition to stimulation and suppression, we have also observed that the AM fungus *G. intraradices* induced no change in the levels of some defense-related gene transcripts, such as CHS in roots and HRGP in the stem over the course of infection by the pathogen. Similarly, unchanged levels of chitinase transcripts and PR protein levels were also reported in bean roots colonized by *G. mosseae* and infected with *F. solani* (Mohr *et al.*, 1998), and in tomato roots colonized by *G. mosseae* and infected with *P. parasitica* (Dassi *et al.*, 1998), respectively. Taken together, the results of the current study along with those of previous studies indicate that a clear trend is not evident on how the AM symbiosis affect the plant's defense responses temporally and spatially during a pathogenic interaction. Recently, Shaul *et al.* (1999) and David *et al.* (1998) proposed that the AM induced changes to plant defense responses may be mediated by phytohormones.

The observation that the dramatic difference in gene expression between AM and non-AM plants was detected only in the presence of *R. solani,* indicates that many AM-induced changes in plant defense responses may be observed only during a pathogenic interaction. In line with this observation, Pozo *et al.* (1999) detected two glucanase isoforms found only in AM tomato plants post-infected with *P. parasitica.* Taken together, the results of both studies indicate that one cannot extrapolate the effect of the AM symbiosis on plant defense responses during a pathogenic interaction by investigating AM and non-AM plants in absence of a pathogen as many AM induced effects are only present upon challenge with a pathogen.

The results of the present study indicate that the effect of the AM symbiosis on the expression of defense-related genes is complex and may result in varying responses from stimulation to suppression of gene expression depending on the gene, the tissue and the time after infection with the pathogen. Therefore, to fully understand the effect of the AM symbiosis on plant defense responses, it is crucial to investigate the state of the plant's defense mechanisms in various tissues and times after infection with the pathogen. Studies investigating plant defense responses only in the root tissue or at one time after infection with the pathogen do not provide a clear understanding of the effects of the AM symbiosis.

Stimulation of plant defense responses as a result of AM symbiosis, has been postulated as a possible cause for the reduction in disease severity observed in AM plants (Rosendahl, 1985; Caron *et al.*, 1986b; St-Arnaud *et al.*, 1994; Niemira *et al.*, 1996). Although the results of the present study shows that the AM symbiosis systemically and differentially effects the expression of defense-related genes during a pathogenic infection, they do not clearly demonstrate that induced resistance is involved in the bioprotective ability of the AM symbiosis as there was no direct correlation between AM induced defense responses and disease severity. In summary, the results of the present study supports the growing body of evidence that the effect of AM symbiosis on plant defense responses is extremely complex and more research is therefore required to further investigate what if any involvement AM induced alterations of plant defense mechanisms have on reducing disease severity in AM plants.

Chapter VI

Concluding comments

The effect of the AM symbiosis on reducing the severity of diseases caused by soil-borne pathogens has received considerable attention. Numerous mechanisms have been postulated to explain this phenomenon such as the stimulation of plant defense responses by the AM fungus which would allow the plant to more effectively respond to a subsequent pathogenic attack. This has lead many researchers to investigate how the AM symbiosis effects plant defense mechanisms. Initial research focused on how the AM symbiosis alone influences plant defense mechanisms, with more recent studies investigating the defense responses of AM plants during a pathogenic infection. With few exceptions, these studies only investigated the state of the plant's defense responses in the root tissue and only at one time point after infection with the pathogen. The lack of knowledge on the spatial and temporal effects of the AM symbiosis during a pathogenic infection formed the basis of this thesis, which investigated how the AM fungus *G. intraradices* may alter defense responses in *P. vulgaris* during a pathogenic infection by *R. solani,* and if the AM symbiosis affords beans protection against *R. solani.*

The results of the current study indicate that colonization of *P. vulgaris* by *G. intraradices* does not significantly reduce the severity of disease caused by the soil-borne pathogen *R. solani.* As this is to our knowledge the first study to investigate the *P. vulgaris-G. intraradices-R. solani* combination, further research is needed to support this finding, but it does corroborate previous work conducted on soybean by Zambolim and

Schenck (1983) and by Wyss *et al.* (1991). The results of the current study provide evidence that colonization of *P. vulgaris* by *G. intraradices* causes systemic alteration of defense responses as illustrated by the elevated HRGP transcript levels in the leaves. However, the AM symbiosis generally did not cause an alteration in the transcript levels of the other genes studied.

The results of the present study indicate, that during a pathogenic interaction, the presence of an AM symbiosis alters the defense responses in the root tissue but also in distant tissues such as the stem and leaves that are uncolonized by the AM fungus. The effect of the AM symbiosis on the levels of transcripts of defense-related genes is complex and may cause transcript levels to increase, decrease or remain unchanged depending on the time after infection with the pathogen, the plant tissue, as well as the transcript studied. This supports previous work that has shown that the AM symbiosis can have a variety of effects on plant defense mechanisms. This is one of the first studies to investigate the spatial and temporal effects of the AM symbiosis on plant defense responses during a pathogenic infection and the complexity of the results substantiate the need for further studies.

Future studies should also investigate the levels of transcripts of other defense-related genes such as cell wall hydrolases and how they are affected by the AM symbiosis during pathogenic infections, as transcript levels of glucanase and chitinase could not be investigated in the current study. It is also crucial that future research be conducted to investigate through which mechanisms the AM symbiosis alter plant defense responses

and by what mechanisms are the AM induced changes communicated to distant uncolonized regions of the plant. Importantly, as no significant AM induced reduction in disease severity was observed in the present study, further research on AM induced effects on plant defense responses should utilize AM plant-pathogen combinations where the AM symbiosis significantly reduces disease severity. As well, it would be useful to use more advanced and precise methodologies such as reverse transcriptase PCR to quantify transcript levels in the plant tissue. These new techniques are more sensitive than the slot blot technique used in this study. Research should also focus on the other mechanisms that have been proposed to explain reduced disease severity in AM plants, such as AM induced alteration in the soil's microflora which would inhibit pathogen growth (Meyer and Linderman, 1986; Filion *et al.*, 1999) and may be involved in the phenomenon.

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