THE ROLE OF HYDROGEN PEROXIDE IN THE RESISTANCE OF TOMATO TO PENETRATION BY COLLETOTRICHUM COCCODES

by

Inge Viia Foulds

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Botany University of Toronto

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Degree of Master of Science, 2000

Inge Viia Foulds

Department of Botany
University of Toronto

ABSTRACT

The accumulation of reactive oxygen species and subsequent effects were investigated in the interaction between Colletotrichum coccodes, causal agent of anthracnose, and tomato cotyledons. After initiation of penetration the fungus enters a quiescent stage and does not immediately colonize the host. Hydrogen peroxide (H₂O₂), localized with diaminobenzidine, accumulated at sites of attempted fungal penetration. In contrast, superoxide was not detected at these sites as visualized by nitroblue tetrazolium. Injection of catalase or ascorbic acid into tomato cotyledons prior to inoculation, to reduce H₂O₂ accumulation, significantly increased the frequency of fungal penetration (48 hpi) by the pathogen C. coccodes, but not the non-pathogen C. lagenarium. In the host interaction, H₂O₂ accumulation was associated with cross-linking of cell wall proteins at sites of attempted penetration. Transcription and translation inhibitor treatments did not increase fungal penetration, supporting a role for oxidative cross-linking as a resistance mechanism in this interaction.
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<td>AD</td>
<td>actinomycin D</td>
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<tr>
<td>AsA</td>
<td>ascorbic acid</td>
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<td>BS</td>
<td>blasticidin S</td>
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<td>CAT</td>
<td>catalase</td>
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<td>CHS</td>
<td>chalcone synthase</td>
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<td>cv.</td>
<td>cultivar</td>
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<td>DAB</td>
<td>diaminobenzidine</td>
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<tr>
<td>dpi</td>
<td>days post-inoculation</td>
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<td>f. sp.</td>
<td>forma specialis</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<td>GLP</td>
<td>germin-like protein</td>
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<tr>
<td>hpi</td>
<td>hours post-inoculation</td>
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<td>HR</td>
<td>hypersensitive response</td>
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<td>HRGP</td>
<td>hydroxyproline-rich glycoproteins</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<td>PAL</td>
<td>phenylalanine ammonia-lyase</td>
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<td>PR</td>
<td>pathogenesis-related</td>
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<td>PRP</td>
<td>proline-rich protein</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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INTRODUCTION

This thesis focuses on the spatial and temporal pattern of reactive oxygen species (ROS) production as a component of the defense response of tomato against the fungal pathogen, *Colletotrichum coccodes* (Wallr.) Hughes. *Colletotrichum* species infect a multitude of hosts, and commonly cause rot diseases (known as anthracnose) on numerous economically important crop plants, in both tropical and temperate regions (Bailey and Jeger, 1992). This genus of fungal pathogens can exhibit both biotrophic and necrotrophic modes of nutrition, commonly exhibiting both strategies (Skipp et al., 1995; Perfect et al., 1999). Fungal pathogens that begin pathogenesis as biotrophs and proceed as necrotrophs after initial infection are known as hemibiotrophs (Agrios, 1997). As with other *Colletotrichum* species, *C. coccodes*, may enter a stage of quiescence after initial development, i.e., the host may be infected with a pathogen but colonization (and hence symptom development) does not occur until a later time when conditions are appropriate (Skipp et al., 1995; Agrios, 1997). Consequently, tomato anthracnose is particularly a problem on processing tomatoes, as the fruits stay in the field for an extended period, prior to harvest, allowing latent infections to develop (Fulton, 1948; Dillard and Cobb, 1998).

The fungus, *C. coccodes*, was selected as a model pathogen for this study, due to its ease of culturing *in vitro* as well as its synchronous germination and penetration; all of which make it amenable for a study on the timing and role of reactive oxygen species (ROS) production. Tomato cotyledons were selected for inoculation because of their developmental uniformity, their ease of being injected for pharmacological studies, and
their short growth period. The basic approach taken to study the role of ROS in resistance was to compare the frequency of penetration and colonization by *C. coccodes* on tomato cotyledons with, and without, various treatments designed to eliminate ROS from the system. The effects of these treatments on the degree of fungal development were then assayed by the rate of successful penetration of host tissue.
LITERATURE REVIEW

An Overview of Plant-Pathogen Interactions

Most plants have evolved to be successful at resisting infection by an infinite number of potential pathogens. Among the biotic agents causing disease in plants are: bacteria, mollicutes, protozoa, nematodes, viruses, fungi, and even parasitic higher plants (Agrios, 1997). There are numerous processes employed by plants to combat potential infection. A defensive strategy may be classified as constitutive, when it pre-exists an attack by a potential pathogen, or inducible (i.e., it is expressed upon recognition of the pathogen by the host) (Agrios, 1997; Heath, 1997). If a pathogen is able to successfully colonize a plant, the invaded individual is considered a host species. Within a host species that is normally susceptible to infection by a particular pathogen, some genotypes may demonstrate resistance toward specific genotypes of the pathogen. This is known as "host" resistance (Heath, 2000). This is in contrast to "nonhost" resistance, in which a plant species is resistant to all genotypes of the particular species of pathogen (Heath, 1985; Heath 2000).

An intimate relationship is established between plant and pathogen at an early stage of the interaction. For example, fungal development in *C. gloeosporioides* is queued by specific host plant chemical signals (Podila et al., 1993). Waxes from the host avocado fruit, but not from nonhosts, induced *C. gloeosporioides* appressorial formation and avocado waxes did not induce appressorial formation of most *Colletotrichum* species that infect other hosts (Podila et al., 1993). The host surface can determine the specificity of the pathogenic interaction, where *C. graminicola*, a pathogen of barley requires a
hydrophobic surface for germination (Nicholson and Kunoh, 1995). This is contrary to
the barley powdery mildew pathogen that germinates in response to a hydrophilic surface
(Nicholson and Kunoh, 1995). An investigation into the development of powdery
mildew on barley revealed that there is a near immediate recognition of the plant surface
by fungal conidia (Nielsen et al., 2000). In this interaction conidia were found to uptake
low molecular weight anionic compounds prior to the release of an extracellular matrix
from the conidium, which precedes germination (Nielsen et al., 2000). The differentiation
of the rust fungus, *Uromyces appendiculatus*, that penetrates its host via stomata, is
queued by the host surface topography (Hoch et al., 1987). A height variation of 0.5 \(\mu m\)
was found to result in maximal appressorial formation (Hoch et al., 1987). Plants
respond to pathogen attack with a wide array of defensive strategies. The following
section outlines some of the defense mechanisms exhibited by plants in response to
pathogens, subsequent to their recognition.

**Plant Defense Responses**

For a pathogen to be successful at infecting a plant, it must avoid triggering, or
overcome, a battery of plant defense mechanisms that are targeted to restrict microbial
entry and proliferation. Plant defense mechanisms can categorically be thought of as
offensive strategies, that directly weaken or destroy the pathogen, or as defensive
strategies, that strengthen the plant to resist infection by potential pathogens (i.e., the
construction of structural barriers). Constitutive plant defense mechanisms, such as
structural, chemical, molecular, and life history traits (i.e., the time of flowering) may act
to deter successful infection by a potential pathogen. Inducible defenses may also be
engaged in attempts to prevent infection, including the production of defense-related proteins, ROS, antimicrobial compounds, structural changes in the plant cell wall, and death of the plant cell (Heath, 1997). The production of ROS and defense gene induction is of particular interest in this study and are reviewed below.

**Pathogenesis-Related Proteins**

Pathogenesis-related proteins (PR proteins) are potentially fungitoxic products that are found to be induced in plants after attack by pathogens or in related stress situations. PR proteins have been classified into 14 families based on function, sequence, and various other properties (Van Loon and Van Strien, 1999). Typically PR-proteins appear in both resistant and susceptible plant responses, though they appear earlier in the resistant response. For example, upon infection of tomato leaves with the biotrophic fungus *Cladosporium fulvum* (Cooke), there is a differential accumulation of PR proteins, whereby they appear earlier in an incompatible interaction (host resistant) than in a compatible interaction (host susceptible). Chitinases and β-1,3-glucanases have been successfully purified from apoplastic fluids of an incompatible interaction between *C. fulvum* and tomato and these enzymes have been shown to function in degradation of fungal hyphal walls (Joosten and De Wit, 1989).

One family of PR-proteins, PR-1, has an as of yet unknown function (Van Loon and Van Strien, 1999), although transgenic tobacco constitutively expressing PR-1a exhibits a reduced disease severity in response to two oomycete pathogens (Alexander et al., 1993). The tomato PR protein P14, serologically related to the tobacco PR-1, has been shown to have antifungal activity when tested both *in vivo* and *in vitro* against zoospores
of *Phytophthora infestans* (Niderman *et al.*, 1995). Evidence of anti-fungal activity of PR-1 is also supported by the inhibition of differentiation of the rust fungus, *U. fabae*, by treatment with purified PR-1 protein obtained from intercellular fluid extract from induced resistant broad bean plants (Rauscher *et al.*, 1999). I used an isomer of tomato P14, P4, to monitor the characteristic induction of this PR-gene in tomato tissue after pathogen attack.

**Reactive Oxygen Species**

Reactive oxygen species (ROS) are toxic intermediates arising from the reduction of molecular oxygen, and include, superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (·OH); their production in plants has been observed upon both biotic and abiotic stress stimuli. Although accumulation of ROS to high levels is phytotoxic, it has become clear, that in many cases, they play a central role in plant protection against pathogens. Rapid and transient production of ROS in defense is often referred to as the oxidative burst. Various mechanisms have been postulated to be involved in the generation of ROS during plant defense, among them: NADPH-oxidase, cell wall peroxidase, amine oxidase, and germin-like oxalate oxidase (reviewed by Mehdy, 1994; Baker and Orlandi, 1995; Bolwell *et al.*, 1995; Mehdy *et al.*, 1996; Lamb and Dixon, 1997; Wojtaszek, 1997; Bolwell and Wojtaszek, 1997); amine oxidases are common in legumes, and germin-like oxalate oxidases are found primarily in cereals (Bolwell and Wojtaszek, 1997). With over two decades of work in this field, much knowledge has accumulated on the generation and roles of ROS production in plant defense. In
reviewing some of this work, I will focus on the production of ROS as it relates to defense against fungal pathogens.

Initial evidence for ROS generation in plant defense comes from a study conducted in potato tissue in response to *P. infestans*. Potato tissue inoculated with incompatible races of the oomycete *P. infestans*, exhibits a hypersensitive response (HR), which is the death of plant cells at the site of infection that is associated with limitation of pathogen spread (Goodman and Novacky, 1994). Doke (1983a) demonstrated that upon inoculation of potato tubers with an incompatible race of *P. infestans*, the HR was correlated with both cytochrome c and nitroblue tetrazolium (NBT) reduction. This did not occur in the compatible interaction. The reducing activity was inhibited by superoxide dismutase (SOD), indicative of O$_2^-$ production. Reduced NBT was also localized around fungal hyphae prior to host cell death. Treatment with hyphal wall components alone also elicited the same reducing activity as inoculation with the fungus. SOD treatment prior to fungal inoculation also significantly delayed the onset of the HR, thus implicating the involvement of superoxide (O$_2^-$) generation as a part of the defense response (Doke, 1983a). The effect of a SOD pre-inoculation treatment on the success of fungal colonization, however, was not examined. Doke (1983b) also demonstrated that O$_2^-$ generation in potato tuber protoplasts treated with hyphal wall components of *P. infestans*, was enhanced by NADPH, implicating plasma-membrane NADPH oxidation in this reaction.

Various approaches have been used to study ROS, including work on plant tissue and plant cell culture, both having been performed with a variety of pathosystems (reviewed in Doke, 1997). A selection of reports will be reviewed, to compare the
findings from studies conducted on various interactions. Cell suspension culture is amenable for demonstrating the rapid and transient production of ROS in response to pathogen challenge. For example, tomato suspension cells treated with *C. fulvum* race-specific elicitors resulted in the production of ROS in incompatible interactions, but not in compatible interactions (Vera-Estrella *et al.*, 1992). Treatment of French bean cell cultures with *C. lindemuthianum* elicitor results in the production of H₂O₂, but not O₂⁻ (Bolwell *et al.*, 1998), in contrast to the potato-*P. infestans* interaction, and highlights the variability in ROS production among interactions.

In addition to cell culture studies elicitors have been used to examine the accumulation of ROS in whole tissue. Treatment of tomato cotyledons with race-specific elicitor from *C. fulvum* resulted in the production of O₂⁻, as detected by NBT reduction (May *et al.*, 1996). *In planta* production of ROS, both O₂⁻ and H₂O₂, has also been shown in tomato leaves following *C. fulvum* elicitor treatment (Lu and Higgins, 1998). Through the use of a fluorescent probe, the concentration of H₂O₂ production in elicitor treated tomato leaves was estimated to be on the order of 10-20 mM (Lu and Higgins, 1998).

Many studies have measured ROS production by cytochrome c reduction and by chemiluminescence assays, both *in planta* and in cell culture, and often with elicitor treatment, rather than use of the pathogen itself. Extrapolation of these results to an intact system may therefore not be representative of the ROS production at particular plant-pathogen interaction sites. Other studies on ROS have involved the use of a whole plant and pathogen. In the interaction between barley and the barley powdery mildew fungus, H₂O₂ was detected and localized with a histochemical stain (diaminobenzidine) to cells interacting with the fungus at cell wall appositions known as papillae. In this system H₂O₂
accumulation was also detected in whole epidermal cells undergoing the HR (Thordal-Christensen et al., 1997). Close examination of the effect of ROS on the HR has also been conducted in the cowpea-cowpea rust pathosystem (Chen and Heath, 1994; Heath, 1998). The time of cell death, as measured by cell autofluorescence, is extended by the addition of ROS scavengers (Chen and Heath, 1994). However, the use of ROS scavengers to monitor the early stages of cell death by cellular disorganization at the microscopic level suggests that ROS are not involved (Heath, 1998). In addition, cytological staining to localize ROS also failed to show that they were present in the early stages of cell death. Thus, in the cowpea-cowpea rust interaction ROS are only involved in the later stages of the cell death response, after protoplast collapse, and precludes ROS from a role in triggering the HR (Heath, 1998).

The creation of transgenic plants to elevate the constitutive levels of ROS has also been used to study disease development and defense responses. Transgenic potato plants transformed with a glucose oxidase-encoding gene, to elevate levels of endogenous H$_2$O$_2$ production, demonstrated increased resistance to several pathogens (Wu et al., 1995; Wu et al., 1997). Elevating the constitutive levels of H$_2$O$_2$ was found to lead to various changes in the transgenic potato plants, such as an increase in defense-related gene induction, elevated lignin content, increased peroxidase levels, and an increase in total salicylic acid (SA) content (Wu et al., 1997).
Functions of the Oxidative Burst

Current evidence points to various functions for ROS production, including, direct toxicity, cellular signaling, cell wall strengthening, and the induction of programmed cell death (reviewed in Sutherland, 1991; Mehdy, 1994; Baker and Orlandi, 1995; Tenhaken et al., 1995; Mehdy et al., 1996; Doke et al., 1996; Lamb and Dixon, 1997; Dat et al., 2000).

Antimicrobial Activity

Numerous studies have investigated a potential antimicrobial role for ROS. In vitro generation of H$_2$O$_2$ was found to completely inhibit spore germination of three fungi, at concentrations of 26.1 $\mu$M (Peng and Kuc, 1992). Also in this study, tobacco leaf discs exogenously treated with peroxidase and NADH or NADPH, to generate H$_2$O$_2$ at the time of inoculation with Peronospora tabacina had a reduction in the development of blue mold. This demonstrated the potential inhibitory effects of peroxidase generated H$_2$O$_2$ in disease resistance.

The effect of H$_2$O$_2$ on fungal viability has also been examined in germination and germ tube growth assays with C. fulvum. It was demonstrated that application of 4 mM H$_2$O$_2$ significantly inhibited in vitro spore germination, after a high humidity incubation, whereas 6 mM H$_2$O$_2$ caused an almost complete inhibition of germination (Lu and Higgins, 1999).

The inhibition of germ tube growth rather than the inhibition of conidial germination, was shown to be a factor in tomato resistant to C. fulvum (Lazarovits and Higgins, 1976). The in vitro effect of H$_2$O$_2$ on germ tube elongation was thus examined
on germinated conidia. Germ tube elongation was found to be significantly inhibited at 5 mM H$_2$O$_2$, after an 18 hr post-germination incubation period. Significant inhibition of germ tube elongation was obtained with a 5 mM H$_2$O$_2$ treatment and a complete inhibition of germ tube elongation was obtained with a 20 mM treatment (Lu and Higgins, 1999). This study was extended by use of fluorescein diacetate, to test germ tube viability. Germ tube death was found at concentrations as low as 5 mM H$_2$O$_2$ (~20% germ tube death), and the ED$_{50}$ value for germ tube death was 22.4 mM. This indicates that a comparable concentration of H$_2$O$_2$ is sufficient to almost completely inhibit conidial germination (6 mM), as is required to significantly reduce germ tube elongation (5 mM) (Lu and Higgins, 1999).

Cell Wall Fortification

Among the proposed roles of ROS in disease resistance is strengthening the cell wall against microbial attack via the catalysis of oxidative cross-linking reactions of cell wall proteins. Bradley et al (1992) found that treatments with elicitors obtained from fungal cell walls (P. megasperma or C. lindemuthianum) resulted in a loss of sodium dodecyl sulphate (SDS)-extractability of two proteins (p33 and p100) in soybean cell culture and one (p35) in bean cell culture. Characterization of one of the soybean proteins revealed that it is a proline-rich protein (PRP), which is a cell wall structural protein. H$_2$O$_2$ treatment was also found to induce the same phenomenon as the fungal elicitors, thus showing that ROS treatment could stimulate cell wall protein cross-linking in the same manner as a pathogen-mediated stimulus (Bradley et al., 1992). The insolubility of these proteins could be reduced by an antioxidant treatment, thus reinforcing the idea that
the protein insolubilization was due to H$_2$O$_2$-mediated oxidative cross-linking of these wall proteins (Bradley et al., 1992). In a subsequent study, it was shown that oxidative cross-linking makes soybean cells refractory to cell wall digestion by a protoplasting solution. This protein insolubilization was found to be independent of transcription and translation and mediated by peroxidase (Brisson et al., 1994). Inoculation of soybean leaves with the bacterial pathogen Pseudomonas syringae pv. glycinea, resulted in protein insolubilization in the incompatible interaction, as compared to readily extractable proteins in the compatible interaction, thus demonstrating the occurrence of cross-linking in planta (Brisson et al., 1994).

Similar work conducted on chickpea cell cultures demonstrated an insolubilization of cell wall structural proteins which was activated by an NADPH-dependent elicitor-induced oxidative burst (Otte and Barz, 1996). This study also implicated peroxidas in the catalysis of the oxidative cross-linking reaction. Moreover, protein kinase and phosphatase activity was shown to affect this phenomenon, thus implicating their involvement in a signal transduction pathway mediating oxidative cross-linking.

Of the three cross-linking studies that have just been discussed none demonstrated any direct histological observation of cross-linking in planta, as much of the work was conducted in cell culture. Microscopic examination of the barley-powdery mildew interaction, however, revealed protein cross-linking in papillae and in cells undergoing a HR, with both corresponding to the locations where H$_2$O$_2$ was detected (Thordal-Christensen et al., 1997).
Secondary Signaling

H₂O₂ has been proposed as a signaling molecule in the induction of defense responses. Early evidence for signaling functions of ROS came from monitoring low molecular weight antimicrobial compounds, known as phytoalexins, that are commonly formed by plants in response to pathogen attack (Paxton, 1981). It was observed that glyceollin, a soybean phytoalexin, was rapidly induced in *Verticillium dahliae* elicitor treated soybean cell culture (Apostol *et al.*, 1989). It was shown through H₂O₂ application and catalase sensitivity that H₂O₂ was sufficient and necessary for glyceollin production, suggesting the involvement of H₂O₂ in a defense signaling pathway (Apostol *et al.*, 1989). In a subsequent study, however, by Davis *et al.* (1993) it was shown that the protein and carbohydrate portions of the crude elicitor preparation initially used on the soybean cell culture independently elicited the accumulation of the phytoalexin and of H₂O₂, respectively. After initial reports that H₂O₂ application alone induced phytoalexin production (Apostol *et al.*, 1989), it has been further examined whether this ROS affects the gene expression levels of phytoalexin biosynthetic enzymes. Direct application of H₂O₂ is transient with respect to ROS exposure levels, therefore, treatment with ROS generating systems has proved to be a useful tool.

Application of glucose and glucose oxidase, a H₂O₂ generating system, to soybean cell culture strongly induced transcript accumulation of the cellular protectant gene, glutathione S-transferase (GST) (Levine *et al.*, 1994). Addition of H₂O₂ alone also induced GST transcript accumulation and the accumulation rate was faster than GST gene induction resulting from treatment with elicitor from *Phytophthora megasperma* f. sp. *glycinea* (Pmg). The expression of a cellular protectant gene demonstrates that the
protection of self from potential damage caused by ROS production during the oxidative burst is also at play, not simply gene induction to cause injury to the pathogen. Two other genes, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), which are enzymes involved in the biosynthesis of defense-related compounds in the phenylpropanoid pathway, were also monitored in the study by Levine et al (1994). In contrast to GST, PAL and CHS were only weakly induced in glucose/glucose oxidase treated cell culture, as compared to the Pmg elicitor treatment (Levine et al., 1994). Although the temporal effect of gene induction varied with elicitor treatment or enzymatic generation of H$_2$O$_2$, this is supportive evidence for gene induction by ROS.

As previously discussed, Wu et al (1997), demonstrated that transgenic potatoes expressing glucose oxidase-expressing transgenic potato, had elevated levels of two defense gene transcripts. Other transgenic work has similarly elucidated a role for H$_2$O$_2$-mediated defense gene expression. Transgenic tobacco with antisense expression of a catalase gene has also indicated that pathogen-induced defense gene expression may function via a H$_2$O$_2$ mediated signaling pathway (Chamnongpol et al., 1998). Sublethal accumulation of H$_2$O$_2$ in these plants, generated by photorespiration under high light conditions, resulted in a local induction of acidic and basic PR-proteins, along with an increased tolerance to the bacterial pathogen, Pseudomonas syringae pv. syringae (Chamnongpol et al., 1998).

In an Arabidopsis cell culture, addition of the bacterial elicitor, harpin, triggers the production of H$_2$O$_2$ and results in the initiation of the cell death process (Desikan et al., 1998). This system has been used to study the molecular effects of ROS. It was demonstrated that H$_2$O$_2$ was required to elicit a signal transduction pathway leading to
cell death after harpin treatment. Additionally, there was H$_2$O$_2$-specific gene activation, namely induction of PAL and GST. Gene induction by harpin was shown to act through both H$_2$O$_2$-dependent and independent pathways. The two H$_2$O$_2$ regulated genes both contain a promoter region that bears similarity to the binding sequence of the animal transcription factor, NF-κB, which is sensitive to oxidation status. In animal systems H$_2$O$_2$ is thought to release the IκB inhibitory protein from the transcription factor complex thus implicating oxidation status in the stimulation of gene expression (Desikan et al., 1998).

There are however conflicting results with respect to the role of H$_2$O$_2$ in defense gene expression. A study by Dorey et al (1999) found that addition of glucose/glucose-oxidase to tobacco cell culture did not result in PAL induction, nor did it enhance elicitor induced PAL expression. Nevertheless, evidence is accumulating for direct signaling functions of ROS in plants, as has already been shown in mammals (Schreck et al., 1991; Allen and Tresini, 2000). For example, the GST6 promoter in Arabidopsis has been shown to be induced by H$_2$O$_2$ (Chen and Singh, 1999). In Arabidopsis leaf cells, a mitogen activated protein kinase (MAPK) has been shown to be activated by H$_2$O$_2$ (Kovtun et al., 2000), and a receptor-like protein-kinase (PK) gene in Arabidopsis is activated by both oxidative stress and by pathogen attack (Czernic et al., 1999). Also, in a recent study by Desikan et al (2000) differential display was successfully used in the identification of genes induced by H$_2$O$_2$, with the isolation of several putative signaling molecules. Future work stands to more clearly elucidated the role of ROS in defense signaling among plant-pathogen interactions.
*Colletotrichum coccodes* as a Plant Pathogen

*Colletotrichum* species have proven to be versatile models for a wide range of studies concerning plant-fungal interactions. *C. coccodes*, the causal agent of anthracnose on solanaceous crops, primarily infects the aerial parts of susceptible plants, although root infection has been reported (Kendrick and Walker, 1948; Pantidou and Schroeder, 1955; Dillard, 1992). Leaflet and cotyledon infections begin as small, sunken lesions that expand and eventually become surrounded by a chlorotic halo (Younkin and Dimock 1944; Kendrick and Walker, 1948). Foliar infection was found to be a source of secondary inoculum, potentially leading to the development of anthracnose on tomato fruits (Pantidou and Schroeder, 1955). In a compatible interaction germination and sporulation of the fungus on leaves causes necrotic spots with a chlorotic halo. Fruit infection begins as a small fleck that spreads into a sunken rot lesion that may merge with other lesions (Dillard, 1992). Initial fruit infection may take place in green fruit, but the fruit generally appears asymptomatic as the pathogen enters a quiescent stage until host maturity when visible lesions appear (Fulton, 1948). The plant hormone ethylene induces fruit ripening, while also inducing the development of infection structures in *Colletotrichum* species infecting climacteric fruit (Flaishman and Kolattukudy, 1994). This demonstrates how the pathogen can efficiently use a host signal to time it’s own development (Flaishman and Kolattukudy, 1994).

Conidia of *Colletotrichum* germinate after adhesion to the plant surface, form a germ tube, and subsequently develop appressoria. Although the initial infection strategies may vary somewhat among *Colletotrichum* species, infection develops when a penetration peg, emerging from the penetration pore in the melanized appressorium
directly penetrates the plant cuticle, forming an infection vesicle (IV) and a primary hypha in the epidermal cell. Often, penetration is aided by the activity of fungal hydrolytic enzymes (Knogge, 1998). Initially no damage to the cell is seen in this interaction. This is considered the biotrophic phase, which is followed by the development of secondary hyphae which cause necrosis, initiating the necrotrophic phase (Skipp et al., 1995; Perfect et al., 1999). In tomato, the formation of pigmented appressoria reaches a maximum by about 24 hours post-inoculation (hpi) in *C. coccodes* (Byrne et al., 1997). Melanization, as seen in the appressorium of *C. coccodes* is common among fungal pathogens that directly penetrate the plant cuticle. Among the pathogens with melanized appressoria is the rice blast fungus, *Magnaporthe grisea*. In *M. grisea* the intracellular concentration of glycerol was shown to account for the generation of turgor pressure (De Jong et al., 1997). Melanin is essentially impermeable to glycerol, thus allowing for the development the high pressure and facilitating mechanical entry. Mutant albino *C. lagenarium*, incapable of infecting cucumber, was rescued with a melanin biosynthetic gene and pathogenicity was restored (Kubo et al., 1991), demonstrating the importance of melanized appressoria for successful infection. Elaborate optical measurements have revealed the mechanical force exerted by a *C. graminicola* appressorium to be 17 N (Bechinger et al., 1999). Melanin is also an important pigment in the protection against environmental stresses; whether melanization of appressoria increases their survival during the quiescent stage of infection remains to be studied.
Resistance mechanisms to Colletotrichum Species

Due to the diversity of Colletotrichum species, and their wide host range, there is a diverse array of mechanisms engaged by plants against these fungal pathogens. Lignification is a frequent cell wall response to infection that may serve to fortify the cell wall against potential pathogens. This response has been associated with the attempted penetration of C. lagenarium, where lignification appeared more rapidly and extensively in systemically protected leaves (Hammerschmidt and Kuc, 1982). Lignification may function to protect against enzymatic degradation of plant tissue by pathogens and to strengthen the cell wall against direct penetration. With respect to other structural defenses, the formation of papillae is a response to infection against Colletotrichum species in both susceptible and resistant plant varieties (Esquerré-Tugayé et al., 1992). Papillae are heterogeneous wall appositions, that may be induced upon fungal attack and other stimuli (Aist, 1976). Their formation is induced post-inoculation in the wall beneath the appressorium in resistant oat leaves, in response to C. graminicola (Politis, 1976). Papilla formation has also been reported in cucumber leaves inoculated with C. lagenarium; appearing more frequently in response to penetration in induced resistant than in control plants (Strumm and Glesser, 1986). Within papillae, hydroxyproline-rich glycoproteins (HRGP) have been found to accumulate in bean at sites resistant to infection by C. lindemuthianum (O'Connell et al., 1990). These proteins comprise the extensin family of plant cell wall proteins, and are thought to serve a function in strengthening the plant cell wall (Showalter, 1993). HRGP content has been shown to accumulate in a number of interactions involving Colletotrichum species (Esquerré-Tugayé et al., 1992). Extensin content was shown to increase in melons infected with C.
*lagenarium* (Esquerré-Tugayé and Mazau, 1974), and HRGP enrichment in muskmelon cell walls has been correlated with an increased resistance to *C. lagenarium* (Esquerré-Tugayé *et al.*, 1979). Moreover, HRGP transcript levels in bean cell cultures treated with *C. lindemuthianum* elicitor, accumulated faster in resistant than in susceptible hosts (Showalter *et al.*, 1985). The initial response in HRGP transcripts was slower than accumulation of phytoalexin biosynthetic enzymes, although overall HRGP transcript accumulation was more prolonged (Showalter *et al.*, 1985).

In addition to HRGP mRNA accumulation, other plant defense related transcripts have been demonstrated to increase in response to *Colletotrichum* species. Both chitinase and β-1,3-glucanase are highly expressed in host cells upon attack. These enzymes have been identified and purified from the interaction between *C. lindemuthianum* and French bean (*Phaseolus vulgaris*) seedlings (Daugrois *et al.*, 1990; Daugrois *et al.*, 1992). The accumulation of these enzymes is faster in incompatible, than in compatible interactions (Daugrois *et al.*, 1990). In resistant bean inoculated with *C. lindemuthianum* the defense genes PAL and CHS were also found to accumulate earlier than in a susceptible host (Bell *et al.*, 1986).

With the exception of a couple of studies, the production and effect of ROS have not been well characterized in plant defense against *Colletotrichum* species, or in response to other hemibiotrophic fungal pathogens. Suspension cultured bean cells treated with an elicitor from *C. lindemuthianum* elicitor, to produce an incompatible interaction lead to the expression of various metabolic changes, such as lipid peroxidation (an indicator of ROS), phenolic accumulation, and phytoalexins. Treatment of bean
cotyledons with ROS generators also lead to the accumulation of phytoalexins and was correlated with an increase in lipid peroxidation (Rogers et al., 1988).

**Thesis Objectives**

In the present study the in planta production of ROS was examined during a compatible interaction between tomato cotyledons and the fungal pathogen *C. coccodes*. Specifically, I investigated the production of ROS in response a fungal pathogen that enters a quiescent stage prior to the onset of penetration and colonization. Initial experiments showed that in young (2 wk old) cotyledons, after formation of an appressorium and initiation of a penetration peg the fungus progressed no further until the cotyledons reached maturity (5 wk old). This quiescent stage then ends and is completed with the development of primary hyphae into epidermal cells. The quiescent stage of younger tissue could be circumvented with a pre-inoculation antioxidant treatment. Hence, this histological system was used to more clearly elucidate the role of ROS as direct or indirect effectors in the resistance to fungal penetration.
MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (Lycopersicon esculentum) plants, cv. Moneymaker, were grown in the controlled environment of a growth chamber at 22°C with a 16 hr photoperiod, and a light intensity of ≥200 mol photons m⁻²s⁻¹. Seeds were germinated in 12 cell plastic packs (4 cm x 3 cm; Plant Products Co., Ltd., Brampton, ON), containing sterilized general purpose growing medium (Pro-mix BX, Premier Horticulture Ltd., Rivière-du-Loup, Québec). Plants were watered daily and fertilized once a week with 20:20:20 (N:P:K) fertilizer. Experiments were performed on cotyledons after first leaf emergence at which time the growing tips of the plants were removed so the cotyledons would expand and be retained.

Fungal Growth Conditions and Inoculation

Cultures of Colletotrichum coccodes and C. lagenarium were obtained from infected fruit and conidia were preserved cryogenically at −80°C in 50% glycerol. The fungus was subcultured on V8 medium, by inoculation with a conidial suspension, then incubated (22°C, 16 hr photoperiod). For plant inoculation, two week old cultures, were aseptically suspended by flooding Petri plates with sterile dH₂O and filtering the suspension through four layers of cheesecloth. The inoculum concentration was determined by use of a haemocytometer and adjusted to 5x10⁵ conidia/ml for C. coccodes and 5x10⁶ conidia/ml for C. lagenarium. Prior to inoculation Tween-20, was added to the conidial suspension, to give a final concentration of 0.05% (v/v).
Cotyledons were spray inoculated with an atomizer on their adaxial surface. Inoculated plants were stored in covered plastic tubs to maintain conditions of high humidity for the duration of each experiment.

**Histological examination**

At various times (see below) after inoculation, cotyledons were excised at the petiole, decolourized in boiling 95% (v/v) ethanol for 10 min, cleared in saturated chloral hydrate, and mounted on glass slides in modified Hoyer’s medium (Cunningham, 1972). Tissue was viewed with a light microscope equipped with Nomarski optics (Zeiss Photomicroscope).

For each experiment, 50 sites of attempted penetration were examined on 4 cotyledons from separate plants and each experiment was repeated at least once, with similar results. Each figure is a representative result from one experiment.

**Reactive Oxygen Species (ROS) localization**

*In planta* Detection of H$_2$O$_2$

H$_2$O$_2$ was localized *in planta* using 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Sigma). In the presence of endogenous plant peroxidases, DAB is oxidized by H$_2$O$_2$ to a stable reddish-brown precipitate. An aqueous solution of DAB (2 mg/ml; Heath, 1998) was injected into the intercellular space via the abaxial cotyledon surface, and left to react for 2 hr. Tissue samples were harvested and processed as above at 3 hr intervals from 0 hpi to 24 hpi, and then again at 48 hpi. DAB staining was recorded as
present or absent for each appressorium attempting to penetrate (i.e., with a visible penetration pore).

To ensure that the histochemical localization was specific to H₂O₂, tissue was co-injected with DAB and the H₂O₂ scavenger, catalase (CAT; from bovine liver; Sigma, EC 1.11.16) or with DAB and the superoxide scavenger, superoxide dismutase (SOD; from bovine erythrocytes; Sigma, EC 1.15.1.1). Catalase was dissolved in an aqueous solution of 0.1% thymol to a concentration of 3.7x10⁶ U/ml, this stock was further diluted to 1000 U/ml in sterile Milli-Q H₂O for injection into cotyledons (Wolfe et al., 2000). SOD was dissolved in 10 mM potassium phosphate buffer, pH 7.8, to a concentration of 3.0x10⁴ U/ml, and diluted to 900 U/ml (Heath, 1998) in sterile Milli-Q H₂O prior to injection. Inoculated control cotyledons were co-injected with equal final concentrations of thymol or buffer, as appropriate. These and all subsequent injections were performed with a 30G½ needle on a 1c.c syringe.

As a positive control for SOD activity cotyledons were inoculated with 10⁶ cfu/ml of Pseudomonas syringae pv. morosprunorum PM21 (from Dr. A. Jones, Michigan State University, via Dr. D. Cuppels, Agriculture and Agri-Food Canada) resuspended in 10 mM MgCl₂. Bacteria were pressure infiltrated with a needleless 1c.c. syringe. Cotyledons were then co-injected with 0.05% p-nitroblue tetrazolium (NBT; Calbiochem; see below) and 900U/ml SOD or with NBT alone. The staining intensity was compared in NBT versus SOD/NBT co-injected cotyledons.
**In planta Detection of O$_2^-$**

To localize any O$_2^-$ production *in planta* following inoculation with *C. coccodes*, cotyledons were injected with 0.05% NBT in water (Heath, 1998). NBT was left to react for 20 min, whereby a blue formazan reaction product will form from the reduction of NBT by O$_2^-$. Inoculated tissue samples were harvested and processed as above at 3 hr intervals from 0 hpi to 24 hpi, and then again at 48 hpi. Cotyledons were also inoculated with 10$^6$ cfu/ml *P. syringae* pv. *morosprunorum* PM21 and injected with NBT at 24hpi, as a positive control for NBT.

**ROS Scavenger and Antioxidant Treatments**

Catalase was injected (as above) prior to inoculation, to test the effect of scavenging H$_2$O$_2$ on the frequency of fungal penetration. The water soaking was allowed to disappear prior to inoculation of cotyledons with *C. coccodes*. Similarly, an aqueous solution of the antioxidant L-ascorbic acid (AsA; BDH Inc.) was injected prior to inoculation at a concentration of 10 mM (Wolfe *et al.*, 2000), to test for any effect on fungal development. SOD was injected (as above) to examine the effect of superoxide scavenging, as a pre-inoculation treatment, on the frequency of fungal penetration. Control (untreated) cotyledons received injections with the appropriate solvent used in the various treatment regimes, prior to inoculation. All tissue samples were harvested at 48 hpi.
Inhibitor and Peptide Treatments

Aqueous solutions of actinomycin D (AD; from *Streptomyces* species; Sigma) at 10μg/ml and blasticidin S-HCl (BS; from *S. griseochromogenes*; ICN) at 1 μg/ml, inhibitors of translation and transcription respectively, were injected into cotyledons prior to inoculation (Heath, 1979). Sterile MILLI-Q H₂O was injected into control cotyledons. When water soaking disappeared cotyledons were inoculated. Some inhibitor-injected cotyledons were left uninoculated to see if the chemical treatments alone had any effect on the leaves. All tissue samples were harvested at 48 hpi.

As a positive control for the transcription and translation inhibitors, cotyledons were inoculated with bacteria to test for the effect of the chemicals on the HR, that requires active plant metabolism, including gene transcription and *de novo* protein synthesis (Keen *et al.*, 1981; He *et al.*, 1993, 1994; Desikan *et al.*, 1998). When dry, cotyledons were inoculated *P. syringae* pv. *morosprunorum* PM21 resuspended in 10 mM MgCl₂ (as before). Plants were examined for a macroscopic HR after 24 hpi.

Peptides containing the RGD (Arg-Gly-Asp) sequence have been shown to interfere with integrin-mediated extracellular matrix binding in animal systems and more recently RGD-binding sites have been found to play a role plant plasma membrane-cell wall adhesion (Canut *et al.*, 1998). RGDS peptides (Sigma) were injected into cotyledons at a concentration of 500 μM in dH₂O to examine the necessity for plasma membrane adhesion to the cell wall as a part of the defense response (Kiba *et al.*, 1998). RGES peptides, which do not bind interfere with RGD-mediated adhesion were used at the same concentration to serve as a negative control in this study. Tissue was inoculated after water soaking disappeared and harvested at 48 hpi. As a positive control for the
peptides, Hechtian strands, linking the cell wall to the plasma membrane, were observed in living tissue (Canut et al., 1998).

A summary of all chemical treatments applied to cotyledons with application concentration and function is given in Table 1.

**Tissue Preparation for Detection of Cell Wall Protein Cross-linking**

To examine tissue for H\textsubscript{2}O\textsubscript{2}-induced cross-linking of cell wall proteins, cotyledons were inoculated on their abaxial surface to allow use of epidermal peels for observation. Catalase injected (as above) and solvent injected cotyledons were both inoculated to investigate differences in protein cross-linking with or without H\textsubscript{2}O\textsubscript{2} accumulation. Epidermal peels were harvested at 48 hpi. To remove soluble proteins peels were placed on depression slides with 1% SDS in the well and incubated at 80°C for 24 hr at high humidity. As a positive control, cell wall proteins were cross-linked by a 10 hr incubation in 2% gluteraldehyde, and then subject to the same treatment as the experimental slides. Proteins were subsequently stained with 0.1% (w/v) Coomassie Brilliant Blue (Colab Laboratories, Inc.) in 40% (v/v) ethanol and 10% (v/v) acetic acid at 90°C for 10 min, followed by destaining in 40% (v/v) ethanol and 10% (v/v) acetic acid at 90°C for 5 min (Thordal-Christensen et al., 1997). Peels were then examined for cross-linked proteins, by recording the presence or absence of a blue-stained plant cell wall underlying a melanized appressorium.
Lignin Staining

Inoculated plant tissue was stained for lignin, to investigate whether it accumulated in the plant cell wall in response to attempted penetration by the fungus. Tissue for staining was either fixed in 95% (v/v) ethanol or used fresh and subsequently mounted on a slide in a solution of saturated aqueous phloroglucinol (Fisher) in 20% HCl. Tissue was harvested for staining at 24 hpi and at 5 dpi. Tissue was observed with a light microscope, with positive lignin staining indicated by a red-violet colour (Jensen, 1962).

Statistical Analysis

Data are presented as frequency of a given response of the total number of attempted penetration sites observed in a given repeat of an experiment. The arcsine transformation was used to normalize the frequency data obtained in each histological experiment. Comparisons were made by performing the Student’s t-test, where P≤0.05 was considered significant. If data failed a normality or an equal variance test Mann-Whitney rank sum test was performed. All statistical analyses described were performed with SigmaStat Version 2.03 and graphs were created with SigmaPlot Version 5.0.

RNA Isolation

Cotyledons were either inoculated (as previously described) or sprayed with sterile dH2O (0.05% Tween-20). Total RNA was prepared from tomato cotyledons with TRIReagent (MRC, Inc.), using 100mg of tissue for each extraction. The manufacturer’s protocol was followed, including a 12 000 g spin for 10min at 4°C prior to the addition of
chloroform to remove insoluble materials and the addition of a high salt precipitation solution after the addition of isopropanol to reduce contamination by polysaccharides. RNA quality and quantity were determined spectrophotometrically (Hewlett Packard 8452A Diode Array Spectrophotometer) at 260 nm and 260/280 nm respectively.

**Northern Analysis**

Each RNA sample (5 μg of total RNA) was added to sample preparation buffer (21% formaldehyde, 61% formamide, 6% 1X MOPS, and 40% gel loading dye, containing 40 μg/ml ethidium bromide), heated for 10 min at 95°C, and placed on ice for one minute. Samples were then loaded onto a denaturing 1.5% agarose-formaldehyde gel (1X MOPS and 18% formaldehyde) and separated by electrophoresis in 1X MOPS buffer. The gel was blotted overnight to Hybond N+ nylon membrane (Amersham Pharmacia Biotech) by capillary transfer in 10X SSC (Sambrook *et al.*, 1989). RNA was UV crosslinked (UV Stratalinker 2400) to the membrane at 1200 μwatts cm⁻².

After drying, membranes were prehybridized in Church's hybridization solution for 2 hr at 65°C in a hybridization oven (Hybridizer HB-2D, Techne) prior to the addition of the ³²P-labeled probes (preparation described below) for an overnight incubation. Membranes were washed, enveloped in plastic wrap, stored in a phosphor imaging cassette (BIO-RAD), and subsequently visualized using BIO-RAD Molecular Imager FX and Quantity One software.
Preparation of Radiolabelled DNA Probes

Transcript accumulation for the tomato PR-protein P4, an isomer of the tomato P14 PR-protein (van Kan et al., 1992), was monitored. A probe for detection of P4 transcripts was amplified by PCR from tomato genomic DNA. Genomic DNA was extracted from the tomato cultivar Moneymaker carrying the Cf-5 resistance gene. Primers used to amplify the PR-gene, P4, were designed based on the GenBank nucleotide sequences, with the 5' to 3' sequences of GGG GTT GTT CAA CAT CTC and GAA CCT AAG CCA CGA TAC. The PCR annealing temperature was 55°C and was run for 30 cycles. PCR products were run on a 0.7% agarose gel in 1X TAE. The appropriate fragment (706 base pairs) was then excised from the gel to use as a probe for Northern blot analysis. The excised fragment was incubated for 5 min at 100°C in two volumes of dH2O and stored in microfuge tubes in aliquots of about 25 ng. Unlabelled probe was stored at –20°C.

Ribosomal DNA (rDNA) was used as a loading control for Northern analysis. The plasmid containing the 3.7 kb rDNA fragment was isolated from E. coli via alkali lysis (Sambrook et al., 1989), run on a gel and isolated as described above.

Fragment aliquots were boiled in a water bath for 10 min, then incubated at 37°C for 5 min, prior to labelling. The probe was then labelled with α32P dCTP (Amersham Pharmacia Biotech) using the Rediprime Labelling Kit (Amersham Pharmacia Biotech) and incubated at 37°C for one hour. The probe aliquot was then boiled for 5 min, prior to addition into the hybridization tube.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (Source)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ascorbic acid (AsA)</td>
<td>10mM (BDH Inc.)</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>1000 U/ml (Sigma)</td>
<td>H$_2$O$_2$ scavenger</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>900 U/ml (Sigma)</td>
<td>O$_2^-$ scavenger</td>
</tr>
<tr>
<td>Actinomycin D (AD)</td>
<td>10 μg/ml (Sigma)</td>
<td>Transcription inhibitor</td>
</tr>
<tr>
<td>Blasticidin S (BS)</td>
<td>1 μg/ml (ICN)</td>
<td>Translation inhibitor</td>
</tr>
<tr>
<td>RGD(E)S-peptides</td>
<td>500 μM (Sigma)</td>
<td>Interferes with plasma</td>
</tr>
<tr>
<td></td>
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<td>membrane-cell wall adhesion</td>
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</tbody>
</table>
RESULTS

Temporal Pattern of Reactive Oxygen Species (ROS) Accumulation

The production of ROS was examined in tomato cotyledons in response to inoculation with conidia of *C. coccodes*. Diaminobenzidine (DAB) was used to detect \( \text{H}_2\text{O}_2 \) production during attempted penetration. In tissue inoculated with *C. coccodes*, reddish-brown DAB staining was primarily localized to the anticlinal plant cell wall subtending an appressorium; staining was also frequently visible in the periclinal cell wall (Figure 1A). At 24 hours post-inoculation (hpi) DAB staining was present at a high percentage of the sites (Figure 2).

The specificity of DAB for \( \text{H}_2\text{O}_2 \) was ensured by its co-injection with the ROS scavengers catalase or superoxide dismutase (SOD). Co-injection of catalase with DAB significantly decreased the frequency of DAB staining at sites of attempted penetration, as compared to injection with DAB alone (Figure 1B and 2). In contrast, co-injection of cotyledons with SOD and DAB did not result in a significant difference in DAB staining frequency as compared to tissue injected with DAB alone (Figure 2). As a positive control for SOD activity, cotyledons were injected with nitroblue tetrazolium (NBT), a histochemical stain to localize \( \text{O}_2^- \) production, or co-injected with NBT and SOD, 24 hpi with the non-pathogen, *P. syringae* pv. *morosprunorum* PM21. When NBT was co-injected with SOD, there was a visible reduction in blue staining as compared to tissue injected with NBT alone, as observed macroscopically, indicating that the SOD was active at the concentration used.
Figure 1. Localization of $H_2O_2$ production at sites of attempted penetration. Cotyledons were inoculated with *C. coccodes* (5x10$^5$ conidia/ml) and injected with DAB (2 mg/ml) (A) or co-injected with DAB and CAT (1000 U/ml) (B) 2 hr prior to harvest at 24 hpi. Tissue was examined by light microscopy, equipped with Nomarski optics (magnification X 360). The appressorium (a) on DAB injected tissue has staining in the anticlinal (thick arrow) and periclinal (thin arrow) cell walls. The bars in the lower right corners indicate 20 $\mu$m.
Figure 2. Frequency of H$_2$O$_2$ accumulation at sites of attempted penetration at 24 hpi and the effect of ROS scavengers on DAB staining frequency. Tomato cotyledons were inoculated with C. coccodes (5x10$^5$ conidia/ml). Two hours prior to harvest tissue was injected with DAB (2 mg/ml) or co-injected with DAB and the ROS scavenger CAT (1000 U/ml) or SOD (900 U/ml). Tissue was harvested at 24 hpi. The values represent the mean ± SD of four replicates, with 50 sites each. The asterisk represents a significant difference between control and experimental treatments, where P≤0.05.
DAB staining was monitored from 0 to 24 hpi, sampling every 3 hr, and again at 48 hpi. From 0 to 6 hpi no DAB staining was observed in the tissue. At 9 hpi staining was observed in the anticlinal cell walls of the tissue. Since C. coccodes conidia do not necessarily adhere to the tissue at such an early developmental stage, during tissue fixation, it was not possible to tell if this staining was in response to conidal germination. Occasionally, however, the DAB staining was in the vicinity of a conidium. The first appressoria began to form at 12 hpi; by 15 hpi a significant number of appressoria had formed to count the frequency of DAB (Figure 3). Staining frequency was found to increase to 24 hpi. By 48 hpi the frequency of DAB staining was once again reduced to levels seen at 21 hpi.

A time course experiment (similar that described above) was also performed with NBT stained tissue to detect any O$_2^-$ production in response to inoculation with C. coccodes. There was no detectable blue formazan staining at any of the time points examined from 0 hpi to 48 hpi. As a positive control for NBT, greatly increased blue formazan staining was visible in cotyledons after bacterial inoculation with P. syringae pv. morosprunorum PM21, as compared to mock-inoculated tissue.

**Effect of Transcription and Translation Inhibitors on H$_2$O$_2$ Accumulation**

Actinomycin D and blasticidin S, inhibitors of transcription and translation, respectively, were employed to test the effect of these processes on H$_2$O$_2$ accumulation in response to challenge by C. coccodes. Leaves were inoculated after the inhibitor treatment and injected with DAB prior to harvest at 24 hpi. There was no significant effect of either treatment on H$_2$O$_2$ accumulation (Figure 4).
Figure 3. Frequency of \( \text{H}_2\text{O}_2 \) accumulation following inoculation. Tomato cotyledons were inoculated with \textit{C. coccodes} \( (5\times10^5 \text{conidia/ml}) \) and injected with 2 mg/ml DAB prior to the indicated harvest time. DAB staining frequency at appressoria was recorded at 15, 18, 21, 24, and 48 hpi. The values represent the mean ± SD of four replicates with 50 sites each.
Figure 4. Effect of the transcription inhibitor, actinomycin D (AD), and the translation inhibitor, blasticidin S (BS), on H$_2$O$_2$ accumulation. Tomato cotyledons were injected with 10 $\mu$g/ml AD or 1 $\mu$g/ml BS and control cotyledons were injected with water. After water soaking disappeared cotyledons were inoculated with C. coccodes (5x10$^5$ conidia/ml). Prior to harvest, at 24 hpi, tissue was injected with DAB (2 mg/ml). The values represent the mean ± SD of four replicates with 50 sites each.
**Effect of ROS Scavenger Treatments**

In the natural situation, initial successful penetration of tomato cotyledons by *C. coccodes* seemed a rare, if almost absent event on healthy cotyledons, even by 48 hpi. In a study on tomato foliage most appressoria had already formed, melanized, and attempted penetration by 24 hpi. yet no successful penetration was observed (Byrne et al., 1997). To investigate the importance of H$_2$O$_2$ production in limiting the early development of infection by *C. coccodes*, ROS scavengers were applied to prevent their accumulation and the effects on penetration were examined.

The H$_2$O$_2$ scavenger, catalase, the antioxidant, L-ascorbic acid, and the O$_2^-$ scavenger, SOD, were injected into cotyledons, and water soaking was allowed to disappear, prior to inoculation. The effect of each treatment was determined from the frequency of successful penetrations at 48 hpi. Successful penetration resulted in hyphal growth that either penetrated into both the epidermal and mesophyll cell layers, or into the epidermal cell layer alone (Figure 5A and 5B). Growth was intracellular with no evidence of intercellular colonization by 48 hpi. At later points when tissue was highly damaged, it became difficult to determine the specifics of infection histologically. Both the catalase and ascorbic acid treatments resulted in a significant increase in the penetration efficiency of *C. coccodes*, as compared to controls (Figure 6). There was a rise in penetration efficiencies from about 5% penetration to over 30% with each of these treatments. A pre-inoculation treatment with SOD, however, did not result in a significant increase in the penetration efficiency, as compared to the control. This indicates that the accumulation of H$_2$O$_2$, but not O$_2^-$, is critical to maintaining resistance to penetration.
Figure 5. Successful penetration of tomato cotyledons following antioxidant treatment. Tissue was injected with ascorbic acid (10 mM) and when water soaking disappeared the tissue was subsequently inoculated with *C. coccodes* (5x10⁵ conidia/ml). Penetration was monitored at 48 hpi. Hyphal (h) growth originating from an appressorium (a) in an epidermal (A) or mesophyll cell (B). An appressorium that did not successfully penetrate the host (C). Tissue was viewed by light microscope equipped with Nomarski optics (magnification X 360). The bars in the lower right corners indicate 20 μm.
Figure 6. Effect of ROS scavengers on penetration frequency. Catalase (CAT), L-ascorbic acid (AsA), and superoxide dismutase (SOD) were injected into tomato cotyledons at concentrations of 1000 U/ml, 10 mM, and 900 U/ml, respectively. Control tissue (CAT-; AsA-; SOD-) was injected with the appropriate solvent for each treatment. When water soaking disappeared, cotyledons were inoculated with C. coccodes (5x10^5 conidia/ml). Tissue was harvested at 48 hpi. The values represent the mean ± SD of four replicates each with 50 sites each. The asterisks represent a significant difference between control and experimental treatments, where P≤0.05.
The cotyledons injected with catalase prior to inoculation showed disease at a macroscopic level, as indicated by the presence of brown necrotic lesions by 48 hpi (Figure 7). Inoculated, untreated and uninoculated, catalase treated leaves remained symptomless at 48 hpi. SOD treated leaves, both inoculated and uninoculated, appeared symptomless at a macroscopic level.

Effect of Scavenging H2O2 After Appressorial Formation

To examine how quickly the effect of H2O2 is engaged in establishing a restriction of visible infection, H2O2 was removed from the system by a catalase injection at 24 hpi and subsequent fungal development was examined at 72 hpi (i.e., 48 hours post-catalase treatment, as in Figure 6). In this manner I could check whether a later addition of catalase would still allow for successful penetration, as it did when it was applied prior to inoculation. When catalase was applied at 24 hpi there was no significant increase in fungal penetration as compared to the control tissue that had been injected with the solvent alone (Figure 8). This indicates that the effects of H2O2 on the restriction of penetration in this system are established within the first day post-inoculation.

Defense Gene Accumulation Post-Inoculation

Changes in gene expression profiles are a common response by plants in a pathogenic situation. Defense gene expression was monitored to examine whether tomato cotyledons exhibit an induced response to C. coccodes inoculation at a transcriptional level. P4 is a basic isomer of the tomato PR-protein, P14, that has been shown to have antifungal activity (van Kan et al., 1992; Niderman et al., 1995).
Figure 7. Macroscopic symptoms of disease after a pre-inoculation catalase treatment. Tomato cotyledons were injected with 1000 U/ml catalase (CAT) or control solution (CTR). When water soaking disappeared tissue was inoculated (in) with *C. coccodes* (5x10^5 conidia/ml) or mock-inoculated (un). Tissue was harvested at 48 hpi.
Figure 8. Effect of catalase (CAT) treatment after appressorial formation on the penetration frequency. Cotyledons were inoculated with *C. coccodes* (5x10^5 conidia/ml), and at 24 hpi injected with CAT at 1000 U/ml or with control solution (CAT-). Tissue was harvested at 72 hpi. The values represent the mean ± SD of four replicates with 50 sites each.
Upregulation of P4 expression has been confirmed in tomato foliage inoculated with the biotrophic fungus, C. fulvum (van Kan et al., 1992), the oomycete, P. infestans (Fidantsef et al., 1999), and with the bacterium P. syringae pv. tomato (Fidantsef et al., 1999). P4 transcript levels were monitored, via Northern analysis, in C. coccodes inoculated and mock-inoculated tissue. In a time course study conducted post-inoculation, tomato cotyledons had an upregulation of P4 transcripts by 36 hpi and this expression was increased by 48 hpi (Figure 9). Mock-inoculated tissue did not exhibit comparable levels of P4 induction. The same membrane probed with rDNA is shown as a reference for the relative loading of RNA among samples (Figure 9).

**Effect of Transcription and Translation Inhibitor Treatments**

To examine the effect of transcription and translation on the success of C. coccodes penetration of tomato cotyledons by 48 hpi, an inhibitor for each of these two processes was employed. As the fungus developed similarly on inhibitor treated and on control tissue, there appeared to be no direct effect of the inhibitors on fungal development. The transcription inhibitor, actinomycin D, was applied prior to inoculation to examine the role of transcription in the early restriction of fungal development. There was no significant increase in the frequency of fungal penetration as compared to water injected control tissue (Figure 10). Treatment of cotyledons with the protein synthesis inhibitor, blasticidin S, also did not result in a significant increase in fungal penetration frequency as compared to water injected control cotyledons (Figure 10).
Figure 9. P4 expression in tomato cotyledons. Cotyledons were inoculated with C. coccodes (5x10^5 conidia/ml) or mock-inoculated (control), and tissue was collected at -2, 0, 6, 12, 24, and 48 hpi. Total RNA (5 μg) was used for RNA gel blot analysis and membranes were hybridized with a tomato P4 DNA probe. As a reference for relative loading for the quantity of RNA between lanes membranes were probed with rDNA.
Figure 10. Effect of the transcription inhibitor, actinomycin D (AD), and the translation inhibitor, blasticidin S (BS), on penetration frequency. Tomato cotyledons were injected with 10 μg/ml AD or 1 μg/ml BS and control cotyledons (AD-; BS-) were injected with water. After water soaking disappeared, cotyledons were inoculated with C. coccodes (5x10^5 conidia/ml). Tissue was harvested at 48 hpi. The values represent the mean ± SD of four replicates with 50 sites each.
To ensure that the inhibitors were active *in planta* in this experimental system, they were tested for their ability to delay a bacterially induced HR. At 24 hpi with the non-pathogen, *P. syringae pv. morosprunorum* PM21, there was no macroscopic HR response in AD or in BS treated tissue, whereas tissue that was not treated with the inhibitor did exhibit an HR.

**Effect of RGD-Peptide Treatment**

Peptides containing the RGD (Arg-Gly-Asp) sequence have been shown to disrupt plant plasma membrane-cell wall adhesion (Canut *et al.*, 1988). Such adhesion has been shown to be involved in the induction of defense responses associated to pathogens (Kiba, 1998; D.G. Mellersh and Dr. M.C. Heath, University of Toronto, unpublished data). The effect of RGDS peptides on the success of penetration of tomato by *C. coccodes* was examined. When tissue was observed from samples harvested at 48 hpi, no significant difference in the frequency of penetration was found between cotyledons injected with RGDS and RGES (control) peptides (Figure 11). As a positive control for the effectiveness of the batch and the concentration of peptides, a loss of Hechtian strands in RGDS-treated tissue was observed in living, plasmolysed cells, as compared to RGES-treated tissue in both pea and cowpea tissue (D.G. Mellersh and Dr. M.C. Heath, University of Toronto, unpublished data).

**Cross-linking of Cell Wall Proteins**

To examine whether structural changes in the cell wall were a possible early defense response induced by H$_2$O$_2$ production, protein cross-linking was examined in
Figure 11. Effect of peptide treatments on the penetration frequency. RGDS-peptides, with an affinity for plasma membrane RGD-binding sites or RGES-peptides, which do not bind at these sites, were injected into tomato cotyledons at a concentration of 500 μM. After water soaking disappeared, cotyledons were inoculated with C. coccodes (5x10³ conidia/ml). Tissue was harvested at 48 hpi. The values represent the mean ± SD of four replicates with 50 sites each.
both catalase treated and control tissue. Soluble cell wall proteins were removed with SDS and the remaining cross-linked proteins were visualized with Coomassie Blue staining (Thordal-Christensen et al., 1997). Tissue that was treated with gluteraldehyde, to induce cell wall crosslinking, prior to incubation in SDS had blue staining throughout the anticlinal cell walls (Figure 12C) as compared to untreated tissue that was unstained after the SDS removal of unbound proteins (Figure 12A). Inoculated tissue often had visible staining in the anticlinal cell wall adjacent to appressoria (Figure 12B). The presence or absence of blue staining in the cell wall adjacent to where appressoria had formed was recorded (Figure 13). At 48 hpi, there was a significant difference between control and catalase treated tissue, with a reduced number of attempted penetration sites showing protein cross-linking when H$_2$O$_2$ was removed from the system.

**Absence of Lignification at Sites of Attempted Penetration**

Inoculated tissue, both fixed and fresh, was stained with phloroglucinol-HCl to investigate whether there was an accumulation of lignin at sites of attempted penetration. Only the vascular tissue stained positively with phloroglucinol, and no lignin was seen to accumulate at sites of attempted penetration. Any structural reinforcement of the cell as an induced response to *C. coccodes* inoculation of tomato cotyledons is, therefore, not due to lignification.

**Accumulation of H$_2$O$_2$ in a Nonhost Interaction**

Tomato is a host for *C. coccodes*, albeit one in which latent infection occurs, and in this interaction there was an induced accumulation of H$_2$O$_2$ in response to attempted
Figure 12. Cross-linking of cell wall proteins at sites of attempted penetration. Tissue was inoculated with *C. coccodes* (5x10^5 conidia/ml). At 48 hpi abaxial epidermal peels were incubated in 1% SDS for 24 hr (80°C) (A and B), or subjected to a 10 hr 2% gluteraldehyde incubation and then incubated in SDS (C). Subsequent to the SDS incubation, tissue was stained with 0.1% Coomassie Blue. Tissue was viewed by light microscope equipped with Normarski optics (magnification X 360). Sites of attempted penetration, were examined for the absence (A) or presence (B) of cell wall protein crosslinking surrounding appressoria (a). A control gluteraldehyde treatment caused staining of cross-linked proteins (C). The bars in the lower right corners indicate 20 μm.
Figure 13. Effect of catalase treatment on the frequency of cell wall protein cross-linking at sites of attempted penetration. Tomato cotyledons were injected with 1000 U/ml CAT, or appropriate control solvent (CAT-). After water soaking disappeared, cotyledons were inoculated with C. coccodes (5x10^5 conidia/ml). At 48 hpi unbound proteins were removed with a SDS incubation, and Coomassie Blue was used to stain remaining cross-linked proteins. The values represent the mean ± SD of four replicates with 50 sites each. The asterisk represents a significant difference between control and experimental treatments, where P≤0.05.
penetration by the fungus. To examine whether tomato cotyledons respond in the same manner to a related non-pathogen of tomato, cotyledons were inoculated with the cucumber pathogen, *C. lagenarium*. Subsequent DAB staining revealed that there was no significant difference in the frequency of sites with H$_2$O$_2$ accumulation as compared to inoculation with *C. coccodes* at 24 hpi (Figure 14).

**Effect of Scavenging H$_2$O$_2$ in a Nonhost Interaction**

Since the frequency of attempted penetration sites showing an accumulation of H$_2$O$_2$ were comparable in tomato cotyledons inoculated with the pathogen, *C. coccodes*, or the non-pathogen, *C. lagenarium*, the effects of H$_2$O$_2$ scavenging in the nonhost interaction was tested. A comparison of catalase and control (solvent injected) treated tissue, subsequently inoculated with *C. lagenarium*, resulted in no significant difference in penetration efficiency at 48 hpi (Figure 15).
Figure 14. Frequency of $H_2O_2$ accumulation after inoculation at sites of attempted penetration with a pathogen or a non-pathogen. Tomato cotyledons were inoculated with *C. coccodes* (Cc: $5 \times 10^5$ conidia/ml) or with *C. lagenarium* (Cl; $5 \times 10^6$ conidia/ml) and injected with DAB (2 mg/ml) 2 hr prior to tissue harvest at 24 hpi. The values represent the mean ± SD of four replicates with 50 sites each.
**Figure 15.** Effect of a catalase treatment on the penetration frequency of tomato cotyledons by a non-pathogen. Tomato cotyledons were injected with 1000 U/ml CAT, while control cotyledons were treated with the appropriate control solvent (CAT-). After water soaking disappeared, cotyledons were inoculated with *C. lagenarium* (5x10⁵ conidia/ml). Tissue was harvested at 48 hpi. The values represent the mean ± SD from four replicates with 50 sites each.
DISCUSSION

The primary objective of this study was to develop a system that would be amenable to the study of ROS production in a whole plant-fungus interaction, in contrast to numerous previous studies which have made use of fungal elicitor treatments and/or plant cell suspension cultures. Use of tomato cotyledons allowed for ease of injecting chemicals and cotyledons could be efficiently grown to a uniform stage for experimentation. *Colletotrichum coccodes* also exhibits reasonably synchronous germination and colonization, allowing for temporal studies on ROS accumulation and subsequent effects. The quiescent stage which *C. coccodes* enters after an initial penetration attempt proved to be a distinct phase which could be overcome by treatments with H$_2$O$_2$ scavengers, allowing colonization by this fungus which normally had a very low penetration frequency. Hence, the importance of ROS production in the resistance to penetration in this interaction was established. The spatial and temporal pattern of ROS accumulation in this system was studied in relation to its role(s) in the initial limitation of pathogen growth.

Histological staining was performed to reveal ROS accumulation patterns. Initial distinct DAB staining in association with an appressorium was detected by 15 hpi and the frequency of such staining increased until 24 hpi; this corresponds to the time at which the maximum number of appressoria have become melanized (Byrne et al., 1997). The DAB staining frequency values are indicative of an overall increase in H$_2$O$_2$ accumulation at sites of attempted penetration over the first day post-inoculation, but do not represent the time course for a single site. The specificity of H$_2$O$_2$ as the substrate for
DAB in this interaction was established with the co-application of ROS scavengers. There was a significant decrease in the DAB staining frequency of sites when catalase was injected at the same time as the DAB. In contrast, there was no significant difference between tissue co-injected with SOD and DAB versus DAB injected tissue, although SOD was able to cause a visible reduction in NBT staining during a bacterially induced HR. The co-injections confirmed that O$_2^-$ cannot act as a DAB substrate in this experimental system and that DAB staining was specific for H$_2$O$_2$. NBT staining of inoculated tissue did not localize any detectable levels of O$_2^-$ production after inoculation with C. _coccodes_. The positive NBT staining during bacterially induced HR, however, indicates that the absence of staining after inoculation with _C. coccodes_ was not due to failure of the NBT assay.

Similar DAB staining was seen at interaction sites between barley and the barley powdery mildew fungus (Thordal-Christensen _et al._, 1997). Specifically, DAB staining was detectable in papillae, which formed at sites of attempted penetration, and in whole epidermal cells undergoing HR (Thordal-Christensen _et al._, 1997). In both interactions, therefore, there is a localized accumulation of H$_2$O$_2$ at points where the fungus is attempting to gain entry into its host. As with _C. coccodes_ and tomato, in the barley-powdery mildew interaction, resistance to penetration by the fungus was associated with the accumulation of H$_2$O$_2$, but not with O$_2^-$ accumulation (Hückelhoven and Kogel, 1998; Hückelhoven _et al._, 1999). The authors hypothesize, based on the accumulation of O$_2^-$ at sites that were successfully penetrated by the powdery mildew fungus, that fungal contact with the host plasma membrane is required for O$_2^-$ generation.
Additionally, H₂O₂ accumulation in the C. coccodes-tomato interaction was shown to be independent of transcription and translation, as actinomycin D or blasticidin S, treatments did not affect this response. This is contrary to a report by Chai and Doke (1987a) where blasticidin S inhibited an oxidative burst induced by attempted penetration of potato leaves by P. infestans. Also Fauth et al. (1996) found that in cucumber hypocotyls protein synthesis was required for H₂O₂ production following C. lagenarium elicitor treatment; a pre-inoculation treatment of cycloheximide yielded hypocotyls that were compromised in their ability to produce ROS (Fauth et al., 1996). This result, however, may have been affected by their use of elicitor, rather than the whole fungus, and by the gentle cuticle abrasion used to mimic cuticle penetration to allow for elicitor application.

Although the mechanism of H₂O₂ generation in this experimental system was not directly investigated, some inferences can be made from the observed patterns. NADPH-oxidase generates O₂⁻ that may subsequently be dismutated into H₂O₂. Although short lived, it is unlikely that O₂⁻ is produced in this interaction, as it was not detected over the first 48 hr after inoculation. NBT proved sensitive enough to detect O₂⁻ produced during a bacterially induced HR in this study, and previously in tomato cotyledons after injection with race-specific elicitors of C. fulvum (May et al., 1996). Endogenous activity of scavenging enzymes may affect the detection levels of both H₂O₂ and O₂⁻. If O₂⁻ is produced in this interaction, it is being converted to H₂O₂ before it can be detected. The absence of any O₂⁻ localization during attempted penetration by C. coccodes makes NADPH-oxidase an unlikely candidate for ROS generation in this particular interaction. H₂O₂ detection in the absence of O₂⁻ production is suggestive of a peroxidase-mediated
generation of ROS in this interaction. Peroxidase has been implicated in \( \text{H}_2\text{O}_2 \) generation in other interactions, such as the response of suspension-cultured French bean cells to \textit{C. lindenmuthianum} elicitor (Bolwell et al., 1998). Inhibitors of the various ROS generators might clarify the origins of the \( \text{H}_2\text{O}_2 \) involved in limiting penetration of \textit{C. coccodes}.

Inhibitory effects of \( \text{H}_2\text{O}_2 \) accumulation on fungal development were measurable after appressorial formation. When tissue was treated prior to inoculation with the \( \text{H}_2\text{O}_2 \) scavengers, catalase or ascorbic acid, this resulted in a significant increase in penetration by \textit{C. coccodes}. As expected due to the absence of \( \text{O}_2^- \) localization, a SOD pre-inoculation treatment did not have any effect on the resistance to penetration. This suggests that, unlike \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \) accumulation is not necessary to restrict fungal development, and that there is a potential for \( \text{H}_2\text{O}_2 \) to act directly on the restriction of fungal growth.

A study conducted by Beno-Moualem and Prusky (2000) similarly supports a role for ROS production in relation to the maintenance of quiescence and inhibition of fungal development. In avocado cell cultures treated with an elicitor from the avocado pathogen, \textit{C. gloeosporioides}, \( \text{H}_2\text{O}_2 \) production was detected by a fluorescent probe. \textit{In vivo} studies also detected an increase in DAB staining intensity of pericarp tissue over a 20 hr period after inoculation. In contrast to the \textit{C. coccodes}-tomato system, however, superoxide production was also detected during this interaction in plasma membranes isolated from inoculated pericarp tissue. It is also interesting to note that in the \textit{C. gloeosporioides}-avocado interaction, plasma membranes isolated from pericarp of unripe fruits had over 2-fold higher ROS production than those from ripe fruit did, as detected by cytochrome \( c \) reduction. Although individual fruit-fungal interaction sites were not
examined, elevated ROS production in unripe fruit, correlates with the resistance of unripe fruit to *C. gloeosporioides*, as compared to ripe fruit which is vulnerable to infection by the fungus (Beno-Moualem and Prusky, 2000).

Though H$_2$O$_2$ accumulation was detected in the barley-barley powdery mildew interaction (Thordal-Christensen *et al.*, 1997; Hückelhoven *et al.*, 1999), a treatment with the antioxidant ascorbic acid did not result in an increased penetration rate by an avirulent race of the fungus (Zhou *et al.*, 2000). There was, however, a reduction in the number of cells per cm$^2$ undergoing the HR (Zhou *et al.*, 2000). Interestingly, when the HR was reduced by application of a pH 5.5 buffer, which is presumed to prevent the pH drop proposed to activate ROS, the fungus was no longer restricted (Zhou *et al.*, 2000). Unfortunately no DAB staining was reported for this particular study and so it was not directly shown that the effect of the treatment was via a reduction in ROS accumulation.

A role for ROS in the restriction of fungal colonization has also been documented for nonhost resistance. Results similar to those obtained for *C. coccodes* and its host, tomato, have been found in nonhost interactions between *U. vignae* (cowpea rust fungus) and pea plants as well as with *E. cichoracearum* (powdery mildew) and cowpea (D.G. Mellersh and Dr. M.C. Heath, University of Toronto, unpublished data). In the *U. vignae*-pea interaction H$_2$O$_2$ accumulation was detected by DAB staining and a catalase treatment prior to inoculation resulted in a significant increase in intracellular fungal growth. In the *E. cichoracearum*-cowpea interaction, both H$_2$O$_2$ and O$_2^-$ were found to accumulate. A catalase treatment resulted in an increase in fungal penetration efficiency, however, a SOD treatment had no such effect (D.G. and Dr. M.C. Heath, University of Toronto, unpublished data).
Inoculation of tomato with the non-pathogen, *C. lagenarium*, induces \( \text{H}_2\text{O}_2 \) production, however, a catalase treatment did not allow for successful penetration by the fungus. This is quite unlike the situation of a pre-inoculation catalase treatment in the host situation, or the two nonhost interactions just discussed. This suggests that the mechanics of host and nonhost resistance to *Colletotrichum* species, in tomato at least, operate differently in restricting penetration.

DAB staining, indicative of \( \text{H}_2\text{O}_2 \) production was visible in the cotyledons prior to 15 hpi, and may correspond to \( \text{H}_2\text{O}_2 \) production in response to conidial germination. There is other evidence for elicitation of an oxidative burst in response to fungal germination, for example, intact potato leaf tissue, inoculated with both compatible and incompatible races of *P. infestans* zoospores caused an increase in \( \text{O}_2^- \) production as detected by cytochrome c reduction (Chai and Doke 1987a, 1987b). An in planta effect of \( \text{O}_2^- \) scavenging, by SOD, was found to result in an increased germination rate of *E. cichoracearum* conidia (D.G. Mellersh and Dr. M.C. Heath, University of Toronto, unpublished data), suggesting a direct toxic effect of \( \text{O}_2^- \) on conidia prior to germination. In vitro inhibition of *C. fulvum* conidial germination and germ tube elongation, has also been demonstrated by exogenous application of \( \text{H}_2\text{O}_2 \) (Lu and Higgins, 1999). Peng and Kuc (1992) reported an inhibition of *C. lagenarium* germination in vitro, among spores from other fungal species, with 26.1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). As \( \text{H}_2\text{O}_2 \) concentrations generated in tomato cotyledons in response to *C. coccodes* inoculation were not measured, it is difficult to infer whether this would be a restrictive effect in planta. It was not apparent that the initial \( \text{H}_2\text{O}_2 \) production detected had any inhibitory effects on *C. coccodes* germination, however, as there was no visible difference in the number of germinated
conidia between catalase treated and control tissue. This observation was not quantified, as ungerminated conidia are frequently washed off during the fixation process.

The observed effect of H$_2$O$_2$ accumulation on the inhibition of fungal penetration of tomato by $C$. coccodes suggested that the mechanism(s) involved deserved further attention. H$_2$O$_2$ is a diffusible and relatively long lived ROS (Dat et al., 2000) that has been implicated in various plant defense responses, as a direct toxin to the pathogen, a secondary signaling molecule, a facilitator of plant cell wall structural changes, and an inducer of the HR (Sutherland, 1991; Mehdy, 1994; Baker and Orlandi, 1995; Tenhaken et al., 1995; Mehdy et al., 1996; Doke et al., 1996; Lamb and Dixon, 1997; Dat et al., 2000). After demonstrating that scavenging of H$_2$O$_2$ increased penetration, the first three of the above were considered for the potential role(s) of H$_2$O$_2$ in the restriction of fungal penetration. As there is no HR in this interaction, that mechanism was readily eliminated.

If the function of the oxidative burst is at least in part antimicrobial, it is important to consider whether this action is due to H$_2$O$_2$ being fungistatic thus restricting fungal growth, or fungitoxic, thus injuring or killing the fungus. In this particular system, though H$_2$O$_2$ seems to accumulate early at most plant-fungal interaction sites, eventually successful fungal colonization still proceeds (i.e., latent infection). This alone suggests that H$_2$O$_2$ is most likely to act in a fungistatic manner.

The timing of the restrictive effect of H$_2$O$_2$ at penetration sites was tested with catalase application at the onset of the quiescent stage (~24 hpi). When tissue was examined, after the same time lapse that permitted fungal colonization in a pre-inoculation catalase or ascorbic acid treatment (48 hr), there was no significant increase in fungal penetration. This indicates that the accumulation of H$_2$O$_2$ by 24 hpi, is
sufficient to restrict fungal development. Removal of H₂O₂ after attempted penetration, did not reverse the inhibitory effect of ROS production, suggesting that the inhibitory effect of H₂O₂ on *C. coccodes* development is achieved early but is not reversible, eliminating solely a fungistatic role for H₂O₂. The role of H₂O₂ in PR gene induction and/or in cell wall cross-linking was subsequently examined.

Initially defense gene induction was confirmed through examination of the tomato P4 gene, which is serologically similar to the tobacco PR-1 gene (van Kan *et al.*, 1992). P4 induction has previously been shown in tomato interactions with both bacteria and fungi (van Kan *et al.*, 1992; Fidantsef *et al.*, 1999). P4 gene expression was upregulated by 36 hpi in response to *C. coccodes* inoculation. Treatments with transcription or translation inhibitors, however, did not result in increased fungal penetration, suggesting that in the *C. coccodes*-tomato interaction, maintaining resistance to penetration was not dependent on these metabolic processes. This indicates that ROS activity, in conferring resistance to penetration, likely does not act via secondary signaling in this system. In addition, in a preliminary test for P4 induction after a catalase pre-inoculation treatment, there was an immediate upregulation of P4, most likely due to microbial contaminants in the catalase solution, indicating early defense gene activation. Despite the early P4 induction, fungal colonization still occurred in this tissue.

The fungus, *C. coccodes*, directly penetrates the plant cuticle to obtain entry into the plant cell. It follows then, that signals from the extracellular matrix to the plasma membrane and into the cytosol would potentially be important in mediating the plant defense response. RGD-binding sites have been shown to link the cell wall and plasma membrane (Canut *et al.*, 1998), and RGD-mediated signaling has been implicated in the
defense response of pea cell cultures treated with *Mycosphaerella pinodes* elicitor (Kiba et al., 1998). RDG-peptide treatment was therefore used to determine whether blocking the plasma membrane RGD-binding sites affected the frequency of penetration, and if so, if it operated through an effect on H$_2$O$_2$ accumulation. In the tomato-*C. coccodes* interaction, however, RDG-peptide treatment did not increase the frequency of fungal penetration. This contrasts with other interactions that have been examined, namely, the nonhost interactions between *Uromyces vignae* and pea, and in host and nonhost interactions between *Erysiphe* species and cowpea plants. In these interactions many defenses were effected by a pre-inoculation treatment with RGD-peptides (e.g., a reduction in autofluorescent phenolic compounds, decreases in the generation of extracellular H$_2$O$_2$, and decreases in callose deposition). These changes were paralleled by a corresponding increase in penetration efficiency (D.G. Mellersh and Dr. M.C. Heath, University of Toronto, personal communication). The absence of an effect on fungal penetration after a pre-inoculation treatment with RGD-peptides in the *C. coccodes*-tomato interaction suggests that either signaling across the extracellular matrix is not actively involved in resistance to penetration, or that the peptides did not remain bound to their target sites for the duration of the experiment, or perhaps that the concentration of RGD-peptides sufficient to cause changes in the defense response in the aforementioned interactions was insufficient in tomato.

On elimination of a fungitoxic or signaling role for the H$_2$O$_2$ dependent defense, it was important to look for a possible direct role for H$_2$O$_2$ in restriction of fungal growth through the induction of structural changes. In the examination of possible changes effected by H$_2$O$_2$ accumulation to strengthen the cell wall, I explored a role for lignin
formation. H$_2$O$_2$ is required for lignification and its accumulation has been localized to cells undergoing this reaction (Olson and Varner, 1993). In the C. coccodes-tomato interaction, however, lignification was not observed at penetration sites.

Examination of differences between catalase treated and control tissue, revealed a correlation between increased penetration efficiency and a reduction in cross-linking of cell wall proteins in catalase treated tissue. This suggested that the protective role afforded by H$_2$O$_2$ accumulation in the resistance to penetration may be due to strengthening of the cell wall. This is an important defense due to the speed at which it initially happens, as cross-linking occurs with pre-existing cell wall proteins to provide a physical barrier (Bradley et al., 1992; Brisson et al., 1994). Later, upregulation and de novo synthesis of HRGP, for example, may provide additional substrates for oxidative cross-linking. In this work a reciprocal approach was taken to previous techniques that have demonstrated increased cross-linking with elicitor treatment or H$_2$O$_2$ application (Bradley et al., 1992; Brisson et al., 1994), i.e., H$_2$O$_2$ removal, via catalase, lead to a reduction in cross-linking. The association of walls with increased cross-linking with sites of attempted penetration further supports this mechanism. A functional role for oxidative cross-linking in resistance has been implicated with extensins and PRP, as previously discussed, due to their participation in oxidative cross-linking. Notably addition of P. syringae pv. glycinea to soybean cell culture resulted in protein insolubilization during an incompatible interaction but not the compatible interaction (Brisson et al., 1994). Also, as reported by Thordal-Christensen et al (1997), cross-linking was found in barley at sites of attempted penetration by barley powdery mildew.
In addition to extensins and related proteins, insolubilization of cell wall germin-like proteins (GLP) in the wheat-wheat powdery mildew interaction has also been reported (Schweizer et al., 1999). Two inducible GLPs, lacking oxalate oxidase activity demonstrated by germin, were found to be pathogen inducible. When these two GLPs were transiently expressed in wheat epidermal cells, there was a significant reduction in penetration efficiency. Moreover, these two GLPs were found to be insoluble at sites of attempted penetration in the vicinity of papilla formation, implicating a function for GLP in structural reinforcement in cereals (Schweizer et al., 1999). The involvement of \( \text{H}_2\text{O}_2 \) in the cross-linking of cell walls in the \textit{C. coccodes}-tomato interaction, is further supported by the fact that inhibitors of transcription and translation were insufficient to affect fungal penetration efficiency. Cross-linking, as a mechanism to toughen the cell wall and deter pathogen ingress, has been shown to decrease plant cell protoplast release by digestive enzyme treatment, and this process is insensitive to transcription and translation inhibitors (Tenhaken et al., 1995, from Brisson et al., 1994). Future work in this system should determine if there is a reduction in cell wall cross-linking in older leaves in response to inoculation, which allow successful fungal penetration, as compared to younger tissue, which is more resistant to penetration.

\textit{Conclusions}

The accumulation of ROS in tomato cotyledons in response to inoculation with \textit{C. coccodes} was examined, where \( \text{H}_2\text{O}_2 \) was localized with the DAB histochemical stain. The effects of ROS scavenger and inhibitor treatments were examined for their effect in
the resistance to fungal penetration. The following conclusions can be made from this study:

1) H$_2$O$_2$ is produced around the site of attempted penetration as a part of the early defense response of tomato against *C. coccodes*.

2) H$_2$O$_2$ accumulation is independent of transcription and *de novo* protein synthesis.

3) H$_2$O$_2$ accumulation at sites of penetration is associated with resistance to penetration and the establishment of fungal quiescence.

4) The resistance to penetration of tomato cotyledons to the non-pathogen, *C. lagenarium*, was not affected by scavengers of H$_2$O$_2$.

5) Early resistance to penetration is not dependent on active transcription and translation.

6) H$_2$O$_2$ production at sites of attempted penetration is associated with increased cross-linking of the plant cell wall proteins.
REFERENCES


Chai HB and Doke N. 1987a. Activation of the potential of potato leaf tissue to react hypersensitively to *Phytophthora infestans* by cytopore germination fluid and the enhancement of the potential by calcium ions. *Physiological and Molecular Plant Pathology* 30: 27-37.


Doke N. 1983a. Involvement of superoxide anion generation in the hypersensitive response of potato tuber issues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. Physiological Plant Pathology 23: 345-357.


Joosten MAHJ and de Wit PJGM. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with Cladosporium fulvum (syn. Fluvia fulva) as 1,3-β-glucanases and chitinases. Plant Physiology 89: 945-951.


Younkin SG and Dimock AW. 1944. Foliage infection of *Lycopersicon esculentum* by *Colletotrichum homoides*. *Phytopathology* 34: 976-977.