CONTROL OF FUSARIUM CROWN AND ROOT ROT ON TOMATO SEEDLINGS USING SYNTHETIC IRON CHELATORS AND PHENOLIC COMPOUNDS FOUND IN LETTUCE ROOTS

by

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ABSTRACT

The role of iron chelation by ortho-dihydroxyphenolics in *Lactuca sativa* was investigated as an allelochemical mechanism of control of Fusarium crown and root rot and damping-off of *Lycopersicon esculentum* in hydroponic culture. The severity of root rot on tomato seedlings was proportional to the iron concentration of the hydroponic growth medium. Addition of the synthetic iron chelators, EDDHA and EDTA, to the growth medium lowered the severity of root rot on tomato seedlings. O-diphenol compounds in lettuce root tissue were identified by chromatography (HPLC and TLC) as caffeic acid, chlorogenic acid and isochlorogenic. Caffeic acid was also detected as a root exudate in the growth solution of lettuce plants. Mycelial growth of the FCRR pathogen was unaffected by caffeic acid or chlorogenic acid at saturation levels in the growth medium. Caffeic acid and chlorogenic acid failed to reduce the severity of FCRR symptoms on tomato seedlings and were found to be phytotoxic to tomato seedlings at the concentrations tested. The companion planting of lettuce seedlings with tomato seedlings failed to control FCRR in hydroponic culture.

Key Words: Tomato, *Lycopersicon esculentum*, Fusarium crown and root rot, damping-off, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Lettuce, *Lactuca sativa,* root exudates, caffeic acid, isochlorogenic acid, allelopathic disease control, iron chelation, EDTA, EDDHA, hydroponic culture

DEDICATION

I would like to dedicate this thesis to my parents

Frances M. MacLeod and **John M. MacLeod**

whose love and understanding made it possible.

Thank you for everything.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. James Traquair for his guidance and support throughout this adventure. I would also like to acknowledge the support, advice and technical assistance given by Betty Singh and Siva Sabaratnam. Finally I thank the staff at the Agriculture and Argi-food Canada Pest Management Research Centre for their assistance in the preparation of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BAW	n-Butanol-acetic acid-water
cv.	Cultivar
DNA	Deoxyribonucleic acid
E	Einstein
EDDHA	Ethylene diamine di(o-hydroxyphenyl acetic acid)
EDTA	Ethylene diamine tetra acetic acid
FCRR	Fusariun crown and root rot
FOL	*Fusarium oxysporum* f. sp. *lycopersici*
FORL	*Fusarium oxysporum* f. sp. *radicis-lycopersici*
FW	Fresh weight
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
i.d.	Inner diameter
M	Molar
mol	Mole
MFW	Methy isobutyl ketone-formic acid-water
N	Normal
n	Sample size
NFT	Nutrient film technique
o	Ortho
OMFRA	Ontario Ministry of Food and Rural Affairs
p	Para
$p<0.05$	Statistically significant at 5% level
$p>0.05$	Statisticallt insignificant at 5% level
PPO	Polyphenoloxidase
rpm	Rotations per minute
RT	Retention time (in minutes)
s	Second
SC	Salts-carbohydrate medium
SII	Suspected isochlorogenic acid isomer
t	Trans
TLC	Thin layer chromatography
UV	Ultra violet
v/v	Volume to volume
w/v	Weight to volume
°C	Degree Celsius

Chapter 1

Introduction

1.1 Fusarium Crown and Root Rot of Tomato and the Damping-Off of Tomato Seedling Transplants

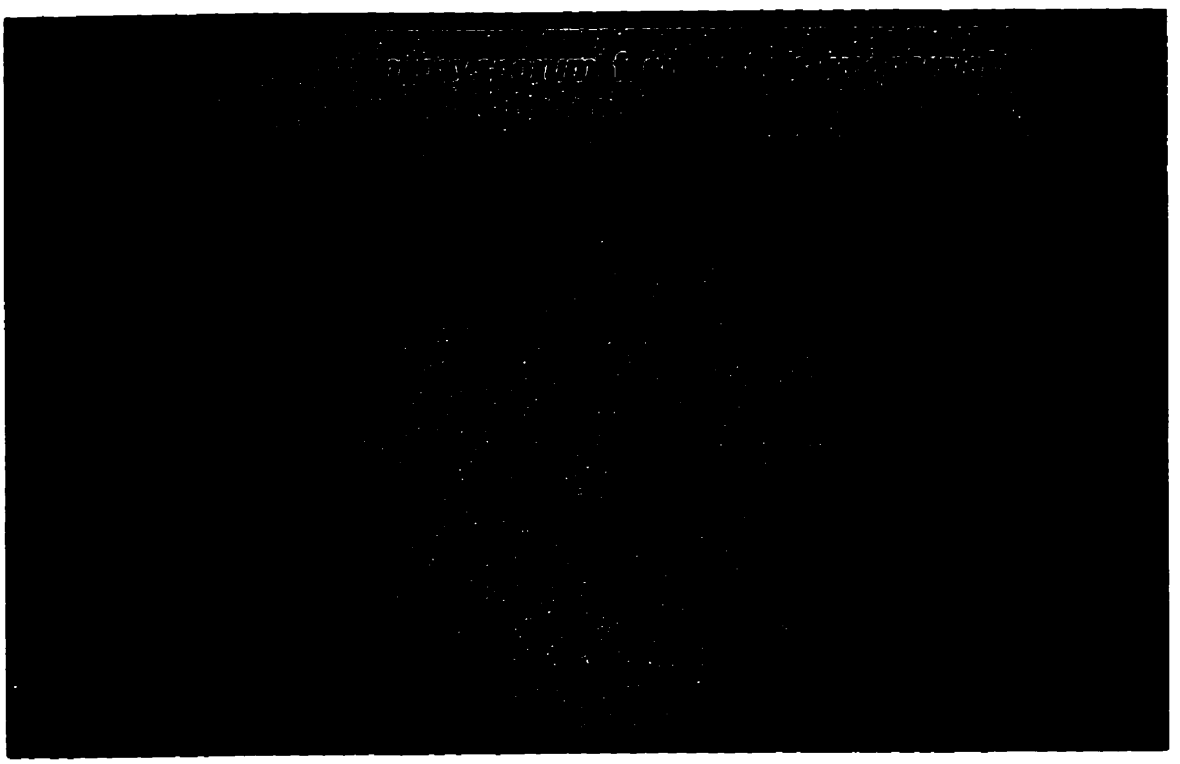
Tomato (*Lycopersicon esculentum* Mill.) is the highest value greenhouse vegetable crop in Canada (Papadopoulos, 1991). In Ontario, the combined farm value for both processing and fresh market tomatoes for 1993 was $96,961,000 (OMFRA Pub. 20). Fusarium crown and root rot (FCRR) of tomato is a serious disease of greenhouse and field grown tomatoes in various regions of the world (Datnoff *et al.*, 1995; Hartman and Fletcher, 1991; Lemanceau and Alabouvette, 1991; Mihuta-Grimm *et al.*, 1990; Sivan and Chet, 1993) including Ontario (Brammall and McKeown, 1989). FCRR is caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis and Shoemaker (FORL) (Jarvis *et al.*, 1975). The name was selected to emphasize the distinctive root-rotting symptom caused by the pathogen on its primary hosts, *Lycopersicon* species (Figure 1.1). FORL was initially identified as a new race (J3) of the tomato wilt causing *Fusarium oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyd. & Hans.(FOL) and is indistinguishable in morphology and cultural characteristics from other *Fusarium oxysporum* isolates (Figure 1.1) (Jarvis, 1988).

FORL is not a true vascular pathogen of tomato such as the tomato wilt pathogen FOL or *Verticillium albo-atrum* but is more similar to *Rhizoctonia* in that root and stem rots are the primary symptoms (Charest *et al.*, 1984). FORL is identified on the basis of

Figure 1.1: Culture of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) on potato dextrose agar medium (top) and the root rot symptom of tomato seedlings caused by FORL infection (bottom).

Top - Perti dish with mycelium of FORL growing on potato dextrose agar medium.

Bottom - Root rot symptom on tomato (*Lycopersicon esculentum*) seedlings caused by FORL infection . A non-infected seedling is on the left (1) while symptom severity increases from left to right (2 to 4).



host specificity tests (Menzies *et al.*, 1990) and the distinctive dark-brown, crown-girdling rot lesion produced on 5-day-old tomato seedlings (Sanchez *et al.*, 1975). The rot symptom produced on a tomato seedling eventually causes the seedling to damp-off (Woltz *et al.*, 1992). The severity of the root rot symptom on tomato seedlings and seedling damping-off are commonly used to evaluate the effectiveness of FCRR control treatments (Benhamou *et al.*,1994; Brammall, 1986; Kasenberg, 1991; Jones *et al.*, 1990).

1.2 Control of Fusarium Crown and Root Rot in Greenhouse Tomato Production

FCRR has the potential to become a severe problem in the Ontario-based tomato transplant production industry (Brammall and McKeown, 1989). Tomato plants often become infected with FORL as young seedlings during transplant production (Woltz *et al.*, 1992). During the transplantation and potting of tomato seedlings roots are often broken and these breaks are sites of entry for FORL (Papadopoulos, 1991). The hypocotyls (crowns) of severely infected seedlings become completely rotted and the seedlings damp-off (Woltz *et al.*, 1992), but less severely infected seedlings may continue to grow and show no disease symptoms prior to transplantation into the field or greenhouse, by which time control is impossible (Brammall and McKeown, 1989). Keeping tomato seedlings for transplantation free from FORL infection is considered to be of utmost importance to reduce losses caused by FCRR (Mihuta-Grimm *et al.*, 1990). Maintaining the pH of the growth medium above pH 6 (Jones *et al.*, 1990) and using nitrate instead of ammonia fertilizers (Woltz *et al.*, 1992) help alleviate FCRR severity on seedling transplants but the search for better control measures continues.

The use of hydroponic systems for the growth of greenhouse tomatoes from seedling transplants is becoming more widespread (Mihuta-Grimm *et al.*, 1990) because these systems provide optimum nutrient levels and minimize the impact of soil-borne pathogens (Papadopoulos, 1991). The nutrient film technique (NFT) is a hydroponic system in which plants are grown with their root systems contained in a trough through which nutrient solution is continuously circulated (Resh, 1991). NFT is potentially the most efficient method of greenhouse tomato production since it allows for maximum efficiency of fertilizer use, and permits the greatest control over the root environment (Resh, 1991). A factor limiting the use of NFT systems by greenhouse operators is the danger that recirculation of nutrient solution might spread and enhance the development of root diseases including FCRR (Papadopoulos, 1991). Once FORL is introduced into a hydroponic system it is extremely difficult to control and often leads to serious yield losses (Mihuta-Grimm *et al.*, 1990; Rowe and Farely, 1981; Vanachter *et al.*, 1988). This situation makes the use of FORL free transplants essential for control of FCRR.

The best control for any plant disease is the use of resistant varieties and a limited number of FCRR single dominant gene resistant varieties are now available (Menzies and Jarvis, 1994). However, as recently as 1995, FCRR was still considered to be a serious problem for the tomato industry in Florida and other regions where the use of susceptible tomato varieties continues (Datnoff *et al.*, 1995).

Conventional methods of disease control in greenhouses depend primarily on the use of disinfectants to sterilize equipment and the growth medium (Menzies and Belanger, 1996). Disinfection procedures are largely ineffective against FORL in

commercial greenhouses (Jones *et al.*, 1993) because of the rapid reinfestation of treated greenhouses and growing media by airborne microconidia (Marois and Mitchell, 1981). Proliferation of FORL is then rapid because of the absence of competitive microorganisms in the sterilized growing medium (Sivan and Chet, 1993). Biological controls for FCRR have been developed which involve the use of one or combinations of non-pathogenic *Fusarium* species which stimulate host defence mechanisms (Brammall, 1986; Lemanceau and Alabouvette, 1991) and hyperparasitic fungal antagonists such as *Trichoderma harzianum* Rifai which directly attacks FORL and saprophytic microbes which compete for nutrients and root colonization sites (Marois and Mitchell, 1981; Sivan and Chet, 1993). Vesicular-arbuscular mycorrhizal fungi such as *Glomus intraradices* Schenck and Smith have also been reported to lower the severity of FCRR (Sivan and Chet, 1993; Datnoff *et al.*, 1995). Unfortunately none of these biological control measures is available as a commercial products in Canada. Pre-transplantation drenches of seedlings with available biological control products based on *Streptomyces griseoviridis* (Mycostop®) and *Pseudomonas putida* (Trevisan®) have proven ineffective for controlling FCRR (Jones *et al.*, 1993).

1.3 Allelopathic Biological Control of Fusarium Crown and Root Rot

The term allelopathic control is used to describe the control of plant diseases involving either the direct incorporation of plant residues into the growth substrate or the simultaneous growth (companion planting) of another plant species with the species for which disease control is desired (Patrick, 1986). The current societal and

environmentalist pressure to reduce the use of synthetic chemicals has stimulated research towards the development of agricultural commercial products based on natural chemicals, including plant extracts (Menzies and Belanger, 1996).

The use of allelopathic control for plant disease is an ancient practice, even though the mechanisms by which allelochemicals reduce disease are complex and not fully understood (Menzies and Belanger, 1996). Aside from having direct effects on the host and pathogen, allelochemicals also affect the saprophytic microflora on and around the root and the nutritional status of the rhizosphere (Inderjit, 1996; Siqueira *et al.*, 1991).

Lettuce leaf and root tissues incorporated into the soil reduce FCRR severity on tomato and thus act as allelopathic control agents (Jarvis and Thorpe, 1981). Dandelion *(Taraxacum officinales* L.) is closely related to lettuce and both plants have similar root phenolic compositions (Bennett *et al.*, 1996; Williams *et al.*, 1996). The incorporation of dandelion residues into tomato soil and the companion planting of dandelion with tomato in soil beds or sawdust bags have been shown to be effective at controlling FCRR (Jarvis, 1988). Hartman and Fletcher (1991), however, found that lettuce and tomato companion planting in rock wool actually increased FCRR severity on the tomato plants. The reasons for the contradictory results from the companion planting studies are not known.

The chemical nature of the lettuce-mediated allelopathic control of FCRR was studied by Kasenberg (1991) but still remains unclear. He detected high levels of ortho-dihydroxy substituted phenolics (o-diphenols) in lettuce (cv. Grand Rapids) tissue. Lettuce extracts were found to demonstrate iron chelation ability. Caffeic acid, which was used as a model o-diphenol compound, reduced germination of FORL microconidia

and the growth of FORL germ tubes *in vitro*. Kasenberg (1991) found that this inhibitory effect on FORL was enhanced in an iron-limited medium. The synthetic iron chelator ethylene diamine di(o-hydroxyphenyl acetic acid) (EDDHA) was also found to limit FORL germ tube growth. Caffeic acid, lettuce leaf ethanol extracts and the synthetic iron chelator EDDHA were all found to reduce FCRR severity. Kasenberg (1991) suggested that direct toxicity of o-diphenols to FORL and iron chelation by o-diphenolics in lettuce leaf and root tissues are two possible mechanisms responsible for the allelopathic control of FCRR by lettuce residues incorporated into soil. However, the mechanism(s) of control by companion planting in hydroponic systems remains unclear.

1.4 Thesis Objectives

I hypothesize that phenolic compounds exuded from living lettuce roots lower FCRR severity by inhibition of FORL growth caused by a combination of restriction of iron availability to FORL and direct toxicity towards FORL growth. The objectives of this thesis were to evaluate the effectiveness of synthetic iron chelators and lettuce root phenolic compounds in controlling FCRR on tomato seedlings. To fulfill this objective experiments were performed to 1) determine the influence of iron deprivation of FORL on FCRR severity, 2) identify phenolic compounds in lettuce root tissues and root exudates and determine how exposure to FORL effects the phenolic content of tomato and lettuce roots, 3) determine the influence of phenolic compounds detected in lettuce root tissue on FCRR severity.

Chapter 2

Control of Fusarium Crown and Root Rot by Limiting Iron Availability to FORL

2.1 Introduction

2.1.1 Response of Fungi and Plants to Iron Limitation

Under iron limiting growing-conditions, fungi release low molecular weight compounds called siderophores into the environment (Winkelmann, 1992). Siderophores chelate soluble iron directly or competitively remove iron from less stable organic-iron complexes (Loper and Buyer, 1991). The siderophore-iron complex binds to a specific cell membrane receptor which transports the iron across the membrane (Winkelmann, 1992). The stability constant (K) of a siderophore is defined as the equilibrium constant for the formation of the siderophore-iron complex (Raymond *et al.*, 1984). The stability constant represents the affinity for ferric iron and is normally expressed in logarithmic form ($\log_{10} K$). Iron chelation is essentially the binding of a Lewis acid (iron) to a Lewis base (siderophore) to form an organic-iron complex (Manahan, 1984). Iron chelation by siderophores is highly pH dependent because iron must compete with hydrogen ions for binding to the siderophore (Raymond *et al.*, 1984).

Dicotyledonous plants do not respond to iron stress by producing iron chelating compounds to scavenge iron but instead they alter the chemical environment of the rhizosphere in order to increase iron solubility (Romheld and Marschner, 1981). Under iron stress plant roots exude organic acids and hydrogen ions into the rhizosphere (Marschner *et al.*, 1982). This exudation lowers the pH and redox level of the rhizosphere

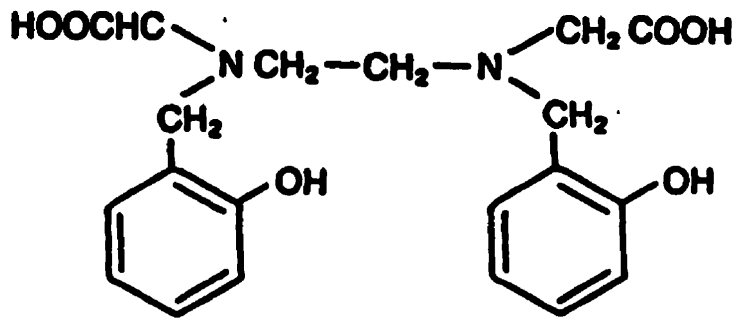
thereby increasing iron solubility (Jugsujinda and Patrick Jr., 1977). Iron uptake then occurs by means of low specificity membrane-bound reductases which transport the iron across the membrane into the root cell (Holden *et al.*, 1991).

The most abundant organic acids exuded by iron-stressed tomato roots are the ortho-dihydroxy phenolic compounds caffeic acid and chlorogenic acid (Olsen *et al.*, 1981). Olsen *et al.* (1981) found a correlation between increased iron reducing capacity and increased caffeic acid levels in the growth solutions of iron stressed tomato plants. They suggested that exuded caffeic acid may have a significant role in the up-take of iron by plant roots. Romeld and Marschner (1983), using one colorimetric assay to detect exuded o-diphenols and another colorimetric assay to detect iron reduction, concluded that o-diphenols do not contribute significantly to iron reduction by plant roots. Ortho-dihydroxy phenolic compounds found in plant tissues are however reported to solubilize iron from iron hydroxides *via* iron reduction (Boyer *et al.*, 1989) and to form insoluble iron-phenolic complexes (Brune *et al.*, 1991). Deiana *et al.* (1995) have recently studied the formation of caffeic-iron complexes in aqueous solution and found that at pH values less than 4 iron reduction with concurrent caffeic acid degradation predominates but at pH values greater than 4, caffeic acid and iron hydroxides form soluble complexes. They found that these caffeic acid-iron complexes polymerize and precipitate within 3 hours. The stability of caffeic acid-iron complexes is not known nor is it known whether they occur in soil or have ecological significance with respect to iron availability in the rhizosphere.

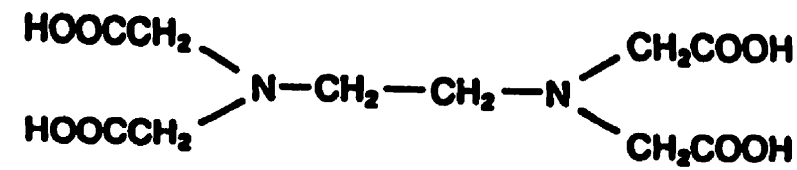
2.1.2 Control of *Fusarium* Diseases by Iron Limitation

Iron limitation not only suppresses mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) but also reduces the ability of FOL culture filtrates to induce wilting of tomato plants (Woltz and Jones, 1971). An iron concentration of 4.5 μM is the minimum requirement for normal growth of FOL mycelium and maximal wilt inducing ability of FOL culture filtrates (Woltz and Jones, 1971). Competition for iron among different microorganisms may be exploited to control soil-borne plant pathogens (Kloepper *et al.*, 1980; Elad and Baker, 1985). Synthetic iron chelators which have a greater affinity for iron than the siderophores produced by *Fusarium* species (*i.e.* larger K values) have been used to control *Fusarium* wilts (Scher and Baker, 1982). The siderophores isolated to date from *Fusarium* species are of the fusarinine type and have stability constants in the order of $\log_{10}K=30$ (Neilands and Leong, 1986). The stability constants for the ferric complexes of the synthetic chelators, ethylene diamine di(o-hydroxyphenyl acetic acid) (EDDHA) and ethylene diamine tetra acetic acid (EDTA), are $\log_{10}K=33.9$ and $\log_{10}K=25$, respectively, and thus lie on either side of that for the fusarinines (Scher and Baker, 1982). At equal concentrations EDDHA (Figure 2.1) should be more effective than EDTA (Figure 2.1) at restricting iron availability to *Fusarium* species. This, in turn, should lead to greater disease suppression by EDDHA than EDTA.

Both EDDHA and EDTA are reported to reduce the growth of germ tubes but not the germination of microconidia of *Fusarium oxysporum* f.sp. *lini* which causes *Fusarium* wilt of flax (Scher and Baker, 1982). EDDHA when added to the soil reduces the incidence of Fusarial wilt on both flax and radish, while EDTA actually increases the



EDDHA
Ethylene diamine di-(o-hydroxyphenyl acetic acid)



EDTA
Ethylene diamine tetra acetic acid

Figure 2.1: Structures of EDDHA and EDTA

incidence of both diseases (Scher and Baker, 1982). Kasenberg (1991) demonstrated that EDDHA could reduce the growth of germ tubes of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) but not reduce the germination of FORL microconidia. The results of Scher and Baker (1982) and of Kasenberg (1991) suggest that the germination of microconidia is insensitive to the addition of chelators to the medium.

Kasenberg (1991) also found that EDDHA when added to the soil reduces Fusarium crown and root rot (FCRR) severity on tomato plants. Kasenberg (1991) suggested that the mechanism of lettuce-mediated allelopathic control of FCRR could be in part the chelation of iron by o-diphenols from lettuce tissues. If iron chelation is responsible for the reduction in FCRR severity, then at equal concentrations EDDHA should have a greater efficacy in reducing FCRR severity than EDTA. The efficacy of EDTA should be reduced at pH values greater than 6 and the efficacy of EDDHA should not be affected by pH. This is because the chelation specificities for iron of EDDHA and EDTA are differentially affected by pH (Lindsay, 1974). EDDHA preferentially binds iron in the pH range of 4 to 10 while EDTA has rapidly decreasing specificity for iron as pH rises above 6 (Lindsay, 1974).

The objectives of this chapter were to assess the effectiveness of iron chelation as a means of controlling FCRR on tomato seedlings. This was accomplished by testing the ability of EDDHA and EDTA to reduce the growth of FORL mycelium and to reduce the severity of FCRR on tomato seedlings at different pHs.

2.2 Materials and Methods

2.2.1 FORL Growth Bioassay

FORL Culture and Preparation of Inoculum

A virulent isolate (cc#082) of *Fusarium oxysporum* f. sp. *radicis-lycopersici* was provided by Dr. W.R. Jarvis, Agriculture and Agri-Food Canada Research Centre, Harrow, Ontario. The fungus was maintained on a "salts-carbohydrate" (SC) medium (Table 2.1) at 4°C and transferred to new SC medium every 3 months (Kasenberg, 1991). One change in the composition of the medium used by Kasenberg (1991) was made in this study, iron was supplied as $FeCl_3$ in order to avoid exposure of FORL microconidia to EDTA prior to their use in experiments.

FORL was grown on solid SC medium at 20 °C and three mycelial plugs (6 mm diameter) from the periphery of actively growing 1 week old FORL cultures were used to inoculate 50 mL of liquid SC medium in 125 mL Erylenmeyer flasks cultures. SC liquid cultures were grown for 2 weeks on a rotary shaker (100 rpm) at 22°C. Cultures were vacuum-filtered through Whatman #3 paper to remove mycelial fragments and the filtrate was centrifuged for 5 minutes at 2700 g to pellet the microconidia. The pellet was washed with sterile water and re-centrifuged twice, and finally re-suspended in sterile water. A haemacytometer was used to determine the microconidial concentration in the FORL microconidial suspensions used as FORL inoculum.

This experiment involved 30 treatments (Figure 2.2 (A)). A total of twelve treatments for each of EDDHA and EDTA (4 concentrations X 3 pH levels) plus six controls (3 pH levels) were tested. The experiment was performed using two

Table 2.1: Composition of SC (salts-carbohydrate) medium used for *Fusarium oxysporum* f.sp. *radicis-lycopersici* propagation (based on Kasenberg,1991) and of the plant nutrient medium used for lettuce and tomato propagation.

SC Medium

1.0 g	K_2HPO_4
0.5 g	$MgSO_4 \cdot 7H_2O$
2.0 g	L-asparagine
10.0 g	D-galactose
2.7 mg	$FeCl_3 \cdot 6H_2O$
1 L	water

The resultant medium had a pH of 4.6 +/- 0.1. Solid medium was prepared by adding 20 g of Agar (BDH, Darmstadt, Germany) to 1 L of liquid medium.

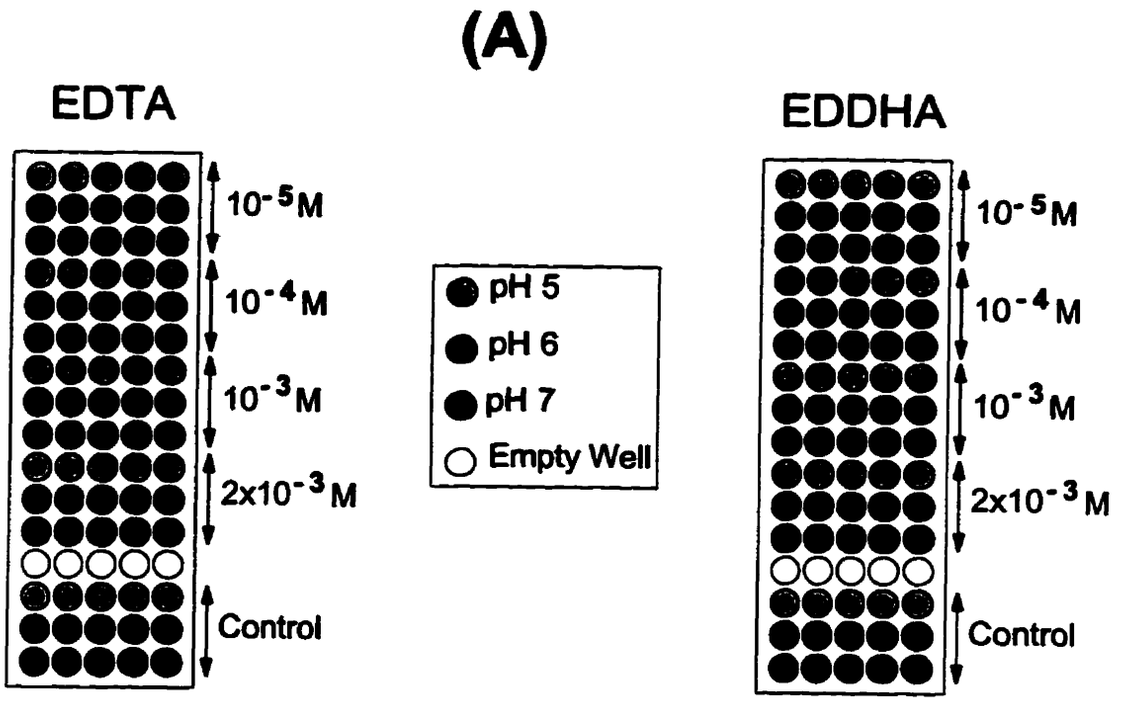
Plant Nutrient Medium

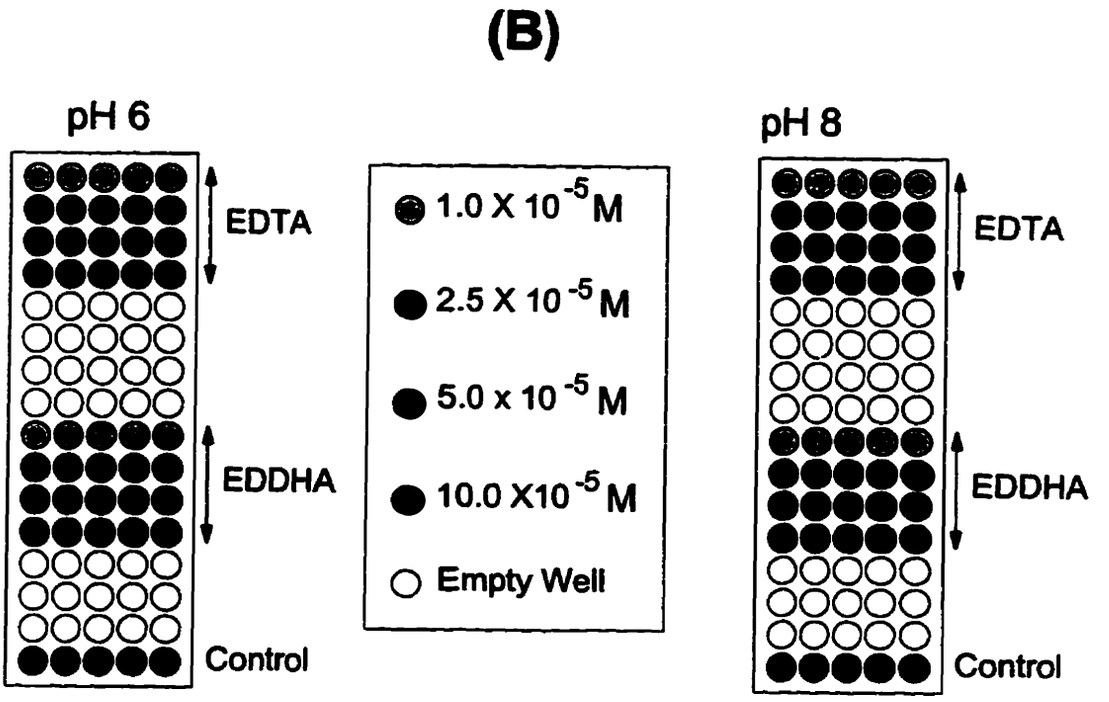
Macronutrients	Concentration
$Ca(NO_3)_2$	5 mM
KH_2PO_4	5 mM
$MgSO_4$	5 mM
KNO_3	5 mM
Micronutrients	
HBO_3	1 µM
$MnCl_2$	1 µM
$CuSO_4$	1 µM
$ZnCl_2$	1 µM
$NaMo_4$	0.1 µM
Iron Source	
$FeCl_3$	10 µM

pH was adjusted to 6.0 with 1N NaOH

Figure 2.2: Bioassays testing the effect of EDTA and EDDHA on (A) the growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) mycelium and (B) the severity of Fusarium crown and root rot (FCRR) on tomato seedlings.

Bioassays were performed in sterile Fisher brand 80® polypropylene 1.5 mL microtube racks. Details of the methodology are given in the text.


(A)
EDTA
EDDHA
10-5M
10-4M
10-3M
2x10-3M
Control
pH 5
pH 6
pH 7
Empty Well


(B)
pH 6
pH 8
EDTA
EDDHA
Control
1.0 X 10-5M
2.5 X 10-5M
5.0 x 10-5M
10.0 X10-5M
Empty Well

polypropylene 1.5 mL microtube racks (Fisher brand 80® microtube racks, Fisher Scientific, Tor., Ont.) each having 80 wells arranged in 16 rows of 5 wells. A row of wells was used as a treatment block in this experiment. The microtube racks had been autoclaved at 121°C for 15 minutes prior to use in the experiment.

Solutions of 0.001, 0.01, 1.0 and 2.0 mM EDTA or EDDHA (Sigma, St. Louis, Mo., USA) were prepared by mixing appropriate volumes of EDTA and EDDHA stock solutions (10 mM in 0.1N NaOH) with water to give final volumes of 150 mL. A 0.02 N NaOH solution (150 mL) was used as a control solution. Treatment media were prepared by adding 2 % agar (w/v) and the ingredients of SC medium (Table 2.1) to each of the solutions. The resultant treatment media were divided into three equal portions (3X50mL). The pH of each third (50 mL) was adjusted to pH 5, 6 and 7 respectively with 8 N HCl. Treatment media were autoclaved at 121° C for 15 minutes before use.

One row of wells in the first microtube rack was filled with treatment media containing each EDDHA concentration at pH 5, 6 and 7 and a row of wells of the second microtube rack was filled using treatment media containing each EDTA concentration at pH 5, 6 and 7 (Figure 2.2(A)). The surface of the medium in each well was inoculated with a 100 µL aliquot from a 10^4 FORL microconidia/mL suspension. The microtube racks were enclosed in sterile transparent plastic boxes (24cm x 14cm x 10.5cm)(Par-Pak Ltd., Canada) in order to prevent contamination. Microtube racks were incubated in darkness at 22° C for 4 days at which time mycelium growth in the wells was assessed using the rating system in Table 2.2.

The experiment was repeated once. Mean FORL mycelial growth ratings for each

Table 2.2: Rating system used to assess mycelium growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* in microtube rack well bioassays.

Index	Growth Description
0	No visible growth to the naked eye
1	Limited mycelial growth not completely covering agar surface
2	Mycelial growth completely covering agar surface
3	Moderate mycelial growth covering agar surface and extending up well walls
4	Dense mycelial growth completely filling well
5	Dense mycelial growth extending beyond well

Table 2.3: Disease index used to assess *Fusarium* crown and root rot symptoms on tomato seedlings (based on Brammall,1986).

Index	Symptoms
0	No disease symptoms
1	1 Discrete dark brown lesion on hypocotyl or radicle
2	> 1 Discrete dark brown lesion on hypocotyl or radicle
3	Numerous coalescing lesions on hypocotyl or radicle
4	Coalescing lesions completely girdling hypocotyl or radicle
5	Seedling dead

EDDHA and EDTA concentration at each pH level were compared statistically using Tukey's multiple comparison test.

2.2.2 FCRR Bioassay 1: Iron Concentration

Growth of Tomato Seedlings

Seeds of the FORL susceptible tomato (*Lycopersicon esculentum* L.) cv. Bonny Best (Stokes Seed Co., St. Catharines, Canada) were surface sterilized in 0.5% sodium hypochlorite for 5 minutes and washed 3 times in sterile water. Seeds were germinated on sterile Whatman #1 filter paper in sterile 9 cm glass Petri plates in darkness (22° C). After 5 days the roots of seedlings were inserted into the centres of sterile 2 cm plastic drinking straw segments which provided support for the seedlings. Seedlings in straw segments were placed in the wells of sterile Fisher brand 80® microtube racks. The microtube rack wells were then filled with autoclaved plant nutrient solution (Table 2.1). The microtube racks were placed in sterile transparent plastic boxes, which were placed in the greenhouse under temperature controlled conditions of 22° C for 16 hours (day) and 18° C for 8 hours (night) until use in FCRR bioassays.

Four sterile transparent boxes were filled with 250 mL of autoclaved plant nutrient medium (Table 2.1) containing 1.8% agar (w/v) at one of four $FeCl_3$ concentrations. The four $FeCl_3$ concentrations tested were 10^{-3}, 10^{-5}, 10^{-7} M and a control not containing an $FeCl_3$ amendment (iron limited).

Eight-day-old tomato seedlings were removed from the straw segments and 15 seedlings were transplanted into the plant nutrient agar medium in each of the four

transparent plastic boxes. For each of the four iron concentrations, boxes with uninoculated tomato seedlings were prepared similarly and used to assess the effect of iron concentration on the growth of tomato seedlings. After 24 hours, a 100 μL aliquot of a 10^6 FORL microconidia/mL suspension was pipetted on the surface of the agar medium proximal to each of the 15 seedlings in one set of boxes. Boxes were returned to the greenhouse and incubated under the conditions described above. No additions of water were made during the duration of the experiment and the boxes were opened daily for 5 minutes to allow aeration. Seven days after inoculation the number of damped-off tomato seedlings in each chamber was noted. At the same time, general observations on the appearance of the uninoculated seedlings were taken.

The mean number of damped-off seedlings for four replicates at each of the four iron concentrations were compared statistically using Tukey's multiple comparison test.

2.2.3 FCRR Bioassay 2: EDDHA and EDTA

This experiment involved 18 treatments, which are depicted in Figure 2.2 part (B) and was performed using two Fisher brand 80® microtube racks. A row of wells was used as a treatment block in this experiment and the microtube racks had been autoclaved at 121°C for 15 minutes prior to being loaded with medium.

Tomato seedlings were germinated as described previously. Five- day-old tomato seedlings were transplanted to the wells of two Fisher brand 80® microtube racks. The wells of the microtube racks had been filled with autoclaved plant nutrient medium (Table 2.1), containing 0.7% agar (w/v). Plant nutrient medium at pH 6 had been used to

the fill the wells of the first microtube rack and plant nutrient agar at pH 8 was used to the fill the wells of the second microtube rack. The microtube racks were kept in separate transparent plastic boxes and placed in the greenhouse under the conditions described previously.

After two days, 20, 50, 100 or 200 µL of EDDHA or EDTA 10mM stock solutions at the corresponding pH of the media (pH 6 or pH 8) were pipetted on to the media surface of one row of wells containing tomato seedlings in each of the two microtube racks. These volumes represent EDDHA and EDTA concentrations of 10, 25, 50 and 100 µM in the wells of the microtube rack wells. A 200 µL aliquot of 0.1 N NaOH adjusted to the pH of the medium was pipetted into one row of wells and acted as the control treatments. An identically prepared set of microtube racks was used to assess the effect of the EDDHA and EDTA treatments on the growth of uninoculated tomato seedlings. After 24 hours seedlings in one set of microtube racks were root-inoculated by pipetting 100 µL of a 10^6 FORL microconidia/mL suspension into each well. The transparent plastic boxes containing the microtube racks were opened daily for aeration and sterile water was added to top up well volumes as needed. FCRR disease symptom ratings were made 7 days after inoculation (seedlings 15 days old) using the disease index system in Table 2.3 (Brammall 1986; Kasenberg, 1991). The experiment was repeated once. The mean FCRR disease index ratings for EDDHA and EDTA concentrations at both pH values were compared statistically using Tukey's multiple comparison test.

2.3 Results

2.3.1 Effect of EDDHA and EDTA on the Growth of FORL Mycelium

The growth of FORL mycelia in control microtube rack wells was not affected by pH (Figure 2.3). EDDHA significantly reduced ($p<0.05$) FORL mycelial growth at 0.1 mM and 1.0 mM at pH 7 only, and at 2.0 mM at pH 5 and pH 6 but not at pH 7. FORL mycelial growth was significantly reduced ($p<0.05$) by EDTA at 2.0 mM at all 3 pH levels tested and by 1.0 mM at pH 5 and pH 6 but not at pH 7. No significant difference ($p>0.05$) was detected in FORL growth between different pH levels at EDDHA or EDTA concentrations lower than 0.1 mM.

2.3.2 Effect of Iron on FCRR Severity

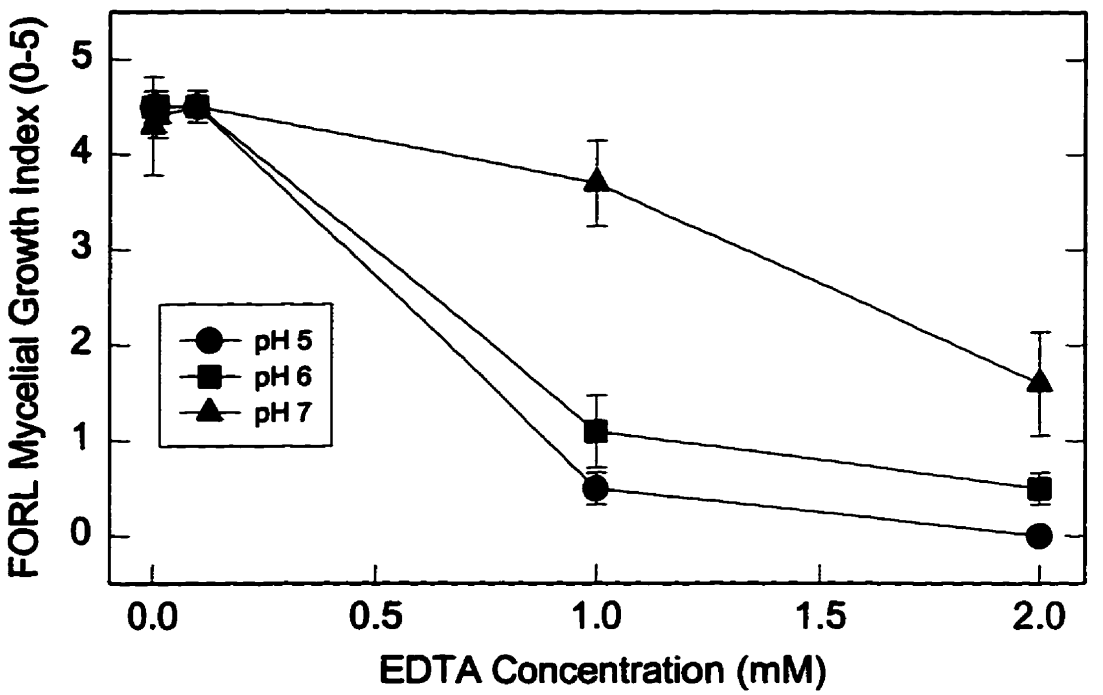
Root rot and damping-off were observed to occur earlier and to develop more rapidly on tomato seedlings grown in the medium containing 10^{-3} M iron compared to seedlings grown at the lower iron concentrations (Figure 2.4). No damping-off or rot symptoms were observed on any uninoculated seedlings and no visual difference in uninoculated seedling development or leaf pigmentation between the various iron concentrations was detected.

The mean number of damped-off seedlings for the four replicates at each of the four iron concentrations *i.e.* no added iron (control) ,10^{-7}, 10^{-5}, and 10^{-3} M $FeCl_3$ were 30%, 63%, 62%, and 82%, respectively, indicating a correlation between FCRR severity and iron availability. However, the number of damped-off seedlings differed significantly ($p<0.05$) between the two extreme iron concentrations only. The number of damped-off

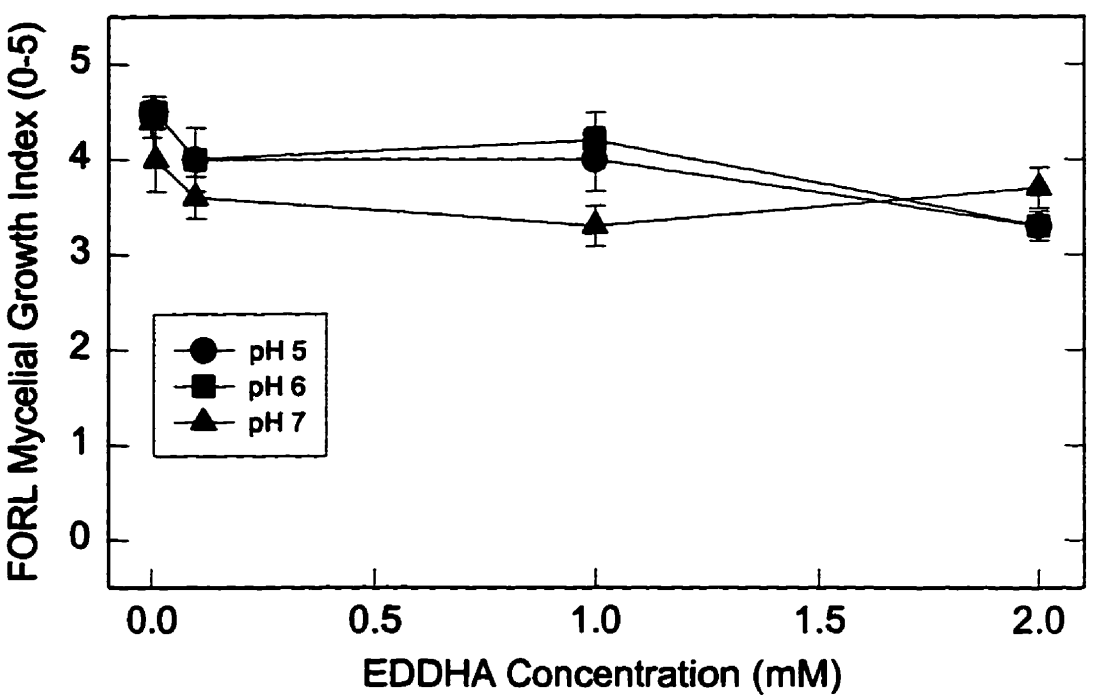
Figure 2.3: Effect of (A) EDTA and (B) EDDHA on the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) mycelium at pH 5,6 and 7.

Growth was assessed on SC media at pH 5, pH6 and pH 7 containing $FeCl_3$ at 10^{-5} M in microtube rack wells. Microtube racks were maintained in darkness at 22° C and growth was assessed 4 days after inoculation with 10^3 microconidia/well. Symbols represent mean growth ratings and error bars represent standard errors from 10 wells (5 wells per replicate).

(A)


FORL Mycelial Growth Index (0-5)
0
1
2
3
4
5
0.0
0.5
1.0
1.5
2.0
EDTA Concentration (mM)
pH 5
pH 6
pH 7

(B)


FORL Mycelial Growth Index (0-5)
0
1
2
3
4
5
0.0
0.5
1.0
1.5
2.0
EDDHA Concentration (mM)
pH 5
pH 6
pH 7

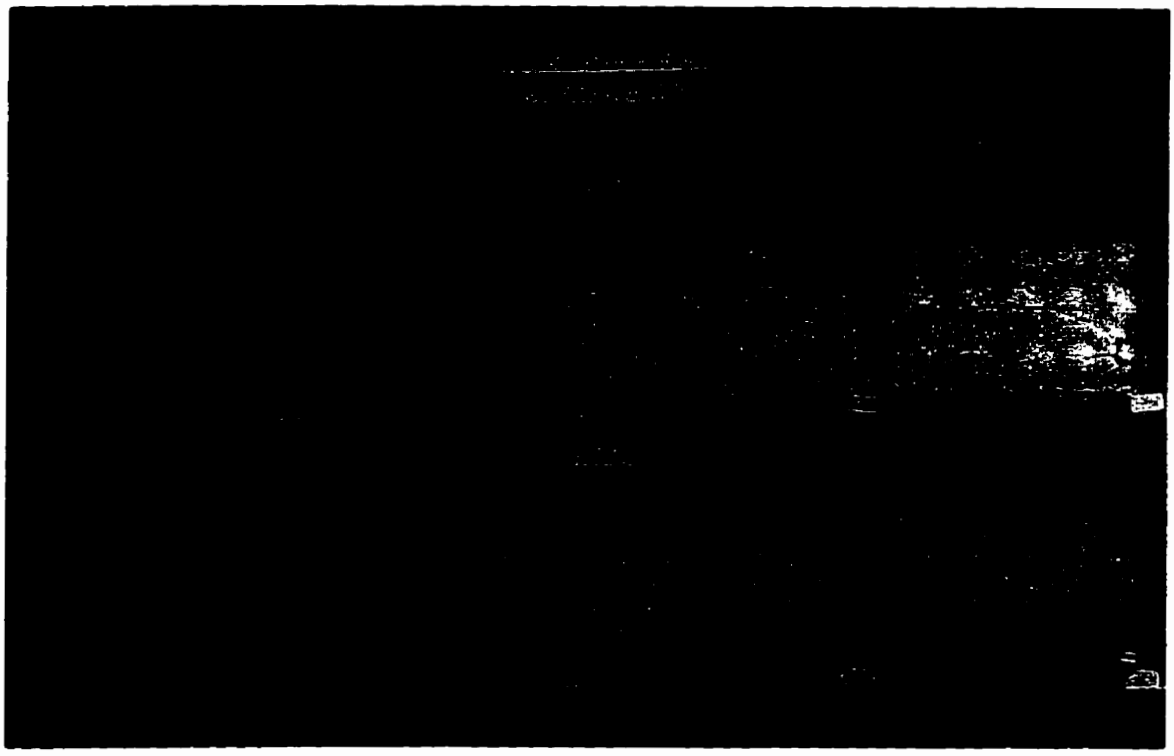


Figure 2.4: Effect of iron concentration on the severity of Fusarium crown and root rot (FCRR) on tomato seedlings. Iron concentration in the medium increases from left to right, no added iron (0 M), 10^{-7} M, 10^{-5} M, to 10^{-3} M. Seedlings in the top row were inoculated with microconidia of *Fusarium oxysporum* f. sp. *radicis-lycopersici* while seedlings in the bottom row were not inoculated. Details of the methodology are given in the text.

seedlings at the standard iron concentration of 10^{-5} M did not differ significantly ($p>0.05$) from the number of damped-off seedlings at other iron concentrations.

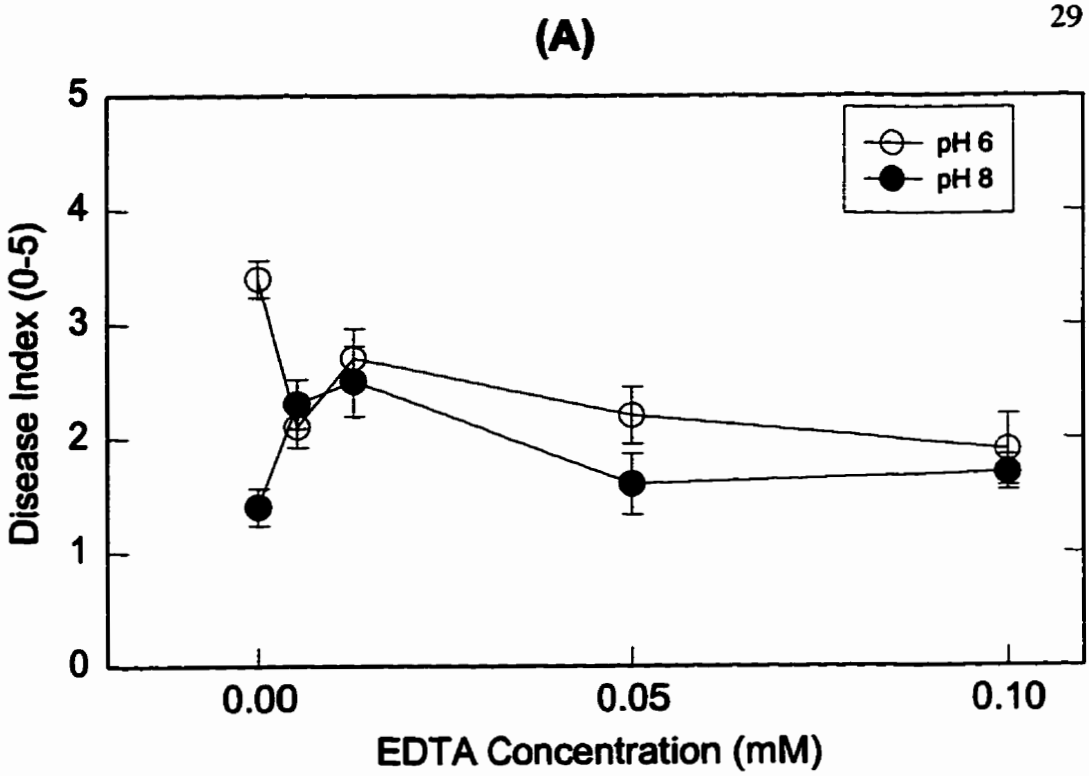
2.3.3 Effect of EDDHA and EDTA on FCRR Severity

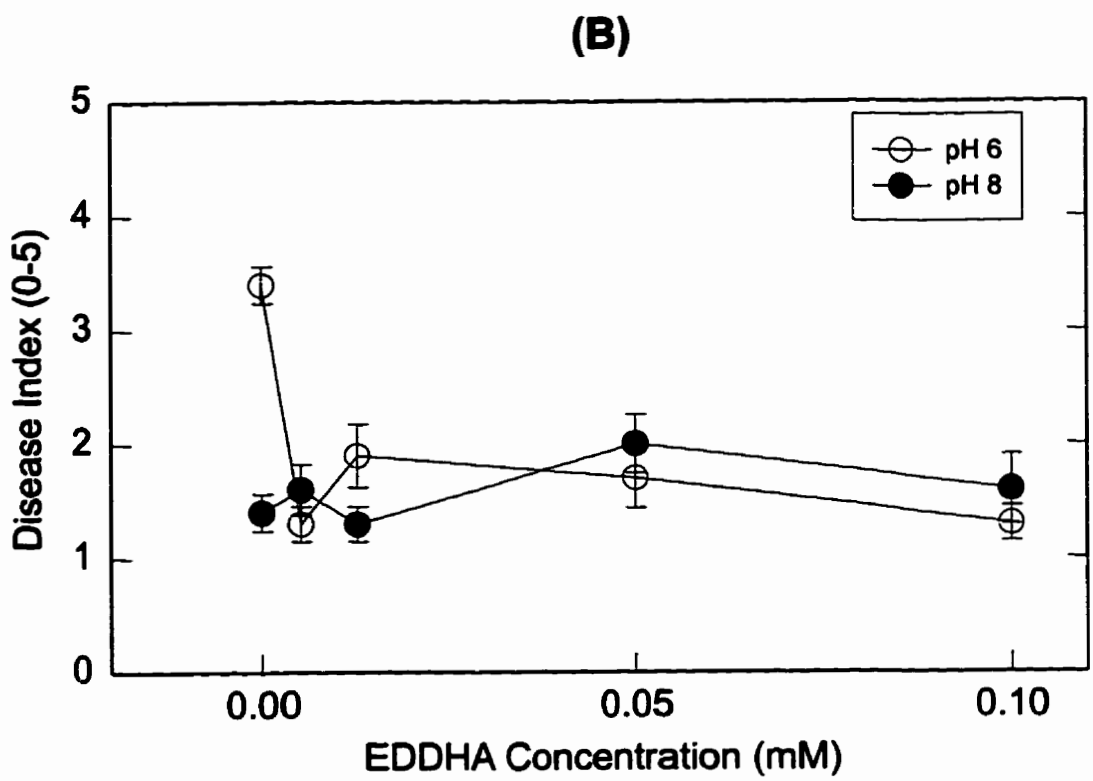
Both EDTA and EDDHA were found to significantly ($p<0.05$) reduce the severity of FCRR on tomato seedlings when compared to control seedlings at pH 6 but not at pH 8 (Figure 2.5). Control seedlings grown at pH 8 had significantly ($p<0.05$) less severe FCRR symptoms than control seedlings grown at pH 6. No significant difference ($p>0.05$) in FCRR severity on seedlings was detected between pH 6 and pH 8 for any of the EDDHA concentrations tested. No significant difference ($p>0.05$) in FCRR severity was detected for any EDTA concentration at either pH 6 or pH 8. Disease symptom ratings for EDTA treatments were not significantly different ($p>0.05$) from disease symptom ratings for EDDHA treatments at equal concentrations at the same pH.

No damping-off or rot symptoms were observed on uninoculated seedlings at any EDTA or EDDHA concentration. Uninoculated seedlings grown at pH 6 and pH 8 were visually indistinguishable from one another with respect to root coloration, shoot height and leaf pigmentation. Uninoculated seedlings grown with EDTA and EDDHA were indistinguishable from the uninoculated control seedlings.

Figure 2.5: Effect of (A) EDTA and (B) EDDHA on Fusarium crown and root rot (FCRR) severity on tomato seedlings at pH 6 and pH 8.

Tomato seedlings were germinated as described in the text. Five-day-old seedlings were transplanted into sterile microtube rack wells (2.2 mL volume) filled with plant nutrient medium (0.7% agar w/v) at pH 6 and pH 8 and containing 10^{-5} M $FeCl_3$. Two days later EDTA, EDDHA or 0.01 N NaOH (control) treatments at the same pH as the medium were applied. The following day each seedling was inoculated with 100 µL of a 10^6 FORL microconidia/mL suspension. Microtube racks were placed in sterile transparent plastic chambers and maintained under a 16 hour (22°C) day and 8 hour (18°C) night regime. The severity of FCRR was assessed after 7 days using the disease index in Table 2.3. Symbols represent mean symptom ratings and error bars represent standard errors for 10 seedlings (5 seedlings per replicate).


(A)
Disease Index (0-5)
0
1
2
3
4
5
pH 6
pH 8
0.00
0.05
0.10
EDTA Concentration (mM)


(B)
Disease Index (0-5)
0
1
2
3
4
5
pH 6
pH 8
0.00
0.05
0.10
EDDHA Concentration (mM)

2.4 Discussion

The rating system for mycelium growth was designed to evaluate the effect of pH on the inhibition of growth of FORL mycelium by EDTA and EDDHA and not to quantify absolutely the inhibition caused by EDTA and EDDHA. In the FORL mycelial growth assay the pH effect on EDTA efficacy and the lack of pH effect on EDDHA efficacy are consistent with iron limitation being the mechanism by which these chelators reduced the growth of FORL mycelia. EDTA was found to dramatically reduce the growth of FORL mycelia and have a higher efficacy in reducing the growth of FORL mycelium than EDDHA even though EDTA is considered a weaker iron chelator (Lindsay, 1974). The higher efficacy of EDTA may be due to its higher polarity and hence greater solubility in the aqueous agar medium (Pribil, 1972). A fraction of the EDDHA in the medium may have been destroyed by the sterilization conditions used *i.e.*, autoclaved at 121° C for 15 minutes. Vanachter *et al.* (1988) found that EDDHA was more susceptible than EDTA to chemical destruction by an ozone based nutrient solution sterilization procedure.

The estimated stability constant for the iron complex ($\log_{10}$K=30) of the fusarinine siderophores produced by *Fusarium* species is higher than that of EDTA ($\log_{10}$ K=25) (Scher and Baker, 1982). However, the relative concentrations of chelators and not just K values influence the mole fraction of iron bound by competing chelators (Raymond *et al.*, 1984). The lowest concentration of EDDHA and EDTA tested was 10^{-4} M. This meant that the concentration of chelator was a minimum of 10 times greater than the concentration of iron. The large excess of free EDTA over free iron would drive the

EDTA chelation equilibrium towards the formation of the EDTA-iron complex. This shift in the EDTA-iron complex equilibrium and a higher concentration of EDTA than FORL siderophores would limit iron availability to FORL. The specificity of EDTA for iron decreases as pH increases (Lindsay, 1974) as did the efficacy of EDTA in reducing FORL mycelial growth. At pH levels of 6, magnesium in the SC medium will begin to replace iron from EDTA. The specificity of EDDHA for iron is pH independent (Lindsay, 1974) as was the efficacy of EDDHA in reducing FORL mycelial growth.

A direct relationship between the absolute iron concentration in the plant nutrient medium and FCRR severity of tomato seedlings was found. This is the first report of a direct relationship between increased iron concentration and increased FCRR severity. The severity of FCRR on tomato seedlings grown at pH 8 was much lower than seedlings grown at pH 6. This finding is consistent with reports of FCRR severity being inversely related to the pH of the growth medium (Jones *et al.*, 1990; Woltz *et al.*, 1992). Unfortunately, simply raising the pH of the medium is not a good control for FCRR because a pH range of 5 to 6 is recommended for maximal growth of tomato (Papadopoulos, 1991). Both EDDHA and EDTA decreased FCRR severity in tomato seedlings grown in plant nutrient medium at pH 6. The degree of protection conveyed was unaffected over the concentrations of 10 μM to 100 μM for both EDDHA and EDTA and the degree of protection did not differ between EDDHA and EDTA. Unlike the mycelial growth effects, the effect of EDTA on FCRR severity was the same at pH 6 and pH 8. The reason for this lack of pH effect is not known and clarification of this phenomenon requires further investigation. Plant roots can affect the pH of the

rhizosphere. An example of the alteration in the pH of the rhizosphere is the raising of the pH of the rhizosphere by plants grown with nitrogen supplied as nitrate as opposed to ammonium (Chaney and Bell, 1987), as was the case in the FCRR bioassay performed in this study. A possible explanation for the lack of pH influence on the effect of EDTA and EDDHA on FCRR severity is that the tomato seedling roots altered the pH of the medium to a level where EDTA and EDDHA formed buffering systems in the plant nutrient medium. EDTA can form a buffering system with its metal salts at pH levels near the pKa's of its last two acid moieties to dissociate *i.e.* pH 6.2 and pH 10.3 (Pribil, 1972). It is possible that EDTA mediated reduction in FCRR severity is not dependent on pH because of buffering of the medium at pH 6. Likewise EDDHA buffering of the medium at approximately pH 8 would explain the similar level of FCRR severity in media initially at pH 6 and pH 8 to that medium used as the pH 8 control. Redox potential of the medium may be involved since plant roots alter it (Marschner *et al.*, 1982). Iron chelation by EDTA and EDDHA is affected by redox potential as well as by the pH of the medium (Schwab and Lindsay, 1989).

In conclusion, both EDDHA and EDTA reduced FCRR severity in tomato seedlings in experimental bioassays. This reduction was based on iron chelation in the medium indicating that iron limitation to FORL is a viable means of controlling FCRR in the bioassay system. If iron chelation is part of the lettuce-mediated allelopathic control of FCRR, lettuce extracts and lettuce-tomato companion planting should display similar control of FCRR using the same bioassay methodology.

Chapter 3

Identification of Phenolic Compounds in Lettuce Roots and Root Exudates

3.1 Introduction

3.1.1 Phenolic Composition of Lettuce Tissues

Literature reports regarding the phenolic composition of lettuce (*Lactuca sativa* L.) are confusing. Monocaffeoylquinic acid (chlorogenic acid), monocaffeoyltartaric acid and dicaffeoyltartaric acid (chicoric acid) have been reported in lettuce leaves (Winter and Herrman, 1986; Bennett *et al.*, 1996). Ke and Saltveit Jr. (1988) reported the presence of chlorogenic acid and isochlorogenic acid but not of caffeoyltartaric acids in lettuce leaf tissue. Isochlorogenic acid was also reported to be the only phenolic compound detected in lettuce roots (Cole, 1984).

The lettuce cultivar Grand Rapids has previously been shown to be an effective allelopathic agent for the control of Fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) (Kasenberg, 1991). Kasenberg (1991) compared lettuce leaf (cv. Grand Rapids) and sweet potato (*Ipomoea batatas* L.) tuber extracts using thin layer chromatography (TLC). He concluded that chlorogenic acid (Figure 3.1(A)) and isochlorogenic acid (Figure 3.1(B)) are constituents of lettuce cv. Grand Rapids leaf tissue. A commercial standard of isochlorogenic was not available at the time of my study. Green coffee beans (*Coffea canephora*) contain high concentrations of chlorogenic acid and isochlorogenic acid isomers (Bicchi *et al.*, 1995; Clifford, 1986; Morishita *et al.*, 1986). Therefore, extracts of coffee bean were used as

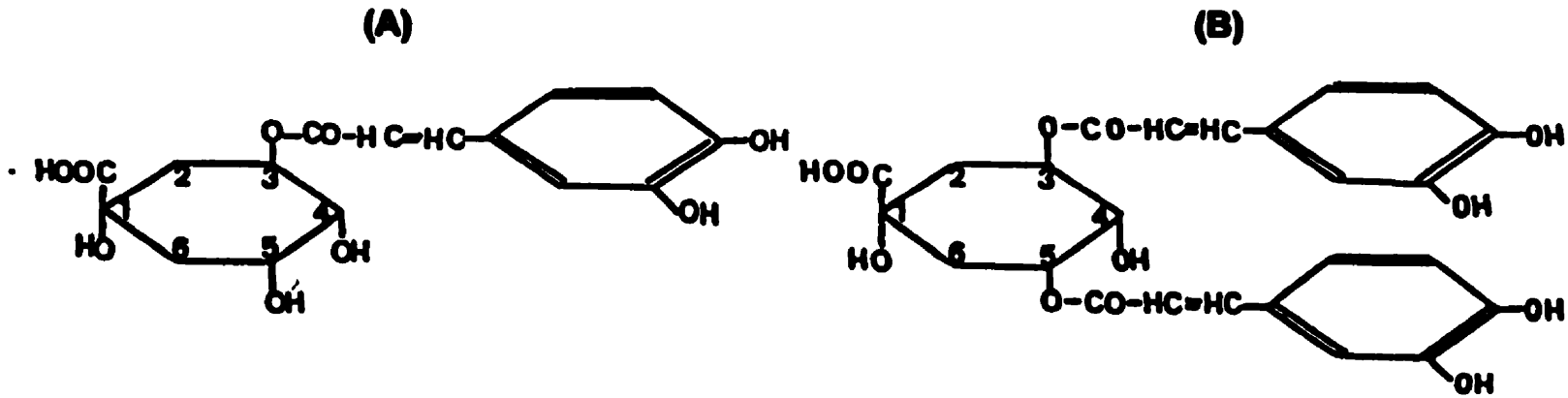


Figure 3.1: Structures of (A) 3-o-caffeoylquinic acid (chlorogenic acid) and (B) 3,5-o-dicaffeoylquinic acid (isochlorogenic acid).

standards for chlorogenic acid and isochlorogenic acid in TLC and high performance liquid chromatography (HPLC) of compounds in lettuce roots.

The objectives of this chapter were the identification and quantification of the ortho-dihydroxyphenolic compounds (o-diphenols) in lettuce root tissues and lettuce root exudates and the determination of the influence of exposure to FORL microconidia on the phenolic composition of tomato and lettuce roots.

3.2 Materials and Methods

3.2.1 Hydroponic Plant Culture

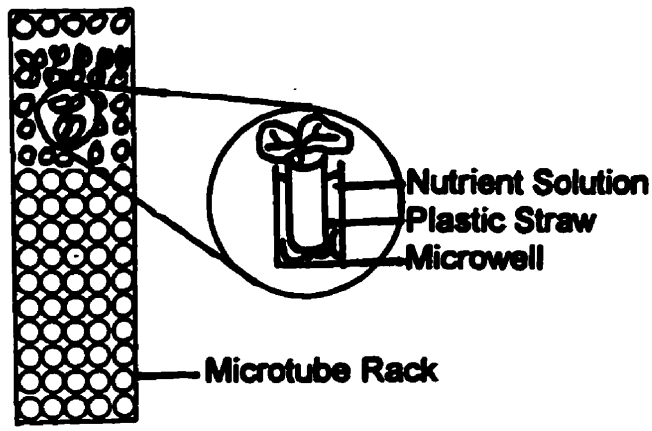
Seeds of lettuce cv. Grand Rapids and FORL susceptible tomato (*Lycopersicon esculentum* Mill. cv. Bonny Best) were purchased from Stokes Seed Co., St.Catharines, Ontario. Seeds were surface sterilized and germinated as described in chapter 2.

Growth in Microtube Racks

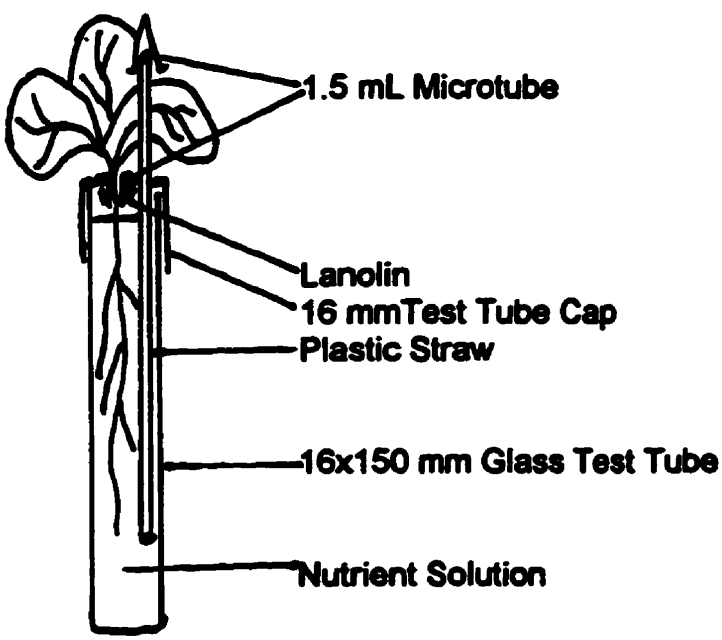
Roots of 3-day-old lettuce seedlings were inserted through 2 cm long segments of sterile plastic straws. The straw segments containing lettuce seedlings were placed in the wells of a sterile 1.5 mL microtube rack (Fisher brand 80® microtube rack, Fisher Scientific) (Figure 3.2 and Figure 3.3 - Stage 1). The microtube wells had been filled with autoclaved plant nutrient solution (Table 2.1). The microtube racks were then placed inside sterile transparent plastic boxes and placed in a walk-in growth cabinet (Constant Temperature Control, LTD., Canada) with a 16 hour day (350 $\mu Em^{-2}s^{-1}$ PAR, 22° C) and 8 hour night (18° C) regime to simulate greenhouse conditions.

Figure 3.2: Three-stage-growth of lettuce plants with axenic root systems and root exudates.

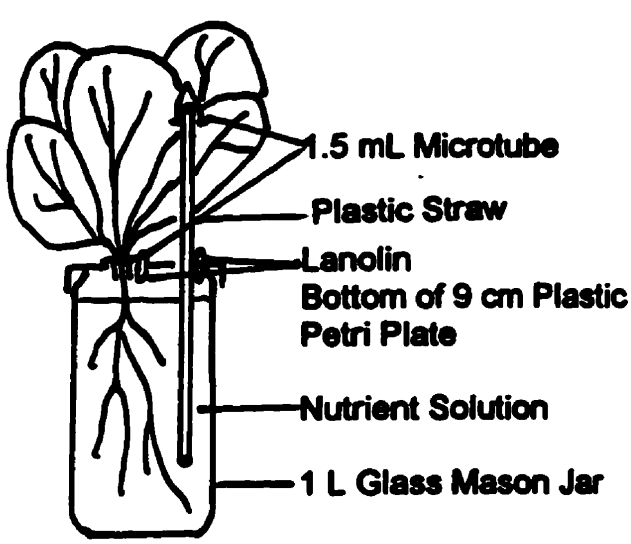
Lettuce seeds were germinated on moistened Whatman #1 filter paper in sterile glass petri plates for 3 days in darkness at 22°C prior to transfer to Stage 1. Plant growth for all stages was inside a walk-in growth cabinet under 16 hours of light ($350\mu Em^{-2}s^{-1}$, 22°C) and 8 hours of darkness (18°C). Sterile lanolin was used to seal plants inside 1.5 mL microtubes and to seal plastic straws in place. Sterile 1.5 mL microtubes were used as caps for the straws. All materials were autoclaved at 121°C for 15 minutes. The composition of the nutrient solution can be found in Table 2.1.



Growth Stage 1
- plants 5-14 days old
- racks are enclosed in plastic chambers

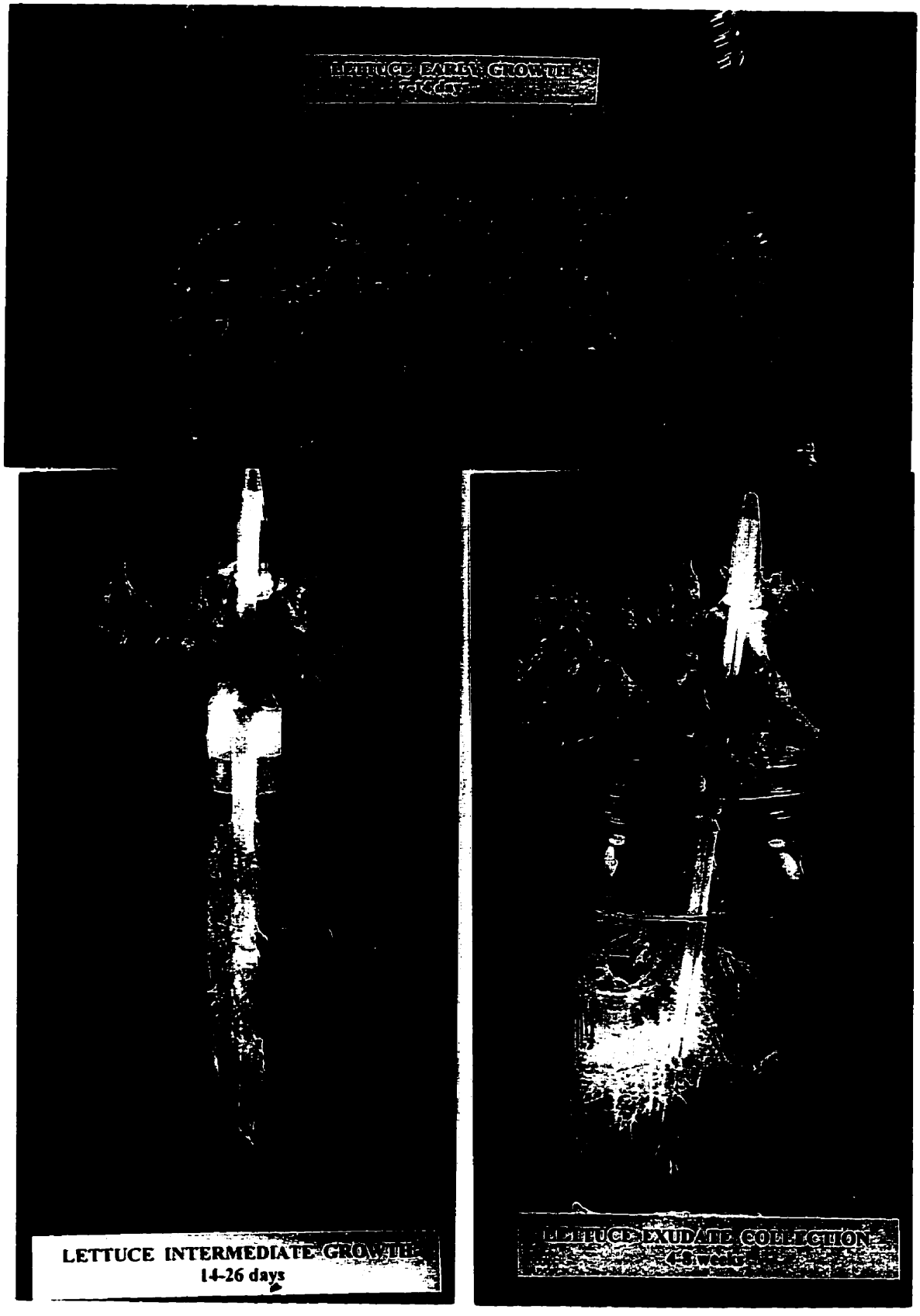


Growth Stage 2
- plants 14-28 days old
- plants are sealed in microtube with lanolin
- water/nutrient solution added through plastic straw



Growth Stage 3
- plants 28-56 days old
- plants sealed in microtube with lanolin
- water/nutrient solution added through plastic straw

Figure 3.3: Lettuce plants of various ages being grown with axenic root systems and root exudates. See the text (page 35) and Figure 3.2 for details of the apparatus and growth conditions.


LETTUCE EARLY GROWTH
LETTUCE INTERMEDIATE GROWTH
14-26 days
LETTUCE EXUDATE COLLECTION

Growth in Test Tubes and Glass Jars

After 14 days, lettuce seedlings at the 4-5 leaf stage were removed from the plastic straws and their root systems were inserted into sterile 16X150 mm glass test tubes containing 20 mL of sterile plant nutrient solution (Figure 3.2 and Figure 3.3 - Stage 2). After 28 days lettuce plants were transferred into sterile 1 L glass Mason jars filled with 950 mL of sterile plant nutrient solution (Figure 3.2 and Figure 3.3 - Stage 3). The test tube caps and Mason jar lids were not sealed, and a gap was left at the top of the nutrient solution for aeration of the nutrient solution. Nutrient solution levels were topped up to initial levels with additions of sterile water as needed. Autoclaved nutrient solution was substituted for water additions once a week.

3.2.2 Thin Layer Chromatography

Lettuce root and coffee bean extracts used in TLC analysis were prepared using an extraction procedure based on Ramirez-Martinez and Luh (1973). All solvents were of HPLC grade (BDH Chemicals, Toronto).

A 25 g fresh weight (FW) sample of 8-week-old lettuce roots (*Lactuca sativa* L. cv. Grand Rapids) or 5 g FW of green coffee beans (*Coffea canephora* var. robusta) were chopped with a razor blade and macerated in 250 mL of 80% methanol with a tissue homogenizer (Polytron® Kimematica Model CH-6010, Switzerland). The slurry was filtered through Whatman #1 paper under vacuum. The filtrate was evaporated to approximately 10 mL at 30°C using a rotary evaporator to ensure removal of the methanol. The volume of the residual aqueous phase was made up to 100 mL by the

addition of distilled water and then it was extracted three times with 30 mL of chloroform. The aqueous phase was saturated with NaCl, adjusted to pH 4 with 1N HCl and then extracted three times with 30 mL of ethyl acetate. The ethyl acetate phase was dried with anhydrous Na_2SO_4 and then evaporated to dryness at 30°C using a rotary evaporator. The residue was redissolved in 5 mL methanol and used for TLC analysis.

A Whatman Microcrystalline Cellulose (250 μm layer) TLC plate (20x20 cm) was spotted with 5μL aliquots from extracts of lettuce root and green coffee bean and with 2μL of 10^{-2} M methanol solutions of caffeic and chlorogenic acid standards (Sigma, St. Louis Mo., USA). The chromatogram was developed using n-butanol / acetic acid / water (4:1:2, upper phase). A second identically spotted TLC plate was developed in 10% acetic acid and a third identically spotted plate was developed in methyl-isobutyl-ketone / formic acid / water (2:1:2, upper phase). Developed chromatograms were air dried and viewed under ultraviolet light (366nm) before and after fuming with ammonia vapour (Ramirez-Martinez and Luh, 1973).

3.2.3 High Performance Liquid Chromatography

HPLC analysis was performed using procedures based on Morishita *et al.* (1986). Equipment used consisted of, two Waters Associates 510 pumps with a U6K manual injector, a Waters Associates Lamda Max Model 481 UV spectrophotometer and Digital Professional 350 software. The column was a Phenomenex Primesphere 5, reverse phase C_{18} (250x4.6(i.d.) mm) with a 5μm particle size. Solvents used were 0.03% H_3PO_4 (solvent A) and HPLC grade methanol (solvent B) and both were de-gassed under

vacuum. Deionized water was used for 0.03% H_3PO_4 (v/v) preparation which had a final pH of 2.5. The elution system was a 40-90% gradient in solvent B over 20 minutes at a flow rate of 1 mL per minute. Peak detection was made using UV absorbance at 320 nm the absorbance maxima for caffeic acid esters (Winter and Herrmann, 1986).

Retention times for caffeic acid and chlorogenic acid standards (Sigma, St. Louis Mo., USA) were used for the identification of these compounds in lettuce root tissue extracts and concentrated lettuce root exudates. Quantitative data on chlorogenic and caffeic acid concentrations in lettuce roots were calculated from a caffeic acid absorption curve (see Figure 3.3) created by plotting the peak area of caffeic acid absorbance at 320 nm against the moles of caffeic acid injected. The assumption was made that the molar absorptivity of the chlorogenic acid at 320 nm is the same as that for caffeic acid (Ong and Nagel, 1978).

A) Plant Extracts

A micro-extraction procedure based on the procedure of Graham (1991) was used for preparation of tissue extracts. All solvents were of HPLC grade (BDH Chemicals, Toronto).

Lettuce root, tomato root or coffee bean tissue was cut into small sections and dried at 60°C for 4 hours. The dried tissues were ground into a fine powder and a 10 mg sample of this powder was placed in a polypropylene 1.5 mL microtube with 1 mL of methanol. The tube was agitated on a gyrorotary shaker for 24 hours and then centrifuged at 16, 500 g for 5 minutes. The upper 0.5 mL was removed and used for HPLC analysis.

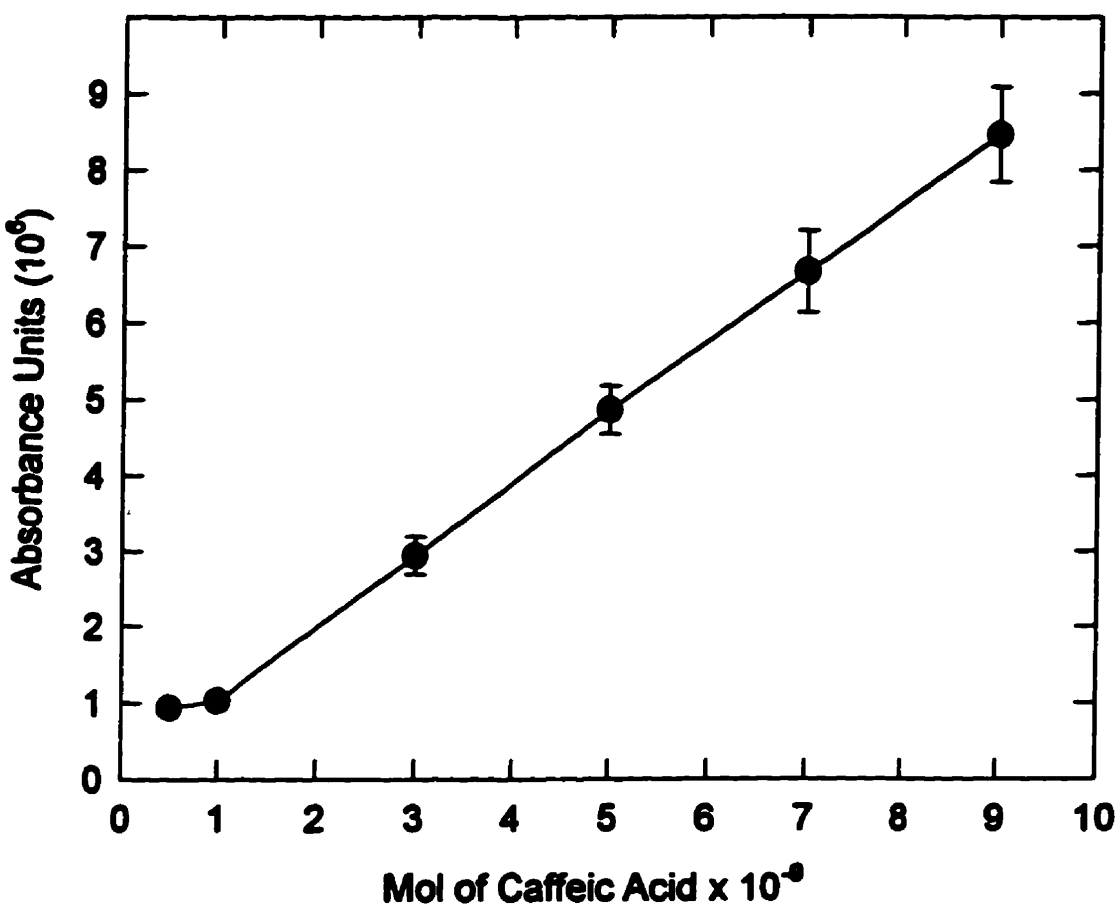


Figure 3.4: Absorbance curve for caffeic acid at 320 nm.
Symbols represent mean absorbance values for 4 injections +/- S.E.

A 10 μL aliquot of the extract was injected into the HPLC column for analysis. The volume of the extract injected (10 μL) contained compounds extracted from 0.1 mg of dried tissue.

Using the above procedure extracts of 8-week-old lettuce roots and green coffee beans were prepared and analysed for the purpose of identifying phenolic compounds in lettuce root tissue. The root extracts of uninoculated and FORL inoculated 3-week-old tomato and lettuce roots were also prepared and analysed in order to quantify phenolics in lettuce root tissue and to detect changes in the phenolic composition induced by exposure to FORL microconidia. The lettuce and tomato seedlings had been inoculated with 100 μL of a 10^6 microconidia/mL suspension when they were 7 days old. Plants were grown in microtube racks for 2 additional weeks (see Figure 3.3 - Stage 1) at which time their roots were excised and extracted as described above.

The mean concentrations of chlorogenic and caffeic acids per gram of 3-week-old lettuce roots were determined from two injections from two different extracts. The observed absorbance was converted to nmol of chlorogenic acid or caffeic acid equivalents using the standard absorbance curve for caffeic acid (Figure 3.3).The number of nmol of chlorogenic acid or caffeic acid injected represented compounds extracted from 0.1 mg of dried tissue. This number was multiplied by 10^4 to give the nmol of chlorogenic acid or caffeic acid equivalents in 1 g of dry tissue and then divided by 10^9 to give the number of moles of chlorogenic acid or caffeic acid equivalents. The number of moles was converted to mg of chlorogenic acid (MW=354.3 g/mol) or caffeic acid (MW=180.1 g/mol) equivalents in 1 g of dry tissue.

HPLC analysis was performed at two temperatures (22° and 26° C) to compare temperature-induced shifts in retention times of standards to those of compounds in samples which had the same retention times as the standards at one temperature. An increase in temperature increases the solubility of compounds in the mobile phase and results in a decrease in retention times. Although completely different compounds can have the same retention time at one temperature, the increase in the solubilities and corresponding decrease in retention times of the two compounds at a higher temperature will likely differ.

B) Root Exudates from 8-Week-Old Lettuce Plants

Lettuce root exudates were collected from lettuce plants that had been grown in 1 L glass Mason jars (Figure 3.2 - Stage 3), 1 plant per jar, in a growth cabinet under conditions described earlier. The plants had been transfered to the Mason jars at 4 weeks of age and were grown in the Mason jar for an additional 4 weeks. The pooled solution thus contained the root exudates which had accumulated during the 4 weeks of growth from four lettuce plants. The liquids (exudate solution) from four Mason jars were pooled giving a total volume of approximately 4 L. This exudate solution was filtered through Whatman #1 filter paper under vacuum.

Concentration Protocol 1) Ethyl acetate Extraction: A 800 mL sample of the lettuce root exudate solution was saturated with NaCl and then extracted four times with 100 mL of ethyl acetate. The ethyl acetate fractions were combined and dried with anhydrous Na_2SO_4. The ethyl acetate extract was then evaporated to dryness at 35° C under vacuum

using a rotary evaporator. The residue was redissolved in 4 mL methanol which was then filtered using a Nalgene™ 0.2 μm cellulose-acetate syringe filter, and used for HPLC analysis using the procedure describe above.

Concentration Protocol 2) XAD-4 Resin Adsorption: XAD-4 resin has been used to isolate phenolic root exudates from the aqueous nutrient solution (Tang and Young, 1982). A 500 mL sample of lettuce root exudate solution was poured into each of two 1000 mL Eyrlenmeyer flasks. A 25 g sample Amberlite XAD-4 anion exchange resin (BDH Chemicals, Toronto) was added to each flask. The flasks were placed on a gyrorotary shaker at 100 rpm for 24 hours. The XAD-4 resin was then removed from the exudate solution by vacuum filtration through Whatman # 1 filter paper. The filtered XAD-4 resin and 200 mL of methanol were added to a 500 mL Eyrlenmeyer flask. This flask was placed on the gyrorotary shaker at 100 rpm for 1 hour. The XAD-4 resin was filtered from the methanol and the filtrate was evaporated to dryness on a rotary evaporator at 35° C under vacuum. The residue was redissolved in 2 mL methanol, filtered using a Nalgene™ 0.2 μm cellulose-acetate syringe filter, and then used for HPLC analysis.

C) Root Exudates from 3-Week-Old Lettuce Plants

The lettuce root exudate solution from ten 3-week-old lettuce plants, which were grown in 16x150 mm glass test tubes (see Figure 3.2), was also analysed using a simple concentration procedure using Waters tC_{18} Sep-Pak Plus cartridges. These cartridges contain packing material similar to that used in the HPLC analytical column.

No additions of water or nutrient solution had been made to the tubes containing the lettuce plants for 1 week prior to exudate collection. The solution in each tube had been allowed to fall to a volume of approximately 5 mL as an initial concentration of the root exudates. The solutions from the 10 plants were pooled and water was added to make a final volume of approximately 50 mL.

Concentration Protocol 3) tC_{18} Sep-Pak Adsorption: A Waters tC_{18} Sep-Pak Plus cartridge was conditioned with 10 mL of methanol then flushed with 10 mL of water. A 1 mL sample of the above exudate solution was loaded into the cartridge. The cartridge was eluted with 10 mL of water followed by 10 mL of methanol to remove phenolic compounds. The methanol was collected as three fractions on the basis of eluent color. The first 2 mL methanol fraction eluted was colorless and designated as Fraction 1 (F1), the next 1 mL fraction eluted was yellow in color and as designated as Fraction 2 (F2), the final 7 mL fraction collected was colorless. This final 7 mL methanol fraction was evaporated to 1 mL with a Savant Speed Vac Concentrator (Model SVC100H) vacuum centrifuge and this was designated as Fraction 3 (F3). A 10 µL sample of each of these fractions (F1,F2 and F3) was injected and analysed by HPLC.

3.3 Results

3.3.1 Thin Layer Chromatography

The R_f values for fluorescent spots detected on TLC chromatograms developed in the three solvent systems are found in Table 3.1. All spots were colorless in visible light. Spot 1 and spot 2 from both lettuce root and coffee bean extracts had R_f values nearly

Table 3.1: Cellulose plate thin layer chromatography of lettuce root and coffee bean 80% methanol extracts.

Extracts were prepared as described in the text. Aliquots (5μL) of lettuce root and coffee bean extracts were spotted on three Whatman microcrystalline cellulose (250 μm thick) TLC plates. Aliquots (10 μL of 10^{-2} M in methanol) of authentic caffeic acid and chlorogenic acid were also spotted on each of the plates. Plates were developed at 22° C using the indicated solvent systems. The developed chromatograms were examined for fluorescence under ultraviolet light at 366 nm. The colors of fluorescent spots were noted before and after exposure to ammonia vapour. The R_f values for fluorescent spots represent the ratio of the distance of spot migration to the distance migrated by the solvent front.

Spot	Spot R_f Values Using Different Solvents*			Spot Fluorescence Color on Dried Chromatogram**	
	BAW	10% HOAc	MFW	UV (366nm)	UV + NH_3
Chlorogenic Acid	0.68	0.71	0.34	Y	Y
Caffeic Acid	0.81	0.33	0.76	B	B
Lettuce Root					
Spot 1	0.68	0.71	0.31	Y	Y
Spot 2	0.79	0.34	0.73	B	B
Spot 3	0.84	0.17	0.88	Y	Y
Coffee Bean					
Spot 1	0.68	0.71	0.29	Y	Y
Spot 2	0.82	0.33	0.77	B	B
Spot 3	0.75	0.17	0.82	Y	Y
Spot 4	-----	-----	0.52	V	V

*BAW (n-butanol/acetic acid/water (4:1:2 upper phase))
HOAc (acetic acid)
MFW (methyl-isobutyl-ketone/formic acid/water (2:1:2 upper phase))
** For BAW plates only B-Blue Y-Yellow V-Violet

identical to chlorogenic and caffeic acid standards, respectively, on all three chromatograms. Spot 3 was detected in both lettuce and coffee extracts and the calculated R_f values for this spot were identical between extracts when 10% acetic acid was used as the developing solvent but varied slightly when n-butanol / acetic acid / water (4:1:2, upper phase) and methyl-isobutyl-ketone / formic acid / water (2:1:2, upper phase) were used as developing solvents. A fourth spot was detected in the coffee bean extract but not in the lettuce root extract.

Spot fluorescence under UV light at 366 nm before and after ammonia fuming is also found in Table 3.1. All corresponding spots from the lettuce and coffee extracts showed similar fluorescence characteristics. Spots 1 and 2 in both extracts had similar fluorescence color properties with chlorogenic and caffeic acids respectively. Changes in fluorescence of phenolic compounds before and after ammonia fuming has been used in compound identification (Ramirez-Martinez and Luh, 1973). Fuming the dried chromatograms with ammonia intensified the fluorescence of spots but did not alter the coloration.

3.3.2 High Performance Liquid Chromatography

The standard absorbance curve for caffeic acid (Figure 3.3, pg. 43) demonstrates that a linear relationship between caffeic acid concentration and absorbance (peak area) at 320 nm occurred in the 1-9 nmol range with the equipment and procedures used. Quantitative data regarding caffeic acid and chlorogenic acid concentrations in lettuce root tissues were determined by injecting sample volumes that gave absorbance values

within this range. All retention times were calculated at maximum absorbance (peak height) and expressed in minutes. The caffeic acid standard curve was generated from 24 injections of caffeic acid made over 2 days with a standard error of +/- 0.18 minutes in the retention time of caffeic acid. The molar absorbance coefficient of isochlorogenic acid will differ significantly from those of caffeic acid or chlorogenic acid due to the presence of two chromatophores. Therefore, the proportion of total extract absorbance for peaks tentatively identified as isochlorogenic isomers, and designated as suspected isochlorogenic acid isomers (SII), is not directly related to the proportion of the total concentration for these compounds in the extract.

A) HPLC of Plant Extracts

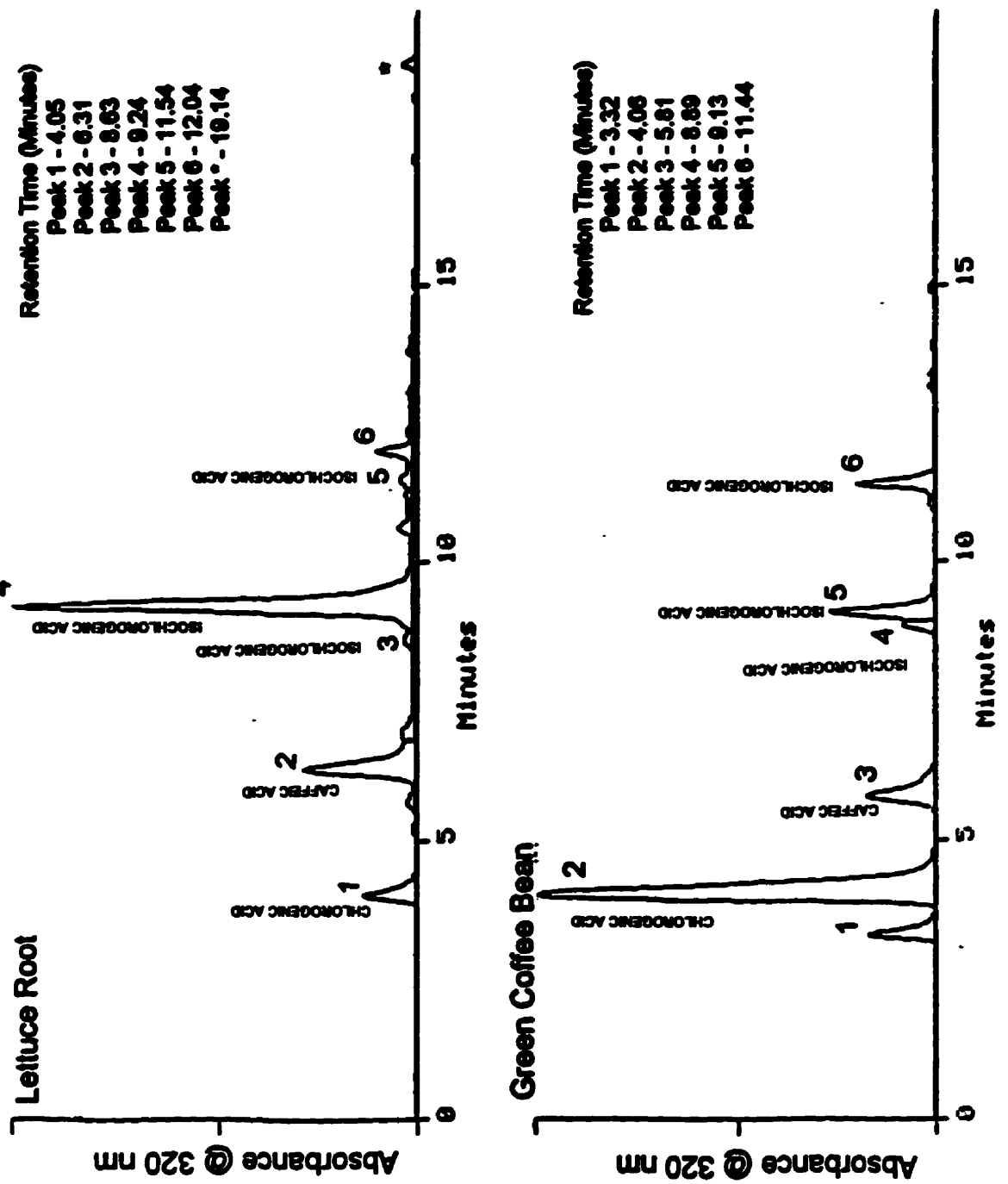
Lettuce Root Tissues

For purposes of describing the HPLC chromatograms of tissue extracts and exudate solutions in the following figures, the term major will be used to describe compounds with large proportions of the total extract absorbance and the term predominant will be used to describe the compound with the greatest proportion of total extract absorbance.

HPLC chromatograms, at 22° C, of 8-week-old lettuce root and green coffee bean methanol extracts can be seen in Figure 3.4. A total of 4 injections were made from a single lettuce root and a single coffee bean extract. Peak retention times for each injection were all within +/- 0.18 minutes of each other (*i.e.* within the standard error for retention time of the caffeic acid standard). The three major compounds that were detected in the

Figure 3.5: Reproduction of HPLC chromatogram of lettuce root and coffee bean methanol extracts.

Extracts were prepared as described in the text. HPLC was performed using a Phenomenex Primesphere 5, C_{18} reverse phase column (250x4.6(i.d.)mm) with a 5 μm particle size. The elution system used was a 40-90% methanol gradient in 0.03% H_3PO_4 at a flow rate of 1 mL per minute over 20 minutes. The sample volume injected was 5 μL for both extracts. The absorbance scale is not the same for both HPLC chromatograms.


Lettuce Root
Retention Time (Minutes)
Peak 1 - 4.05
Peak 2 - 6.31
Peak 3 - 8.63
Peak 4 - 9.24
Peak 5 - 11.54
Peak 6 - 12.04
Peak * - 19.14
CHLOROGENIC ACID
CAFFEIC ACID
ISOCHLOROGENIC ACID
ISOCHLOROGENIC ACID
ISOCHLOROGENIC ACID
Absorbance @ 320 nm
Minutes
Green Coffee Bean
Retention Time (Minutes)
Peak 1 - 3.32
Peak 2 - 4.08
Peak 3 - 5.81
Peak 4 - 8.89
Peak 5 - 9.13
Peak 6 - 11.44
CHLOROGENIC ACID
CAFFEIC ACID
ISOCHLOROGENIC ACID
ISOCHLOROGENIC ACID
ISOCHLOROGENIC ACID
Absorbance @ 320 nm
Minutes

lettuce root extract represented 8%, 16% and 55% of the absorbance of the entire extract. The first two of the major compounds in the lettuce extract were identified as chlorogenic acid and caffeic acid based on the similarity of their retention times (4.05 and 6.31) to those of the chlorogenic acid standard and the caffeic acid standard, respectively (Table 3.2). The predominant compound in the lettuce extract (55% of absorbance) had a retention time of 9.24. Three compounds eluted from the lettuce extract had retention times (8.63, 9.24 and 11.54) similar to the retention times for the last three compounds eluted from the coffee bean extract (8.89, 9.13 and 11.44).

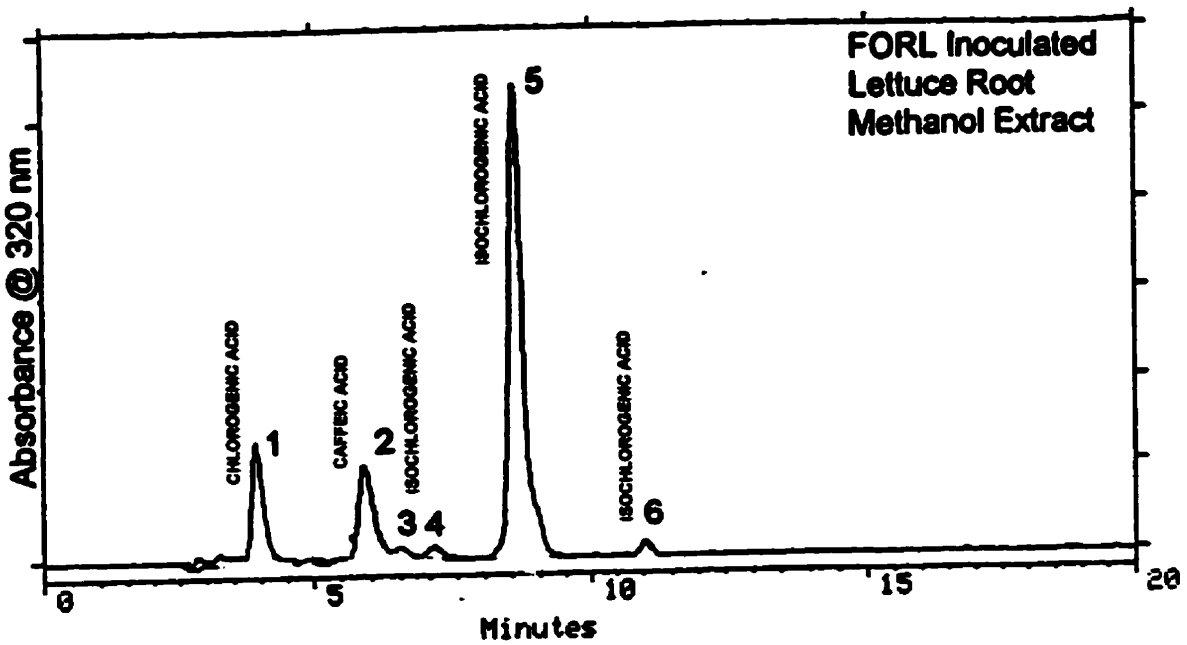
Lettuce Roots Exposed to FORL

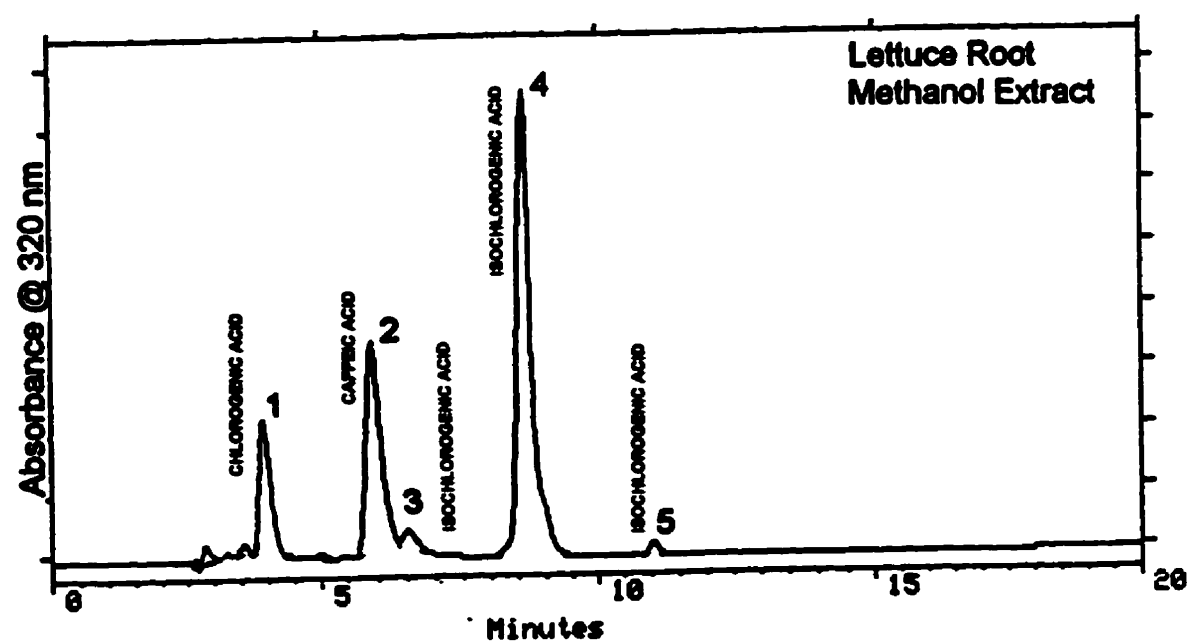
The HPLC chromatogram (at 26° C) of uninoculated and FORL inoculated 3-week-old lettuce root methanol extracts (Figure 3.5) revealed major compounds with retention times (3.90 and 5.89) corresponding to those of chlorogenic acid and caffeic acid, respectively (Table 3.3). The predominant compound in inoculated lettuce root extracts had a retention time (8.56) similar to the predominant compound in uninoculated lettuce roots (8.59).

Increasing the temperature from 22° C to 26° C during HPLC chromatography caused a decrease in retention times for the caffeic acid and chlorogenic acid standards and for all compounds in lettuce root extracts (Tables 3.2 and 3.3). The decreases in retention time for caffeic acid and chlorogenic acid standards were identical to those of compounds in the lettuce root extracts that had been identified as caffeic acid and chlorogenic acid. Similarly the temperature-induced reduction in retention time for

Figure 3.6: Reproduction of HPLC chromatogram of uninoculated and FORL inoculated 3-week-old lettuce root methanol extracts.

The inoculation procedure is described in the text. Extracts were prepared as described in the text. HPLC was performed using a Phenomenex Primesphere 5, C_{18} reverse phase column (250x4.6(i.d.)mm) with a 5 μm particle size. The elution system used was a 40-90% methanol gradient in 0.03% H_3PO_4 at a flow rate of 1 mL per minute over 20 minutes. The sample volume injected was 20 μL for both extracts. The absorbance scale is not the same for both HPLC chromatograms.





Retention Time (Minutes)

FORL Inoculated Lettuce Root

- **Peak 1 - 3.90**
- **Peak 2 - 5.89**
- **Peak 3 - 6.50**
- **Peak 4 - 7.07**
- **Peak 5 - 8.56**
- **Peak 6 - 11.02**

Uninoculated Lettuce Root

- **Peak 1 - 3.90**
- **Peak 2 - 5.90**
- **Peak 3 - 6.52**
- **Peak 4 - 8.59**
- **Peak 5 - 11.03**

Table 3.2: High performance liquid chromatography retention times for compounds detected in lettuce root and coffee bean methanol extracts and in concentrated lettuce root exudates at 22°C.

Chromatography was performed using procedues described in the text. Plant extracts and exudate solutions were prepared as described in the text.

Sample	Retention Time (Minutes									
	UP	CHL	UP	CAF	SII 1	SII 2	SII 3	UP	UP	UP
Lettuce Root (8 wks old)		4.05		6.31	8.63	9.24	11.54	12.04		19.14
Green Coffee Bean	3.32	4.04	5.81		8.89	9.13	11.44			
Lettuce Exudate (Protocol 1)						9.14			15.19	19.17
Lettuce Exudate (Protocol 2)						9.15				19.14

UP - Unknown Peak
CHL - Chlorogenic acid
CAF - Caffeic Acid
SII (1-3) Suspected Isochlorogenic acid Isomer (1-3) (see Text)

Table 3.3: High performance liquid chromatography retention times for compounds detected in FORL inoculated and uninoculated lettuce and tomato root methanol extracts and in uninoculated concentrated lettuce root exudates at 26°C.

Chromatography was performed using procedures described in the text. Plant extracts and exudate solutions were prepared as described in the text.

Sample	Retention Time (Minutes)									
	UP	CHL	UP	CAF	SII1	UP	SII2	SII3	UP	UP
Lettuce Root (FORL)		3.90		5.89	6.50	7.07	8.56	11.02		
Lettuce Root		3.90		5.90	6.52		8.59	11.03		
Lettuce Exudate (Protocol 3 F1)	2.72	4.11		6.03			8.80			
Lettuce Exudate (Protocol 3 F2)		4.05		5.87					13.64	18.83
Tomato Root (FORL)		3.96		5.88						
Tomato Root		3.84	4.95	5.90						

UP - Unknown Peak
CHL - Chlorogenic acid
CAF - Caffeic Acid
SII (1-3) Suspected Isochlorogenic acid Isomer (1-3) (see Text)
FORL - Inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*
F - Fraction Number

compounds identified as isochlorogenic acid isomers was identical between coffee bean and lettuce root extracts.

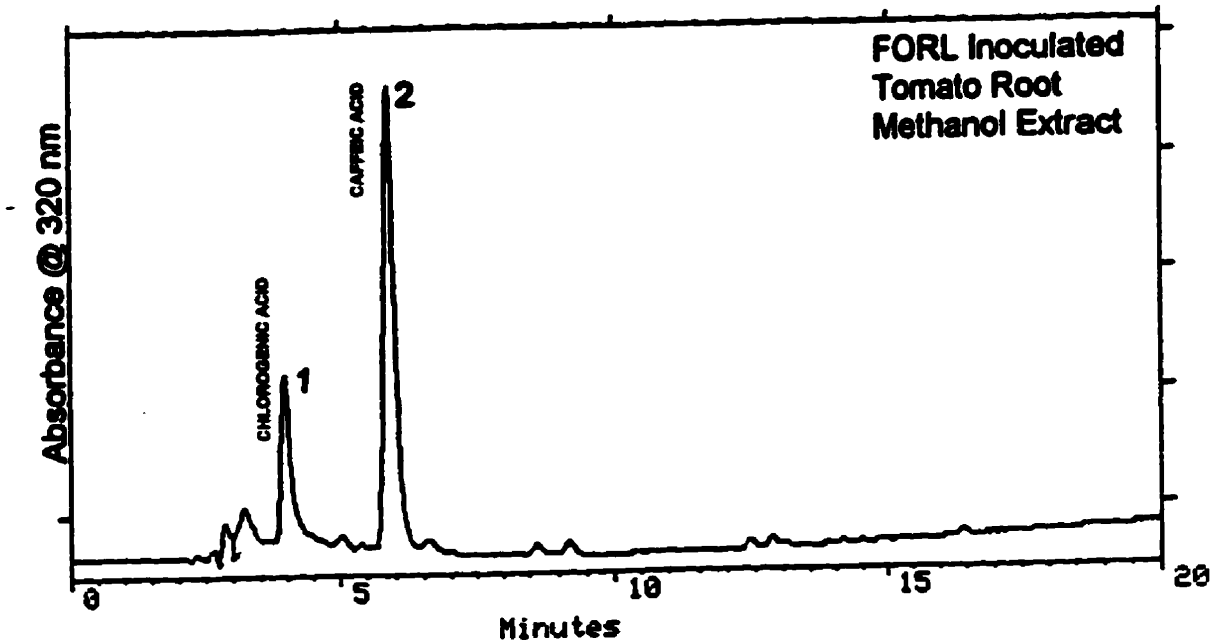
The percentages of total extract absorbance for chlorogenic acid, caffeic acid and the predominant compound in the lettuce roots in the extract from uninoculated lettuce roots were 13%, 25% and 55%, respectively and 14%, 13% and 61% in the extract from FORL-inoculated lettuce roots. Only one new peak with a retention time of 7.07 was detected in the extract of FORL inoculated lettuce root that was not present in the extract of uninoculated lettuce roots. The concentration of chlorogenic acid was higher in inoculated (4.3 +/- 0.6 mg / g tissue) than uninoculated tissue (1.9 +/- 0.4 mg / g tissue) but the concentration of caffeic acid was lower in inoculated than uninoculated roots (1.9 +/- 0.5 mg / g and 2.4 +/- 0.5 mg / g tissue, respectively). The absorbance, and hence concentration, of the predominant compound was approximately 1.75 times higher in the inoculated lettuce root extract than in the extract of uninoculated lettuce roots.

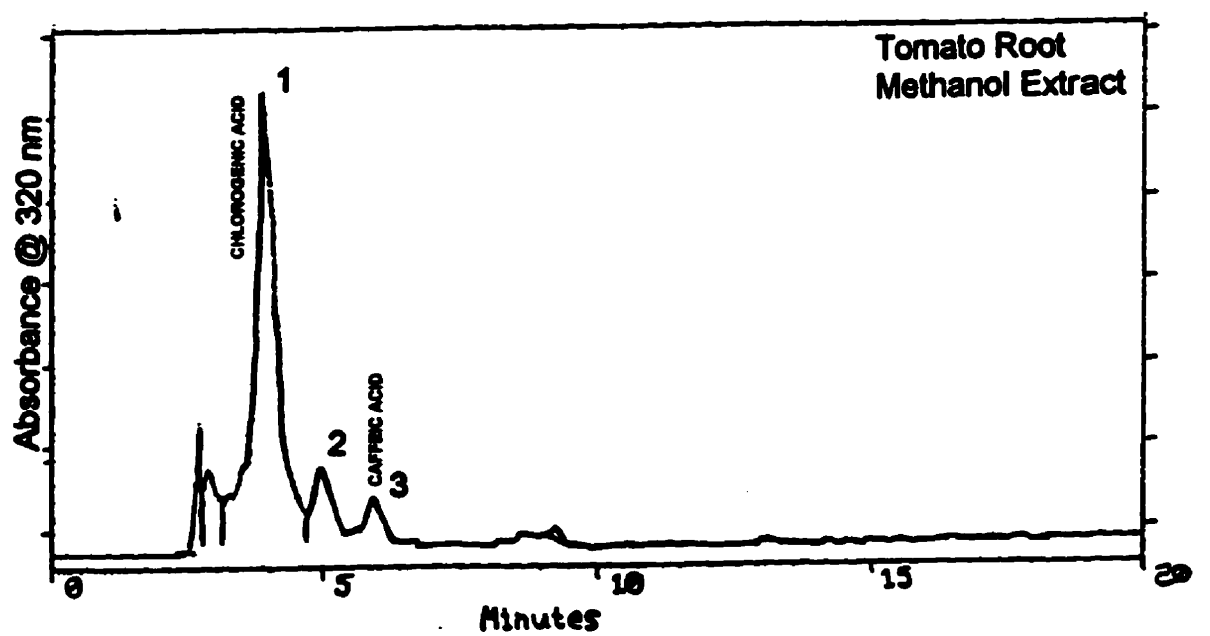
Tomato Roots Exposed to FORL

The HPLC chromatograms (Figure 3.6) of the extracts from uninoculated and FORL-inoculated 3-week-old tomato roots revealed that compounds consistent with chlorogenic acid and caffeic acid are present in tomato roots both prior to and following FORL infection (Table 3.3). The percentages of total extract absorbance for compounds consistent with chlorogenic acid and caffeic acid were 76% and 3% in the extract of uninoculated tomato roots and 21% and 67% in the extract of FORL-inoculated tomato roots, respectively.

Figure 3.7: Reproduction of HPLC chromatogram of uninoculated and FORL-inoculated 3-week-old tomato root methanol extracts.

The inoculation procedure is described in the text. Extracts were prepared as described in the text. HPLC was performed using a Phenomenex Primesphere 5, C_{18} reverse phase column (250x4.6(i.d.)mm) with a 5 μm particle size. The elution system used was a 40-90% methanol gradient in 0.03% H_3PO_4 at a flow rate of 1 mL per minute over 20 minutes. The sample volume injected was 20 μL for both extracts. The absorbance scale is not the same for both HPLC chromatograms.





Retention Time (Minutes)

FORL Inoculated Tomato Root
- **Peak 1 - 3.96**
- **Peak 2 - 5.88**

Uninoculated Tomato Root
- **Peak 1 - 3.84**
- **Peak 2 - 4.95**
- **Peak 3 - 5.90**

B) HPLC of Root Exudates From 8-Week-Old Lettuce Plants

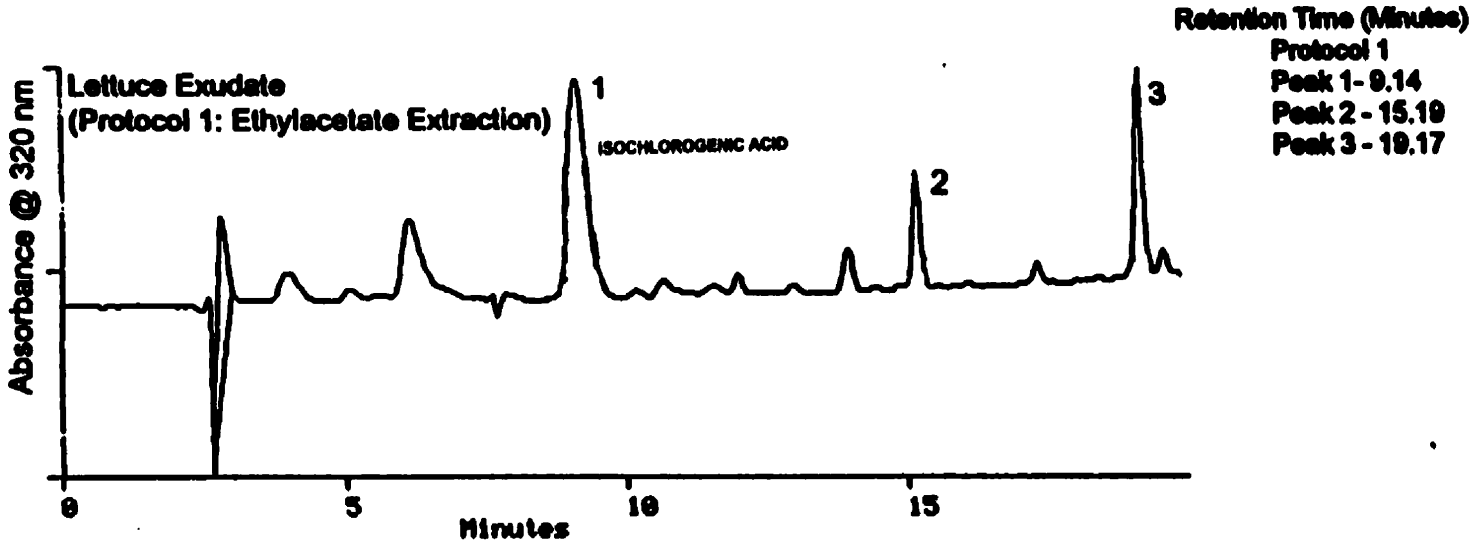
The HPLC chromatograms (at 22 ° C) of the concentrated exudate from 8-week-old lettuce roots from ethyl acetate extraction (Protocol 1) and XAD-4 resin adsorption (Protocol 2) procedures (Figure 3.7) reveal the presence of a compound with a retention time of 9.14 and 9.15 minutes, protocols 1 and 2, respectively similar to the retention time (9.24) of the predominate compound in lettuce root tissues (Table 3.2). A second compound with a retention time of 19.14 and 19.17 minutes, protocols 1 and 2, respectively was also detected in both exudate concentrates. This compound was detected in 8 week old lettuce root extracts (see Figure 3.5 peak marked with asterisk) and in fraction 2 collected in the tC_{18} Sep-Pak Plus cartridge procedure (see below) but not in coffee bean extracts.

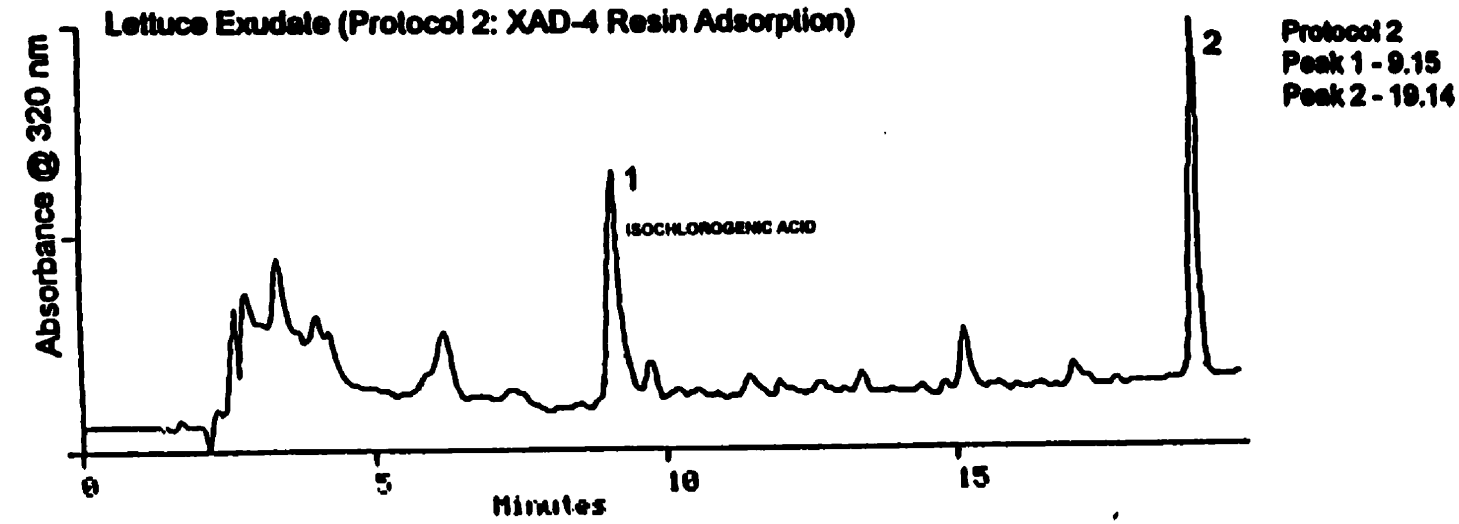
C) HPLC of Root Exudates From 3-Week-Old Lettuce Plants

The HPLC chromatograms (at 26 ° C) of the first two fractions (F1 and F2) from the tC_{18} Sep-Pak Plus cartridge procedure (Figure 3.8) reveal the presence of a compound with a retention time (6.03 and 5.87 minutes, F1 and F2 respectively) similar to the retention time of caffeic acid in root exudates from 3-week-old lettuce plants. No compounds were detected in the third fraction (F3) based on absorbance at 320 nm. The concentration of caffeic acid in the original growth solution was calculated to be $1.8x10^{-7}$ M, using absorbance values of 4 injections, from the standard absorbance curve for caffeic acid (Figure 3.3). Trace amounts of compounds with retention times consistent with chlorogenic acid and the predominant compound in lettuce root tissues were

Figure 3.8: Reproduction of HPLC chromatogram of lettuce root phenolic exudates concentrated by ethyl acetate extraction and XAD-4 resin adsorption procedures.

The concentrated exudate solutions were prepared as described in the text. HPLC was performed using a Phenomenex Primesphere 5, C_{18} reverse phase column (250x4.6(i.d.)mm) with a 5 μm particle size. The elution system used was a 40-90% methanol gradient in 0.03% H_3PO_4 at a flow rate of 1 mL per minute over 20 minutes. The sample volume injected was 20 μL for both extracts. The absorbance scale is not the same for both HPLC chromatograms.

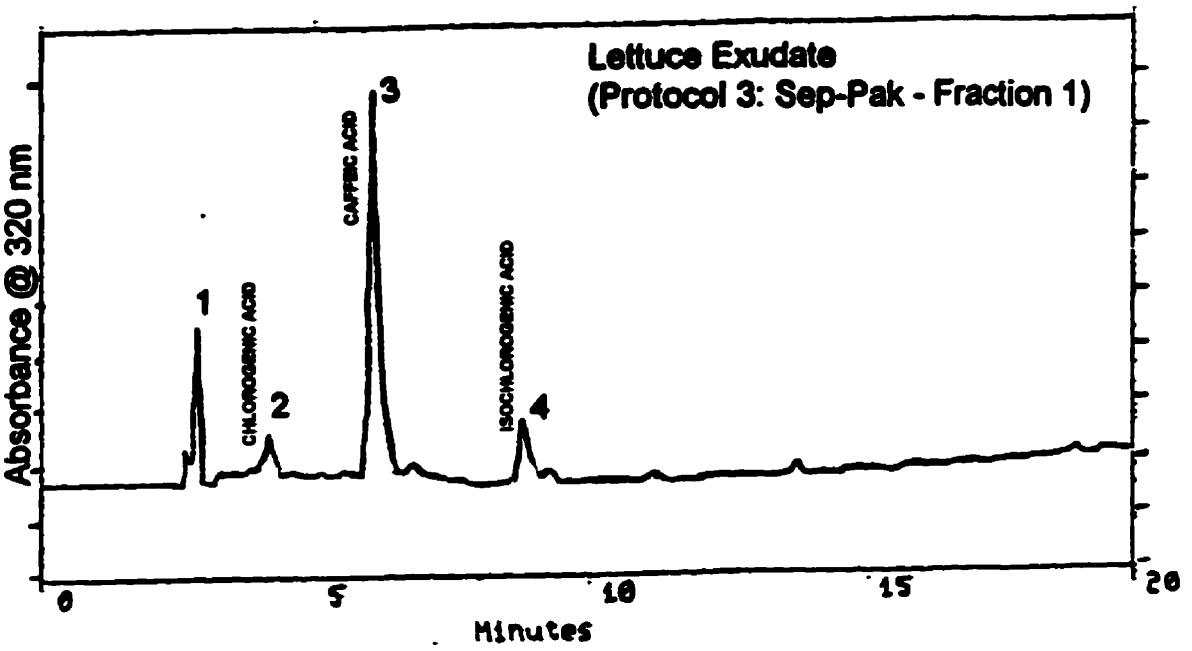


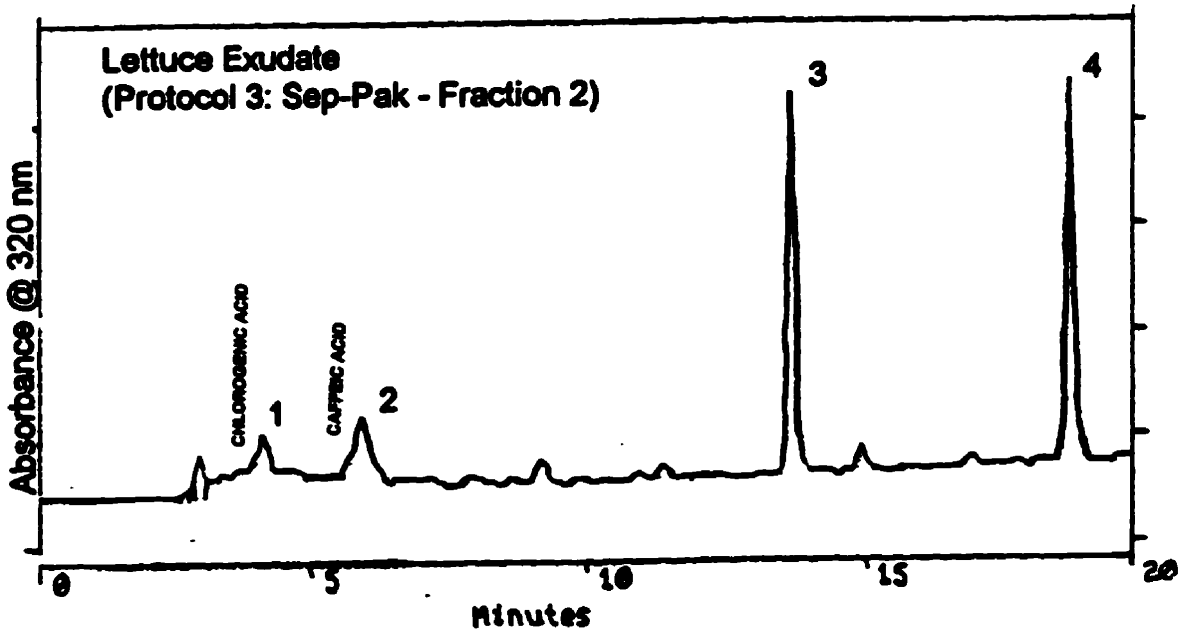


Protocol 2
Peak 1 - 9.15
Peak 2 - 19.14

Figure 3.9: Reproduction of HPLC chromatogram of lettuce root phenolic exudates concentrated by C_{18} Sep-Pak adsorption procedure.

The concentrated exudate solutions were prepared as described in the text. HPLC was performed using a Phenomenex Primesphere 5, C_{18} reverse phase column (250x4.6(i.d.)mm) with a 5 μm particle size. The elution system used was a 40-90% methanol gradient in 0.03% H_3PO_4 at a flow rate of 1 mL per minute over 20 minutes. The sample volume injected was 20 μL for both extracts. The absorbance scale is not the same for both HPLC chromatograms.





Retention Time (Minutes)

Fraction 1
Peak 1 - 2.72
Peak 2 - 4.11
Peak 3 - 6.03
Peak 4 - 8.80

Fraction 2
Peak 1 - 4.05
Peak 2 - 5.87
Peak 3 - 13.64
Peak 4 - 18.83

detected in F1 of the concentrated root exudates (Table 3.3). Fraction 2 of the concentrated root exudates was distinctly yellow in color and had trace amounts of compounds consistent with caffeic acid and chlorogenic acid (Table 3.3). The two major compounds detected in this fraction had retention times of 13.64 and 18.83 minutes. The compound with a retention time of 18.83 minutes was detected in exudates using Protocols 1 and 2 above and in the root methanol extracts of 8-week-old lettuce plants (see Figure 3.3 peak marked with asterisk).

3.4 Discussion

Caffeic acid and chlorogenic acid were identified in the root extracts of lettuce (cv. Grand Rapids) based on the comparison of the R_f values of spots on TLC chromatograms of lettuce root tissue extracts to the R_f values of caffeic acid and chlorogenic acid standards. A third compound present in lettuce root tissue was tentatively identified as isochlorogenic acid by comparing R_f values between lettuce root and coffee bean extracts, the latter is a confirmed source of isochlorgenic acid (Bicchi *et al.*, 1995; Clifford, 1986; Morishita *et al.*, 1986). The lack of close similarity between the R_f values for this spot (spot 3) of the lettuce and coffee extracts (Table 3.1) using a third solvent system (methyl-isobutyl-ketone/formic acid/water (2:1:2, upper phase)) may be due to differing proportions of isochlorogenic isomers in each extract. Chlorogenic acid isomers (monocaffeoylquinic acids), caffeic acid and isochlorogenic acid isomers (dicaffeoylquinic acids) have been eluted consistently in that order from C_{18} reverse-phase HPLC columns (Adzet and Puigmacia, 1985). Results from my HPLC analysis

confirm this pattern and analysis of lettuce root and coffee bean extracts performed at two temperatures indicate that caffeic acid, chlorogenic acid and isochlorogenic acid are present in lettuce root extracts.

Caffeic acid was detected in lettuce root exudates at a concentration of $1.8x10^{-8}$ M while chlorogenic acid and isochlorogenic acid were detected at trace levels. Two unknown compounds were also detected in lettuce exudate which was concentrated using the Sep-Pak procedure. The retention times of these compounds were longer than those of any caffeic derivative identified in this study. A longer retention time is consistent with these compounds having larger molecular weights and/or being less polar compounds than the compounds that were identified. One of these unknown compounds was also detected at trace levels in lettuce root tissue extracts. It is possible that the unidentified compounds are dimers, trimers or polymers of the three principal phenolic compounds found in lettuce roots. Caffeic acid and its esters are highly reactive and are reported to form polymers by the action of polyphenoloxidase catalysed polymerization (Cheynier and Moutounet, 1992) or autocatalysed polymerization (Cillers and Sigleton, 1991). Such polymers would have low solubility and may have formed during the extraction of lettuce tissue or in the lettuce exudate solution prior to concentration. Other possible identities of these molecules include the flavonol quercetin, its corresponding flavone luteolin, or their conjugated forms. These compounds are abundant constituents of the leaves of members of the Lactuceae but have not been reported so far to occur in the roots of these plants (Rees and Harborne, 1984; Williams *et al.*, 1996). Other possibilities are riboflavin or related flavin molecules, which have previously been identified in lettuce

root exudates (Welkie and Miller, 1993).

The presence of caffeic acid and chlorogenic acid in the root tissue extract of 3-week-old tomato seedling (cv. Bonny Best) extracts was confirmed on the basis of HPLC retention-time comparison to authentic standards. Both compounds have previously been reported in tomato roots (Spurr *et al.*, 1965). Bernards *et al.* (1991) reported that the challenge of tomato cells with *Verticillium albo-atrum* induced the synthesis of caffeic acid derivatives not found in unchallenged cells. Infection of tomato roots by FORL did not induce the synthesis of new phenolic compounds but did result in an increase of caffeic acid levels with a corresponding decrease in chlorogenic acid levels. This result is consistent with reports of the infection of tomato causing the conversion of stored chlorogenic acid to more reactive caffeic acid as part of the tomato defense response (Mace *et al.*, 1972) and allows for the possibility that caffeic acid released from lettuce roots is taken up by tomato roots where it augments the level of stored phenolic compounds and then influences the outcome of attempted FORL infection of tomato seedlings.

Chapter 4

Allelopathic Control of Fusarium Crown and Root Rot

4.1 Introduction

4.1.1 Role of Phenolic Compounds in the Browning of Tomato Tissue Caused by *Fusarium* Diseases

Fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) is characterized by the girdling of the hypocotyl and the root cortex of tomato seedlings. The distinctive brown rot lesion eventually causes damping-off of the seedlings (Sanchez *et al.*, 1975). Although more extensive, the biochemical basis for the development of this symptom is similar to the processes leading to browning of tomato vascular tissue caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) the causal agent of *Fusarium* wilt of tomato (Davis *et al*, 1953; Matta *et al.*, 1969).

Pectinases excreted by hyphae of FORL (Benhamou *et al.*,1990; Chamberland *et al.*, 1990) and FOL (Matta and Dimond, 1963) degrade the middle lamella as hyphae grow intercellularly through infected tomato tissue. FORL (Charest *et al.*, 1984) and FOL (Matta and Dimond, 1963) also produce cellulases which destroy tomato cell walls and cell membranes. These destructive processes bring conjugated phenolic compounds normally stored in vacuoles into contact with cytoplasmic transesterases and β-glucosidases. Free phenolics are released by these enzymes from their conjugated forms (Mace *et al* ., 1972). Polyphenol oxidases (PPOs) and peroxidases then oxidize the free phenolic substrates into o-quinones (Beckman, 1980; Retig, 1974).

The brown coloration of *Fusarium*-infected tomato tissue is caused by polymerization of o-quinones into high molecular weight brown pigments and condensation of o-quinones with carbohydrates and proteins (Mace *et al.*, 1972). Production of polyphenol oxidase (PPO) by FORL isolates has been reported *in vitro* and was correlated with pathogenicity of the different FORL isolates (Madhosingh, 1995). It may seem confounding that FORL would produce enzymes for oxidizing and polymerizing host phenolics, a reaction which is normally associated with the plant's defence response (Farkas and Kiraly, 1962). The intrinsic reactivity of o-quinones generates active oxygen species and these create a toxic environment in the area surrounding the site of infection (Leatham *et al.*, 1980). This reaction normally serves as the first line of defence for the tomato plant until other more effective defence mechanisms become fully activated (Bashan *et al.*, 1987). However, the timing and the distance from the infection site determines the efficacy of phenolic oxidation as a resistance or detoxification mechanism (Nicholson and Hammerschmidt, 1992).

Kritzman and Chet (1980) speculated that PPO secreted by *Botrytis allii* (Munn) oxidizes and polymerizes onion phenolic compounds in advance of mycelial growth and that this reduces the risk of pathogen enzymes being inactivated by reactions with host o-quinones. Maraite (1973) found that several PPO isoenzymes are produced by *Fusarium oxysporum* f. sp. *melonis* cultures and also speculated that the different isoenzymes are produced *in vivo* by *Fusarium oxysporum* f. sp. *melonis* to oxidize and hence de-toxify different phenolic substrates found in the host.

Although *in planta* production of PPO by FORL has not been demonstrated it could lead to the premature polymerization of caffeic acid derivatives causing disruption of the tomato defence response. This would leave tomato cells susceptible to FORL enzymes and toxins. The presence of polyphenol oxidase, produced by FORL, would also explain the extensive browning of lesions associated with FORL infection.

4.1.2 Role of Phenolic Compounds in Tomato Resistance to *Fusarium* Diseases

In tomato, fungal infection induces enzymes of carbohydrate metabolism (Benhamou *et al.*, 1991), the shikimate pathway (Dyer *et al.*, 1989), phenylalanine ammonia lyase (Bernards and Ellis, 1989), and enzymes of the phenylpropanoid pathway (Bernards *et al*, 1991; Legrand, 1983). The net result of these inductions of metabolic activity is an increase in the pool of phenolic compounds, principally caffeic acid derivatives, in the cells surrounding the infection site (Matta *et al.*, 1969).

These phenolic compounds are used as precursors for the synthesis of lignin and suberin, which are then incorporated into the cell wall, increasing its mechanical strength and resistance to enzymatic degradation (Matern and Kneusel, 1988). O-quinones inactivate pathogen enzymes directly (Patil and Dimond, 1967) and react with hydrogen peroxide to generate active oxygen species which lead to pathogen enzyme inactivation, lipid peroxidation and DNA cleavage (Baker and Orlandi, 1995; Sutherland, 1991). The oxidation of caffeic acid derivatives initiates the formation of high molecular weight brown pigments formed by caffeoyl quinone polymerization and condensation of caffeoyl quinones with carbohydrates and proteins (Cheynier and Moutounet, 1992; Cilliers and

Singleton, 1991). These barriers act as extracellular hydrophobic barriers to the pathogen because they are highly resistant to enzymatic degradation (Mace *et al.*, 1972). After release from the vacuole,caffeic acid derivatives diffuse through tomato root tissue where they inhibit peroxidase catalysed degradation of indole acetic acid (IAA)(Volpert *et al.*, 1995). This results in increased IAA concentrations in the middle lamella and cell wall which causes increased cell wall plasticity and allows the incorporation of newly formed lignin into the cell wall (Beckman *et al.*, 1974).

The rapid infusion of tomato cell walls with phenolic compounds is a critical factor in resistance to FORL (Benhamou *et al.*, 1994). FORL-resistant cultivars incorporate larger quantities of phenolic compounds in the cell wall more rapidly than FORL susceptible cultivars (Brammall, 1986). Kasenberg (1991) reported that tomato seedlings germinated on an agar medium amended with 0.1 mM and 1.0 mM caffeic acid had less severe FCRR rot symptoms than control seedlings.

4.1.3 Proposed Mechanisms of Action of Lettuce Phenolic Root Exudates in the Allelopathic Control of FCRR

Evidence supporting the proposed role of iron chelation by lettuce root phenolic exudates in the allelopathic control of FCRR was reviewed in chapter 2 and will not be repeated here. In chapter 3, the only phenolic compounds identified in lettuce root tissue were the ortho-dihydroxy phenolic compounds caffeic acid and the caffeoylquinic acids, chlorogenic acid and isochlorogenic acid. Caffeic acid derivatives in lettuce roots and root exudates are expected to affect FCRR by three possible mechanisms not involving

iron chelation (Kasenberg, 1991). These mechanisms may occur within the tomato root rhizosphere and the tomato root apoplastic space.

1) Direct Fungitoxicity of Caffeic Acid Derivatives

Snook *et al.*, (1991) found that a concentration of 4000 ppm (1.1 mM) chlorogenic acid is required for a 25 % inhibition of the growth of *Phytophthora parastica* var. *nictotianae.* Based on the very low inhibitory effect of quinic acid and an inhibitory effect of caffeic acid comparable to that of chlorogenic acid they concluded that the bioactivity of chlorogenic acid is conveyed by the caffeic acid moiety. Chlorogenic acid has been reported to stimulate the growth of some pathogens including the potato pathogen *Fusarium solani* var. *caeruleum* (Waites *et al.*, 1978). Lee and Le Tourneau (1958) reported that chlorogenic acid at 1000 ppm (0.26 mM) inhibits the growth of *Verticillium albo-atrum* Reinke & Berth., but at lower concentrations chlorogenic acid is metabolized and used as a carbon source. Kasenberg and Traquair (1988) reported that at 10 mM both chlorogenic acid and caffeic acid reduce the growth of FORL germ tubes.

2) Inactivation of Fungal Enzymes by Caffeic Acid Derivatives

O-quinones found in plant extracts have long been known to alter the activity of enzymes or completely inactivate them (Pierpoint, 1969). The reaction between o-quinones and enzymes is non-specific (Leatham *et al.*, 1980) and has been implicated in plant resistance (Deverall, 1961; Matern and Kneusel, 1988). Patil and Dimond (1967) reported that caffeic acid and chlorogenic acids inactivate pectinases from *Verticillium albo-atrum*. No reports could be found regarding the inactivation of FORL enzymes by

phenolic compounds but the inactivation of pathogen enzymes by host phenolics is considered to be a general phenomenon contributing to plant resistance (Hunter, 1974).

3) Induced Resistance

Induced resistance is defined as the pre-infection activation of plant defence responses (Benhamou, 1996; Kessmann *et al.*, 1994). However, the mechanisms involved in induced resistance are not fully understood (Fought and Kuc, 1996). Exposure of tomato seedlings to caffeic acid (Kasenberg, 1991), catechol (Retig and Chet, 1974) and phenylalanine (Carrasco *et al.*, 1978) induces resistance to *Fusarium* diseases. In the cases of catechol (Retig and Chet, 1974) and phenylalanine (Carrasco *et al.*, 1978), reduction in the severity of *Fusarium* wilt is correlated with increased concentrations of phenolic compounds and increased peroxidase and PPO activity in seedling roots. Both Retig and Chet (1974) and Carrasco *et al.* (1978) speculated that the treatment compounds are taken-up by the plant roots, augment the store of phenolic compounds and are then used in the synthesis of defensive barriers. However, in their experiments it was not determined whether the plants actually took-up the treatment compounds. Therefore, the possibility exists that the observed responses were simply part of an injury response induced by the test treatments. Fought and Kuc (1996) recently found that extracts from several plants, including lettuce, induce resistance in cucumber to *Colletotrichum lagenarium*. They found no similarity in the chemical composition of plant extracts that induce resistance and could not determine whether any specific compounds were implicated in the induction of resistance.

The objectives in this chapter are 1) to assess the effects of caffeic acid,

chlorogenic acid and lettuce root extracts on the growth of FORL mycelia and on the severity of FCRR on tomato seedlings 2) to determine whether exposure of living lettuce plants to FORL is a requirement for the allelopathic control of FCRR.

4.2 Materials and Methods

4.2.1 FORL Growth Bioassay 1: Inhibition by Various Phenolic Compounds

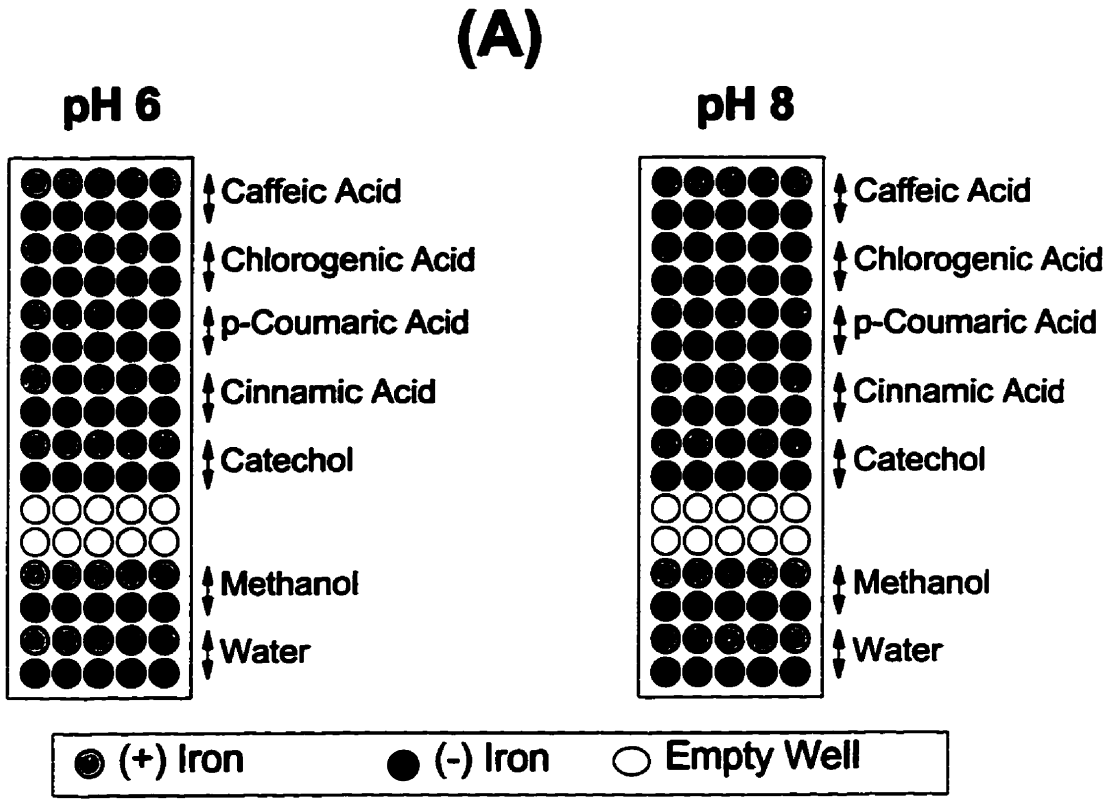
The procedures for the maintenance of FORL cultures and the preparation of FORL microconidia suspensions are described in chapter 2.

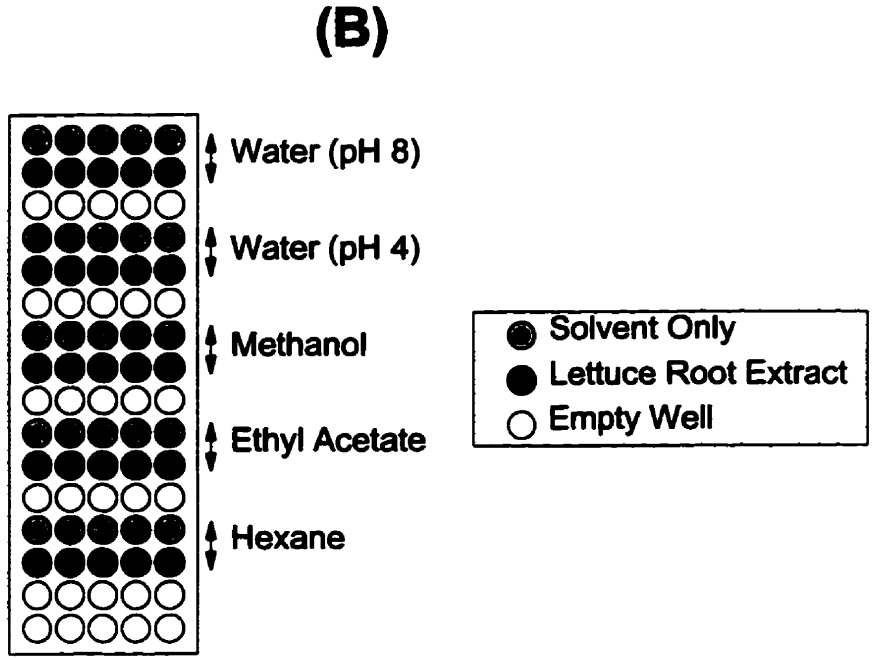
This experiment involved 28 treatments (Figure 4.1 (A)). Caffeic acid, chlorogenic acid, p-coumaric acid, t-cinnamic acid and catechol were tested for inhibition of the growth of FORL mycelia at two iron concentrations and at two pH levels. Both Snook *et al.* (1991) and Ravn et al. (1989) found that non-host phenolics are much more fungitoxic towards pathogens than structurally similar host phenolics. The compounds, p-coumaric acid, t-cinnamic acid and catechol were used as positive controls for the inhibition of the growth of FORL because, unlike caffeic acid and chlorogenic acid, they are not abundant constituents of tomato roots (Spurr *et al.*, 1965). A methanol control and a water control were tested under the same conditions. The experiment was performed using two sterile Fisher brand 80® microtube racks (Fisher Scientific, Toronto, Ont.). The volume of each well was approximately 2.0 mL.

Agar (2 % w/v) was added to two 200 mL volumes of SC medium (Table 2.1) without the iron amendment. The pH was adjusted to pH 6 or pH 8 for each 200 mL of medium using 1 M NaOH and 1 N HCl. The media were autoclaved at 121°C for 15

Figure 4.1: FORL growth bioassays testing the effect of (A) different phenolic compounds and (B) different lettuce root extracts on the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) mycelium.

Bioassays were performed in sterile Fisher brand 80® polypropylene 1.5 mL microtube racks. Absolute phenolic concentrations in the medium were not determined but precipitation of phenolic compounds indicated that they were above saturation levels of the medium. Details of the methodology are given in the text.


(A)
pH 6
pH 8
Caffeic Acid
Chlorogenic Acid
p-Coumaric Acid
Cinnamic Acid
Catechol
Methanol
Water
(+) Iron
(-) Iron
Empty Well


(B)
Water (pH 8)
Water (pH 4)
Methanol
Ethyl Acetate
Hexane
Solvent Only
Lettuce Root Extract
Empty Well

minutes. Fourteen rows of wells of the first microtube rack were filled with the SC medium at pH 6. Fourteen rows of the second microtube rack were filled with the SC medium at pH 8.

Caffeic acid, chlorogenic acid, p-coumaric acid, t-cinnamic acid and catechol (Sigma, St Louis, Mo., USA) were separately dissolved in methanol to make 0.1 M solutions. These solutions were adjusted to pH 6 or pH 8 with 1 N NaOH and 1 N HCl and then filter sterilized using a Nalgene™ 0.2 μm cellulose-acetate syringe filter. A 200 μL aliquot, equivalent to 20 μmol of phenolic compound, of each of the phenolic solutions at the appropriate pH was pipetted onto the surface of the media in 2 rows of wells of the microtube rack filled with medium at the same pH. A 200 μL aliquot of filter-sterilized methanol was pipetted onto medium in 2 rows of wells in both microtube racks to act as one negative control. A second negative control consisted of 2 rows to which filter-sterilized water was added. A 20 μL aliquot of 0.01 M $FeCl_3$ was pipetted in each well of 1 of the 2 rows for each treatment. Wells in this row contained 10 μM iron and were designated as (+) iron while wells in the other row were designated as (-) iron. All phenolic and $FeCl_3$ additions were made while the medium in the wells was still molten (approximately 50° C) to facilitate the diffusion of the compounds into the media and the evaporation of the methanol.

The microtube rack wells were inoculated with FORL by pipetting 100 μL of a 10^4 microconidia/mL suspension onto the surface of the medium. The microtube racks were placed in sterile plastic boxes and incubated at 22° C in darkness. The growth of FORL mycelia in each well was assessed after 4 days using the growth index in Table

2.2. The experiment was repeated once and the mean FORL mycelial growth ratings were analysed statistically using Tukey's multiple paired comparison test.

4.2.2 FORL Growth Bioassay 2: Inhibitory Concentration of Caffeic Acid and Cinnamic Acid

This experiment involved seven treatments. Cinnamic acid and caffeic acid at concentrations of 1, 2 and 5 mM were tested for inhibition of the growth of FORL mycelium on SC medium at pH 6 containing 10 μM $FeCl_3$.

Agar (2 % w/v) was added to SC medium (Table 2.1) which was adjusted to pH 6.0 using 1 M NaOH and 1 N HCl. The medium was then autoclaved at 121°C for 15 minutes. Methanol solutions of 0.1 M cinnamic acid and 0.1 M caffeic acid were adjusted to pH 6 with 1 N NaOH and 1 N HCl and then filter sterilized using a Nalgene™ 0.2 μm cellulose-acetate syringe filter. Appropriate volumes of these solutions were added to 100 mL of the molten SC medium to give the desired phenolic concentrations of 1, 2 and 5 mM. If required, additional filter sterilized methanol was added to each 100 mL of SC media to give final methanol concentrations of 5 % (v/v). The control medium consisted of SC medium at pH 6 containing 5 % (v/v) methanol.

Each of the above media was poured into four sterile 9 cm plastic Petri plates (25 mL / plate). The centre of each plate was inoculated with a 4 mm-diameter plug taken from the actively growing margins of 5-day-old FORL cultures grown on SC medium. The plates were incubated in darkness at 22°C for 1 week. The radius of mycelial growth from the centre plug was measured at 4 locations on each plate. The experiment was

repeated once and the FORL mycelial growth mean radii for treatments were analysed statistically using Tukey's multiple paired comparison test.

4.2.3 FORL Growth Bioassay 3: Inhibition by Lettuce Root Extracts

This experiment involved 10 treatments (Figure 4.1 (B)). Five extracts of dried lettuce root were tested for inhibition of the growth of FORL mycelium on SC medium at pH 6 without the iron amendment (5 treatments). The extraction solvents themselves were tested for inhibition of the growth of FORL mycelium and were used as controls (5 treatments). The experiment was performed using a Fisher brand 80® microtube rack. A row of wells was used as a treatment block in this experiment and the microtube rack was autoclaved at 121°C for 15 minutes prior to use in the bioassay.

Lettuce (*Lactuca sativa* L. cv. Grand Rapids) plants were grown with axenic root systems as described in chapter 3. The roots of four 8-week-old lettuce plants were excised and dried at 60° C for 24 hours. The dried roots were pulverized using a mortar and pestle and the ground samples (10 mg) were placed in five 1.5 mL microtubes. The lettuce root tissue in each microtube was extracted with 1 mL aliquots of water at pH 4, water at pH 8, methanol, ethyl acetate and hexane. These solvents were used to selectively extract basic-water soluble, acidic-water soluble, polar-organic soluble, non-polar-organic soluble and lipid soluble compounds, respectively. The microtubes were placed on a rotary gyrator at 100 rpm for 24 hours at 22° C to ensure complete extraction. The microtubes were then centrifuged at 16, 500 g for 5 minutes. The supernatants were filter-sterilized using a Nalgene™ 0.2 μm cellulose-acetate syringe filter and stored at

4°C until use in the bioassay.

Ten rows of wells in a sterile microtube rack were filled with autoclaved SC medium (Table 2.1) at pH 6 containing 2 % agar (w/v) without the iron amendment. A 100 µL aliquot from each of the above 5 lettuce root extracts was pipetted onto the surface of the medium of 1 row of wells in the microtube rack, a 100 µL aliquot of each of the solvents was also pipetted into a row of wells. The volume of lettuce root extract added to each well contained the equivalent of compounds extracted from 1 mg of dried lettuce root.

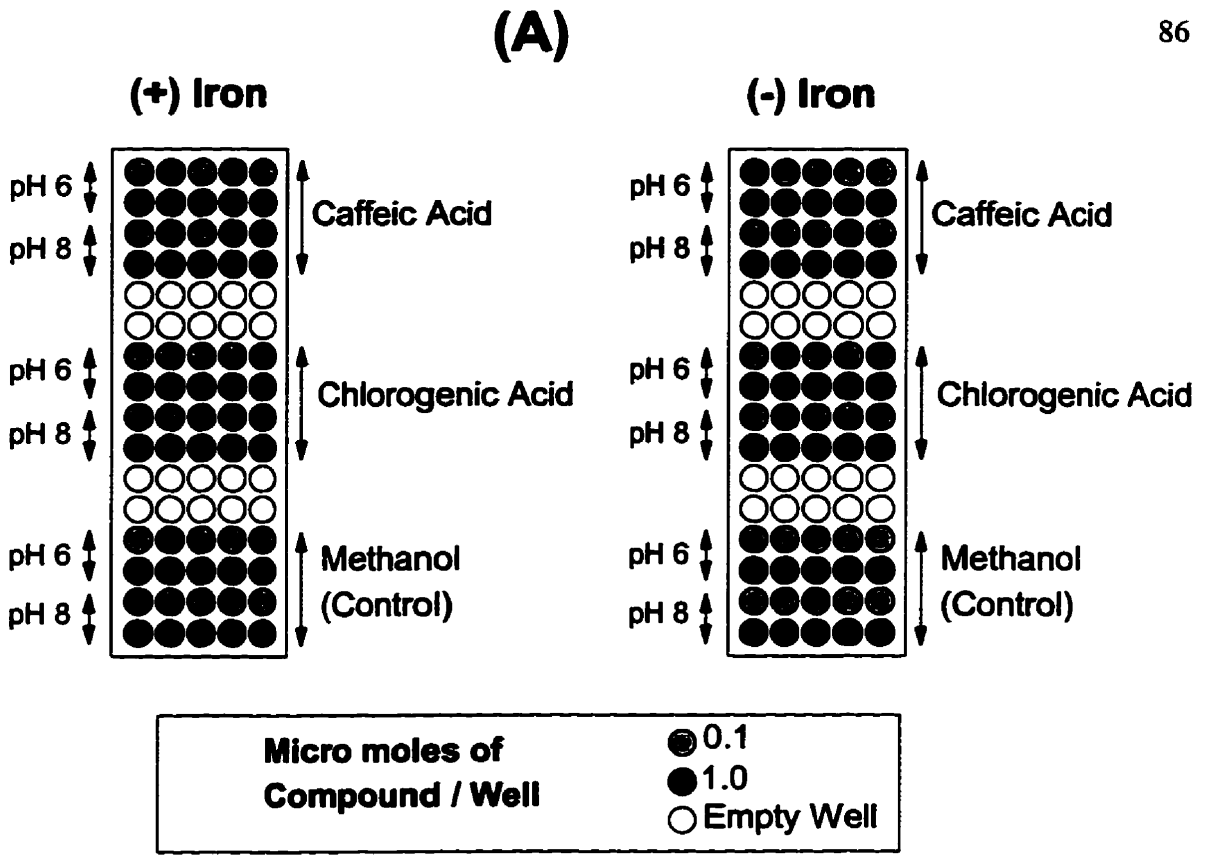
Inoculation, incubation and FORL mycelial growth assessment in microtube racks were performed as in FORL Growth Bioassay 1. The experiment was repeated once and the mean FORL mycelial growth ratings were analysed statistically using Tukey's multiple paired comparison test.

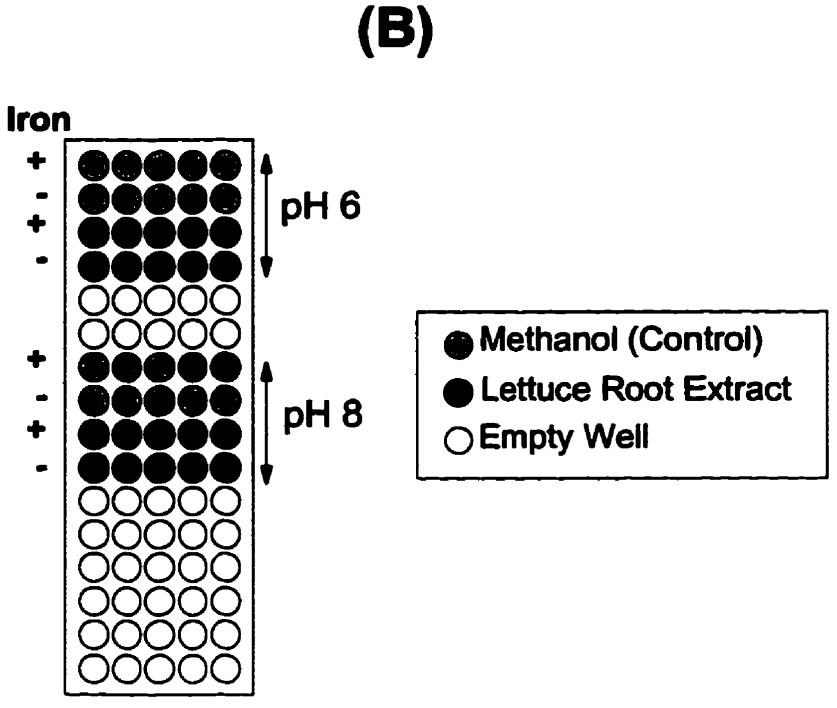
4.2.4 FCRR Bioassay 1: Caffeic Acid and Chlorogenic Acid

This experiment involved 24 treatments (Figure 4.2 (A)). Two concentrations each of caffeic acid and chlorogenic acid were tested for their ability to reduce FCRR severity on tomato seedlings grown at pH 6 and pH 8 and at two iron concentrations (16 treatments). Tomato seedlings grown in the same medium but amended with two different volumes of methanol were used as controls (8 treatments). The experiment was performed using two Fisher brand 80® microtube racks. A row of wells was used as a treatment block in this experiment. The microtube racks were autoclaved at 121° C for 15 minutes prior to use in the bioassay.

Figure 4.2: FCRR severity bioassays testing the effect of (A) caffeic acid and chlorogenic acid and (B) lettuce root methanol extracts on the severity of Fusarium crown and root rot (FCRR) on tomato seedlings.

Bioassays were performed in sterile Fisher brand 80® polypropylene 1.5 mL microtube racks. Details of the methodology are given in the text.


(A)
(+) Iron
(-) Iron
pH 6
pH 8
Caffeic Acid
Chlorogenic Acid
Methanol
(Control)
Micro moles of
Compound / Well
0.1
1.0
Empty Well


(B)
Iron
+
-
pH 6
pH 8
Methanol (Control)
Lettuce Root Extract
Empty Well

In both microtube racks, wells in 2 rows were loaded with 10 µL (0.1 µmol) and 2 rows were loaded with 100 µL (1.0 µmol) aliquots of 0.01 M caffeic acid and chlorogenic acid in methanol. These caffeic acid and chlorogenic acid solutions had previously been filter sterilized using a Nalgene™ 0.2 µm cellulose-acetate syringe filter. In both microtube racks, wells in 2 rows were also filled with 10 µL and wells in 2 rows were filled with 100 µL of sterile methanol. These rows were used as controls to assess the effect of caffeic acid and chlorogenic acid on FCRR development. One of the two rows containing the same treatment (*i.e.* same compound and same concentration) was filled with autoclaved plant nutrient medium (Table 2.1) at pH 6 containing 0.7 % agar (w/v) without the iron amendment, designated as (-) iron medium, and the other row was filled with the same medium but at pH 8. The wells of the second microtube rack were filled similarly, using plant nutrient medium at pH 6 and pH 8 containing 0.7 % agar (w/v) but with the 10 µM $FeCl_3$ amendment, designated as (+) iron medium. A second set of identically prepared microtube racks served as controls to assess the effect of caffeic acid and chlorogenic acid on uninoculated tomato seedling growth.

After the medium had solidified 5-day-old tomato seedlings, grown as described in chapter 2, were placed in each well by inserting their roots into the medium. Sterility of the microtube racks was maintained by placing individual microtube racks in separate sterile transparent plastic boxes. These boxes were kept in a greenhouse under a 16 hour (22° C) light and 8 hour (18° C) dark regime for 2 days. Seedlings from 1 set of microtube racks were then inoculated with FORL by pipetting 100 µL of a 10^6 microconidia/mL suspension into each well. The boxes containing the seedlings were then placed back in

the greenhouse. Boxes were opened daily for aeration and sterile water was added to each well as needed. The severity of FCRR on the tomato seedlings was assessed after 7 days using the disease index in Table 2.3. At the same time the growth of uninoculated seedlings was assessed by measuring shoot heights and visual observations were made of any root rot symptoms, leaf chlorosis and leaf and root necrosis.

The experiment was repeated once and the mean FCRR severity and mean shoot height of uninoculated tomato seedlings were analysed statistically using Tukey's multiple paired comparison test.

4.2.5 FCRR Bioassay 2: Lettuce Root Extracts

This experiment involved 8 treatments (Figure 4.2 (B)). The ability of the methanol extract of dried lettuce root tissue to reduce FCRR severity on tomato seedlings was tested at pH 6 and pH 8 and at 2 iron concentrations (4 treatments). Methanol tested under the same conditions was used as controls (4 treatments). The experiment was performed using a Fisher brand 80® microtube rack. A row of wells was used as a treatment block in this experiment. The microtube racks had been autoclaved at 121°C for 15 minutes prior to use in the bioassay.

Samples of 10 mg of dried 8-week-old lettuce root tissue, prepared as described in the FORL Growth Bioassay 3, were placed in ten 1.5 mL microtubes. The lettuce root tissue in each microtube was extracted with 1 mL of methanol. The microtubes were placed on rotary gyrator for 24 hours at 22° C and then centrifuged at 16, 500 g for 5 minutes. The supernatants were pooled and served as the lettuce root extract. A 100 μL

aliquot of the extract was pipetted into wells of 4 rows in the microtube rack. Each of these 100 µL aliquots contained the equivalent of compounds extracted from 1 mg of the dried lettuce root tissue. These four rows were filled with plant nutrient medium (Table 2.1) containing 0.7 agar (w/v) at pH 6 and pH 8 with 10 µM $FeCl_3$ and without the iron amendment. Controls consisted of wells in 4 rows which had been loaded with 200 µL of methanol and then filled with the above 4 plant nutrient media (1 row/medium). A second identically prepared microtube rack served as a control to assess the effect of the lettuce root methanol extract on the growth of uninoculated tomato seedlings. The transfer of 5-day-old tomato seedlings into the media and inoculation with FORL were performed as described in FCRR Bioassay 1.

The experiment was repeated once and the mean FCRR severity and the mean shoot height of uninoculated tomato seedlings were analysed statistically using Tukey's multiple paired comparison test.

4.2.6 FCRR Bioassay 3: Lettuce and Tomato Companion Planting

Three lettuce allelopathic treatments were tested for their ability to reduce FCRR severity on tomato seedlings. A FORL inoculated tomato seedling was grown on an agar medium at pH 6 containing 10 µM $FeCl_3$. Tomato seedlings were grown with their root systems in direct contact with either the root systems of living lettuce plants (companion planted) or in direct contact with excised lettuce root systems. The aims of this experiment were to determine the effect of exposure of tomato roots to chemicals released from living and dead lettuce root systems and the effect of exposure lettuce plants to

FORL on the severity of FCRR on a tomato companion plant. The following allelopathic treatments were used in this experiment: A) a tomato seedling and a living lettuce plant introduced 3 days prior to introduction of FORL inoculum, B) a tomato seedling and a living lettuce plant introduced the same day as FORL inoculum C) a tomato seedling and a lettuce root which was excised and introduced the same day as FORL inoculum and D) a tomato seedling and FORL inoculum, which acted as the control treatment. Tomato seeds were germinated and grown for 7 days as described in chapter 2 and lettuce seeds were germinated and grown for 30 days as described in chapter 3. Two circular holes approximately 1 cm in diameter and 5 cm apart were cut from the edges of 40 lids of 9 cm plastic Petri plates, using a hot core borer. Each Petri plate bottom was filled with 20 mL of autoclaved plant growth medium (Table 2.1) at pH 6 containing 10 μM $FeCl_3$ and 1 % agar (w/v).

The root system of a 7-day-old tomato seedling was embedded into the medium such that the shoot was outside of the Petri plate bottom. The root system of one living 27-day-old lettuce plant was placed on top of one tomato seedling roots in each of 10 of the Petri plate bottoms (Treatment A). The Petri plate lids were positioned so that the pre-cut holes aligned with the shoot bases of the tomato and lettuce plants. The Petri plates were then sealed with parafilm and the pre-cut holes were sealed with sterile lanolin. The Petri plates were placed in the greenhouse under a 16 hour (22° C) light and 8 hour (18°) dark regime for 3 days. The Petri plates were then re-opened and a root system of one 30 day old lettuce plant was placed in each of 10 of the Petri plates as described above (Treatment B), the axenic root system of each of ten 30-day-old lettuce plants was

excised with a sterile razor blade and placed in one of 10 of the Petri plates on top of the tomato seedling roots (Treatment C). No additions were made to the remaining 10 Petri plates which were used as the control (Treatment D). A 1 mL aliquot of a 10^6 FORL microconidia/mL suspension was pipetted onto the tomato seedling root in 5 of the plates from each of the treatments the other 5 served as uninoculated controls. The plates were re-sealed with parafilm and lanolin and then placed back in the greenhouse. For the following 14 days sterile water was added to each plate by inserting a heated syringe through the lanolin. The volume of water added to each plate varied because of the different transpiration evaporation rates of the plants but sufficient water was added to each plate to prevent the desiccation of the medium.

The severity of FCRR on the tomato seedlings was assessed after 14 days using the disease index in Table 2.3. At the same time visual observations were made of the uninoculated tomato seedlings with regard to root rot symptoms, leaf chlorosis and leaf and root necrosis. The experiment was repeated once and the mean FCRR severity of inoculated tomato seedlings was analysed statistically using Tukey's multiple paired comparison test.

4.3 Results

4.3.1 Effect of Various Phenolics on the Growth of FORL Mycelium

The phenolic compounds added to the FORL growth medium differed dramatically in their inhibition of FORL mycelial growth (Figure 4.3 and Figure 4.4). Saturation of the medium with catechol, cinnamic acid and p-coumaric acid significantly

Figure 4.3: Growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) at pH 6 (top) at pH 8 (bottom) on medium with and without iron and saturated with different phenolic compounds.

Growth of FORL was assessed on SC medium with and without a 10 μM iron amendment. Concentrated methanol solutions of the phenolic compounds were applied to the surface of warm medium (40° C) and the methanol was allowed to evaporate. Absolute phenolic concentrations in the medium were not determined but precipitation of phenolic compounds indicated that they were above saturation levels of the medium. Sterile Fisher brand 80® polypropylene 1.5 mL microtube racks were used for FORL growth. The top 4 rows of the microtube racks were inoculated with 10^3 FORL microconidia per well while the bottom row was not inoculated. Microtube racks were then incubated in darkness at 22° C for 4 days.

CAF - Caffeic acid

CHL - Chlorogenic acid

COU - p-Coumaric acid

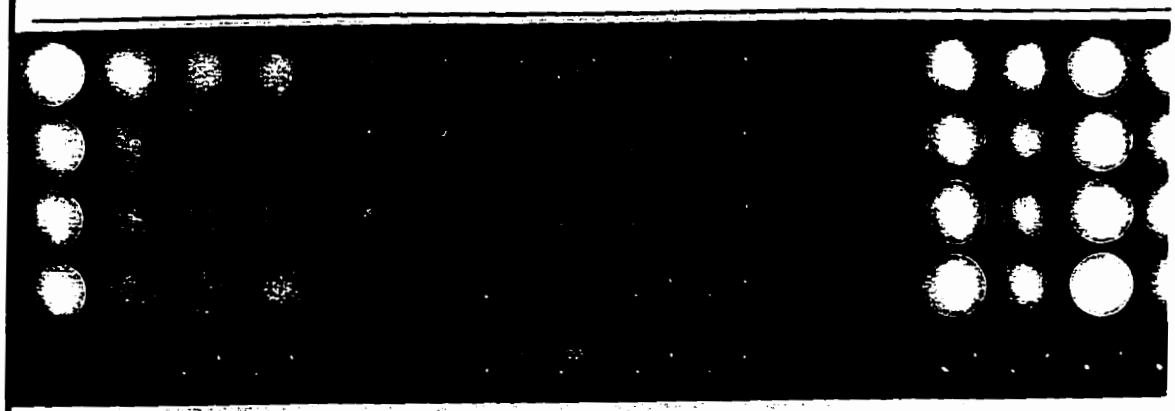
CAT - Catechol

CIN - t- Cinnamic acid

C1 - Methanol (Control 1)

C2 - Water (Control 2)

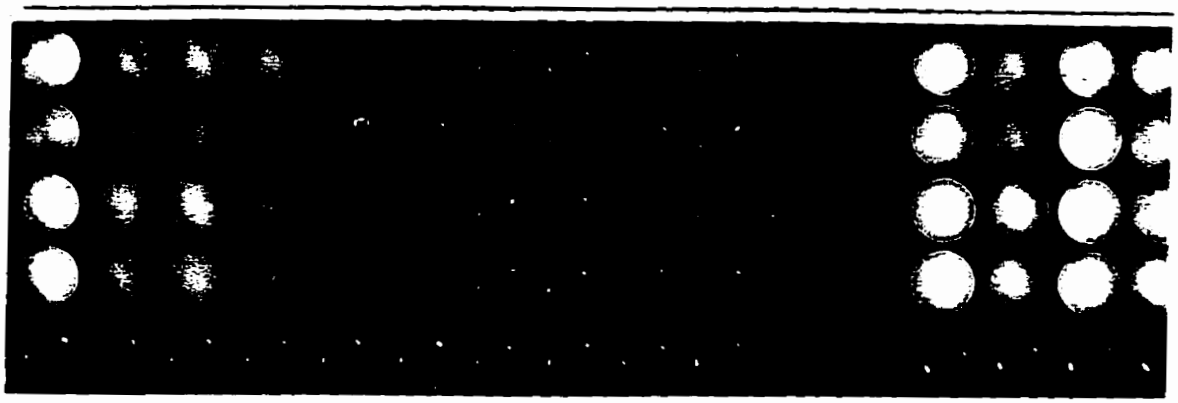
EFFECT OF PHENOLIC ACIDS ON FORL
[$2X10^{-5}$ MOL/WELL] +/- Fe pH 6



+ - + - + - + - + - + - + -

CAF CHL COU CIN CAT C1 C2

EFFECT OF PHENOLIC ACIDS ON FORL
[$2X10^{-5}$ MOL/WELL] +/- Fe pH8



+ - + - + - + - + - + - + -

CAF CHL COU CIN CAT C1 C2

Figure 4.4: Effect of different phenolic compounds on the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) mycelium at (A) pH 6 and (B) at pH 8.

Growth of FORL mycelium was assessed on SC medium with and without a 10 μM iron amendment. Concentrated methanol solutions of the phenolic compounds were applied to the surface of warm medium (40° C) and the methanol was allowed to evaporate. Absolute phenolic concentrations in the medium were not determined but precipitation of phenolic compounds indicated that they were above saturation levels of the medium. Sterile Fisher brand 80® polypropylene 1.5 mL microtube racks were used for FORL growth. Microtube racks were inoculated with 10^3 FORL microconidia per well and then incubated in darkness at 22° C for 4 days. Growth of FORL mycelium was assessed using a growth rating system in Table 2.2. Bars represent mean growth ratings and bars represent standard errors from 10 wells. When error bars are not present the standard error was zero.

CAF - Caffeic acid

CHL - Chlorogenic acid

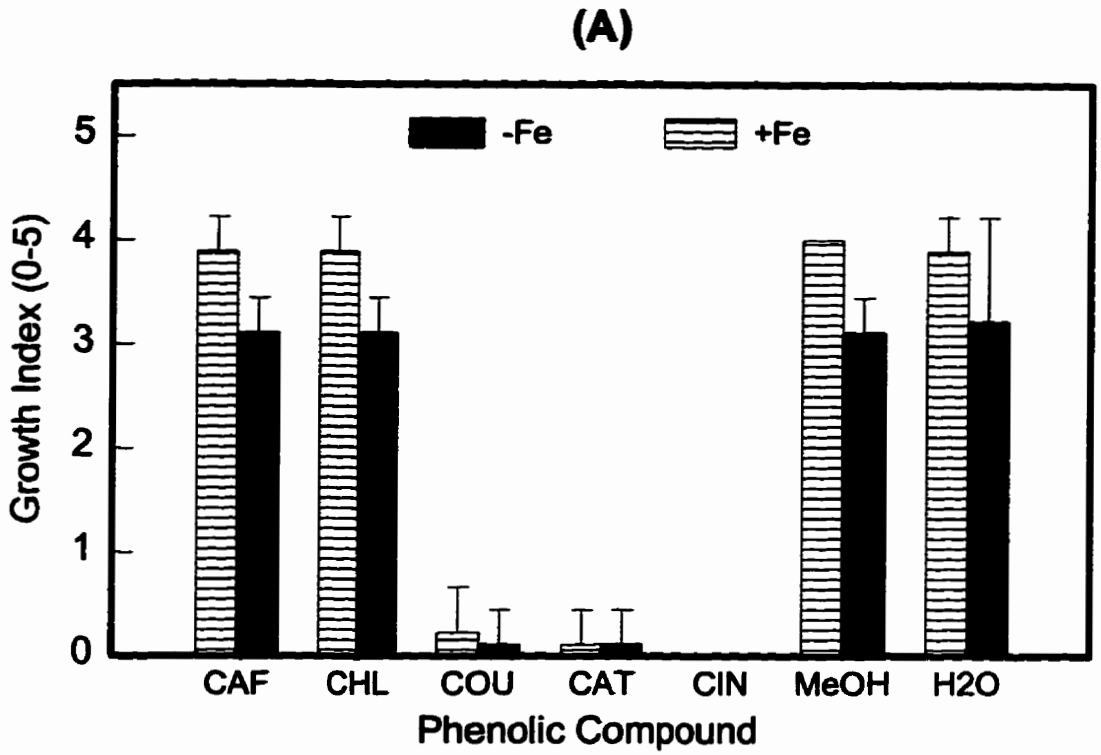
COU - p-Coumaric acid

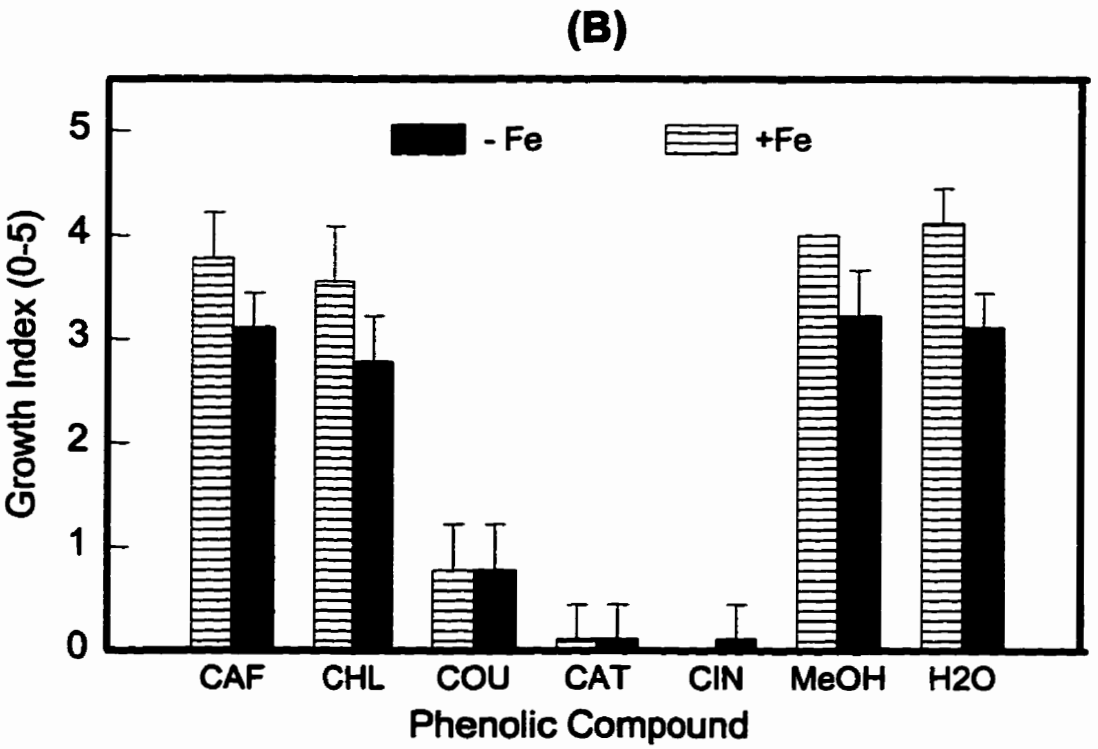
CAT - Catechol

CIN - t- Cinnamic acid

MeOH - Methanol (Control 1)

H_2O - Water (Control 2)


(A)
-Fe
+Fe
Growth Index (0-5)
0
1
2
3
4
5
CAF
CHL
COU
CAT
CIN
MeOH
H2O
Phenolic Compound


(B)
- Fe
+Fe
Growth Index (0-5)
0
1
2
3
4
5
CAF
CHL
COU
CAT
CIN
MeOH
H2O
Phenolic Compound

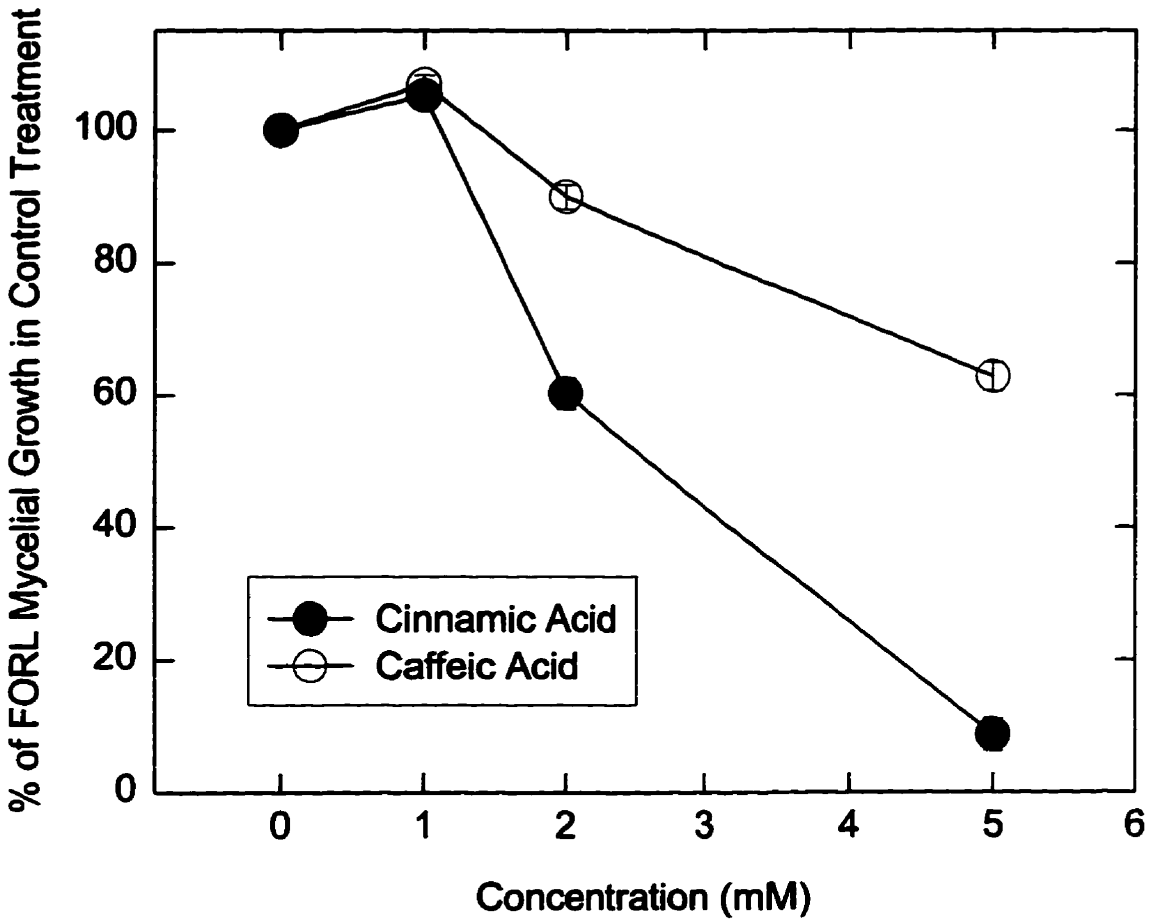
($p<0.05$) reduced the growth of FORL mycelia at both pH 6 and pH 8. Cinnamic acid was the most effective compound tested and almost completely inhibited FORL mycelial growth. Saturation of the medium with caffeic acid and chlorogenic acid did not significantly ($p>0.05$) reduce the growth of FORL mycelium at either pH 6 or pH 8. The growth of FORL mycelium did not differ between the methanol and water control wells. The pH of the medium did not affect FORL mycelial growth in either of the control treatments. In the caffeic acid, chlorogenic acid and control treatments the growth of FORL mycelium was lower in the wells that did not have the iron amendment compared to the wells with the iron amendment but this effect was not statistically significant ($p>0.05$).

4.3.2 Concentrations of Caffeic Acid and Cinnamic Acid Inhibitory to the Growth of FORL Mycelium

The growth of FORL mycelium was not significantly ($p>0.05$) affected by 1 mM caffeic acid or 1 mM cinnamic acid but was significantly ($p<0.05$) reduced at 2 mM and 5 mM of both compounds (Figure 4.5). Cinnamic acid was significantly ($p<0.05$) more inhibitory to the growth of FORL mycelium than caffeic acid at both 2 mM and 5 mM. A concentration of 5 mM caffeic acid caused only a 35 % reduction in the growth of FORL mycelium while 5 mM cinnamic acid caused a 90 % reduction in the growth of FORL mycelium.

Figure 4.5: Effect of caffeic acid and cinnamic acid on the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) mycelium on SC medium at pH 6.

Growth of FORL mycelium in sterile plastic Petri plates was assessed after 7 days of incubation in darkness at 22° C by measuring the distance from a central inoculum plug to the edge of mycelium growth. Four measurement were taken from each of 8 plates per treatment (4 plates per replicate). Symbols represent mean values and bars represent standard errors from 32 growth measurements.


% of FORL Mycelial Growth in Control Treatment
100
80
60
40
20
0
0
1
2
3
4
5
6
Concentration (mM)
Cinnamic Acid
Caffeic Acid

4.3.3 Effect of Lettuce Root Extracts on the Growth of FORL Mycelium

Water at pH 8 significantly ($p<0.05$) reduced the growth of FORL mycelium compared to the water extract of lettuce root made using water at pH 8 but there was no significant ($p>0.05$) difference between the inhibitory effect of any other solvents and corresponding lettuce root extract (Figure 4.6). There was no significant ($p>0.05$) difference in the inhibitory effect between the different lettuce root extracts.

4.3.4 Effect of Caffeic Acid and Chlorogenic Acid on FCRR Severity

Exposure of FORL inoculated tomato seedlings to caffeic acid and chlorogenic acid at both 0.1 µmol/well and 1.0 µmol/well significantly ($p<0.05$) increased the severity of FCRR regardless of the pH or iron status of the medium (Figures 4.7 and 4.8). The iron status of the medium did not significantly ($p>0.05$) affect the severity of FCRR on control seedlings or seedlings exposed to chlorogenic acid at both 0.1 µmol/well and 1.0 µmol/well or caffeic acid at 1.0 µmol/well. Seedlings exposed to 0.1 µmol/well of caffeic acid in (+)iron media had significantly ($p<0.05$) less severe FCRR symptoms than seedlings exposed to 0.1 µmol/well of caffeic acid in (-) iron media. However, the severity of FCRR on seedlings exposed to 0.1 µmol/well in (+) iron media did not differ significantly ($p>0.05$) from that of control seedlings. The severity of FCRR was significantly ($p<0.05$) greater on control seedlings at pH 6 than pH 8 regardless of the iron status of the medium.

Growth of uninoculated tomato seedlings at both pH 6 and pH 8 in both (+) iron and (-) iron medium amended with 1.0 µmol/well of caffeic acid or chlorogenic acid

Figure 4.6 Effects of different lettuce root extracts on the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) mycelium.

Lettuce root extracts were prepared by extracting dried lettuce root tissue in water (pH 8), water (pH 4), methanol, ethyl acetate and hexane. In each case the solvent alone was used as a control to determine the effect of the solvent on FORL growth. Growth was assessed on SC medium at pH 6 without an iron amendment in sterile Fisher brand 80® polypropylene 1.5 mL microtube racks. Microtube racks were incubated in darkness at 22° C and FORL growth was assessed 4 days after inoculation with 10^3 FORL microconidia per well. Bars represent mean growth ratings and error bars represent standard errors from 10 wells. When error bars are not present the standard error was zero.

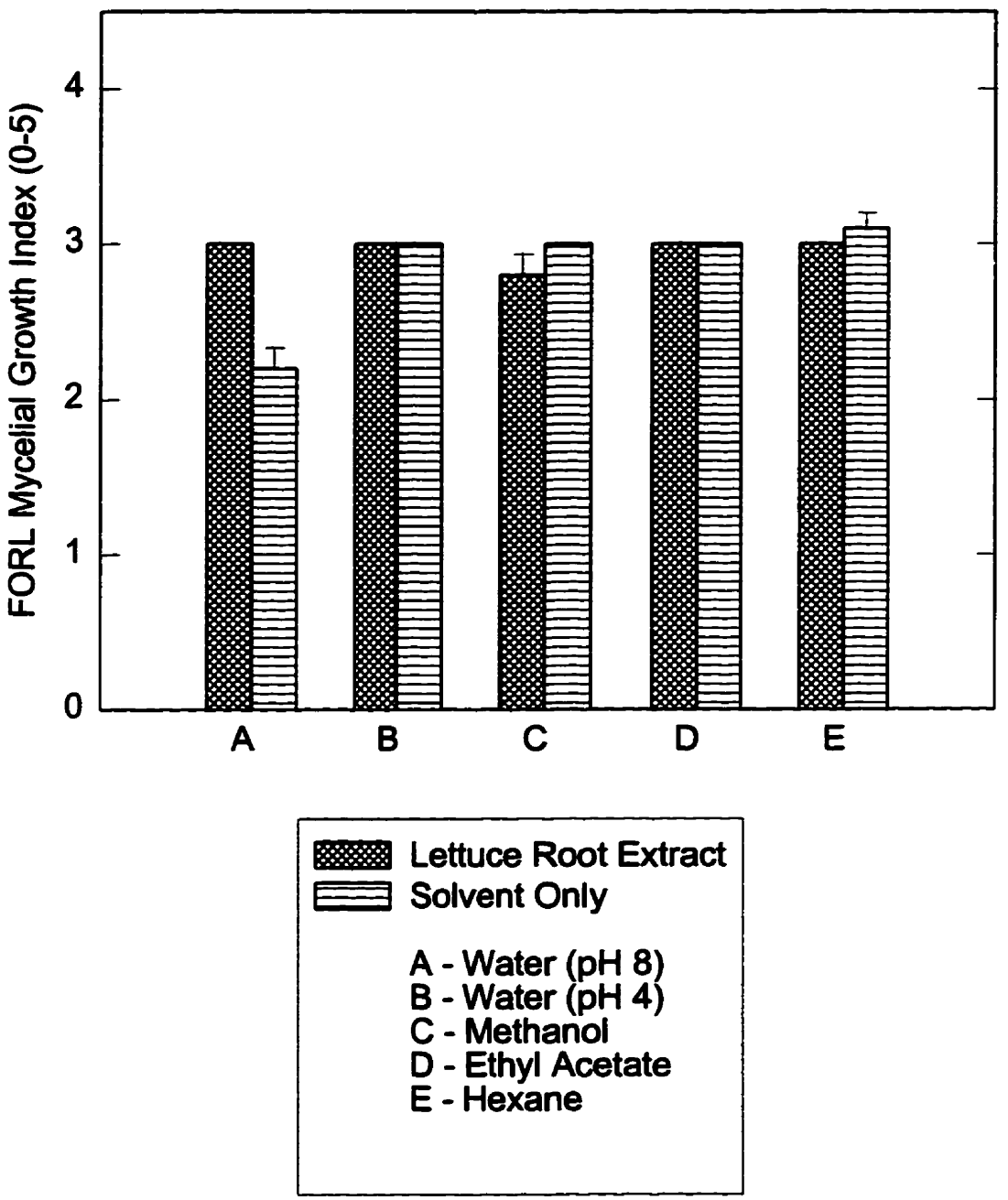
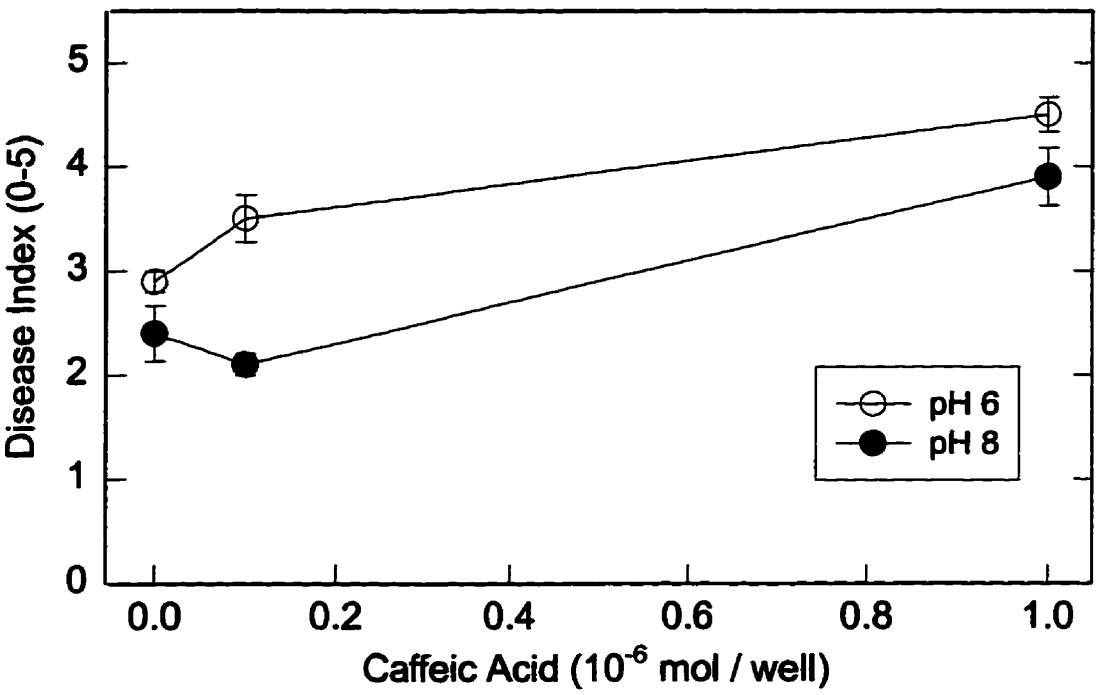

FORL Mycelial Growth Index (0-5)
0
1
2
3
4
A
B
C
D
E
Lettuce Root Extract
Solvent Only
A - Water (pH 8)
B - Water (pH 4)
C - Methanol
D - Ethyl Acetate
E - Hexane

Figure 4.7: Effect of caffeic acid on Fusarium crown and root rot (FCRR) severity on tomato seedlings grown in medium containing (A) 10^{-5} M iron and (B) no iron, at pH 6 and pH 8.

Quantities of 0.1 µmol and 1.0 µmol of caffeic acid in methanol were added to wells of 2 rows in each of 2 sterile microtube racks. Wells loaded with methanol served as controls. The wells of 1 rack were filled with plant nutrient medium containing 10^{-5} M iron and the other rack was filled with medium without iron (2.0 mL/well). Tomato seeds were germinated as described in the text. Five day old seedlings were transplanted into each of these wells. Each seedling was inoculated with 100 µL of a 10^{6} *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) microconidia/mL suspension. Microtube racks were kept in sterile clear plastic boxes and maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. FCRR severity was assessed after 7 days using a disease index in Table 2.3. Symbols represent mean values and bars represent standard errors from 10 seedlings.

(A)


Disease Index (0-5)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Caffeic Acid (10^{-6} mol / well)
pH 6
pH 8

(B)

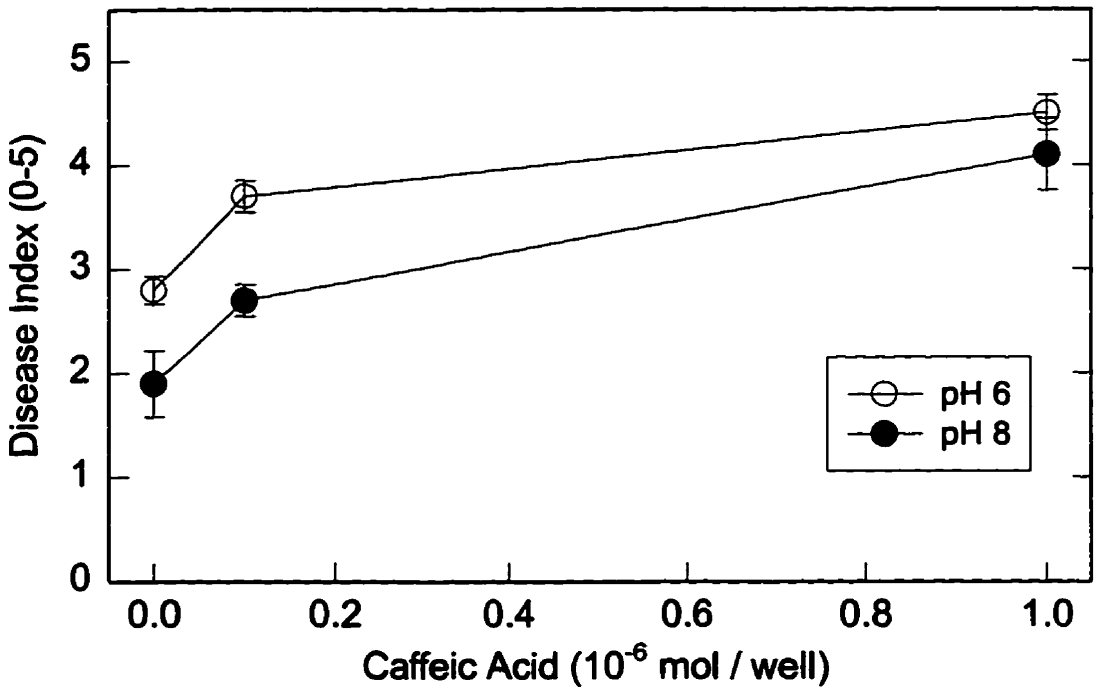
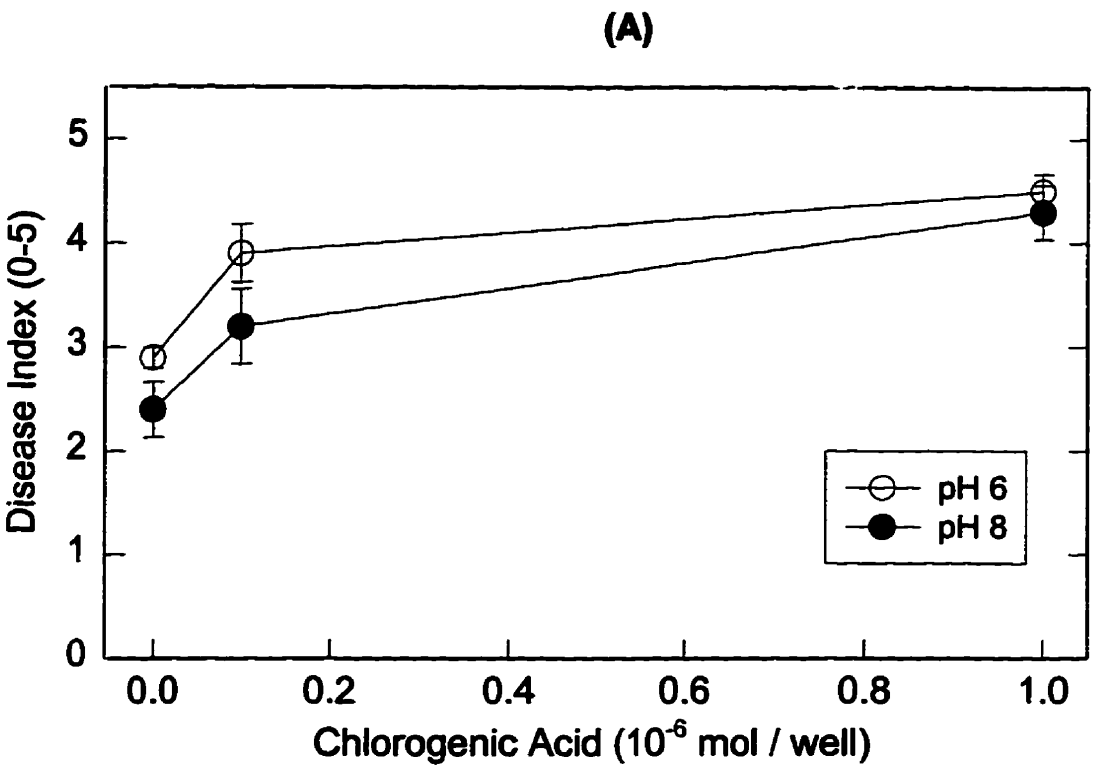

Disease Index (0-5)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Caffeic Acid (10^{-6} mol / well)
pH 6
pH 8

Figure 4.8: Effect of chlorogenic acid on Fusarium crown and root rot (FCRR) severity of tomato seedlings grown in medium containing (A) 10^{-5} M iron and (B) no iron, at pH 6 and pH 8.

Quantities of 0.1 µmol and 1.0 µmol of caffeic acid in methanol were added to wells of 2 rows in each of 2 sterile microtube racks. Wells loaded with methanol served as controls. The wells of 1 rack were filled with plant nutrient medium containing 10^{-5} M iron and the other rack was filled with medium without iron (2.0 mL/well). Tomato seeds were germinated as described in the text. Five day old seedlings were transplanted into each of these wells. Each seedling was inoculated with 100 µL of a 10^{6} *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) microconidia/mL suspension. Microtube racks were kept in sterile clear plastic boxes and maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. FCRR severity was assessed after 7 days using a disease index in Table 2.3. Symbols represent mean values and bars represent standard errors from 10 seedlings.


(A)
Disease Index (0-5)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Chlorogenic Acid (10^{-6} mol / well)
pH 6
pH 8

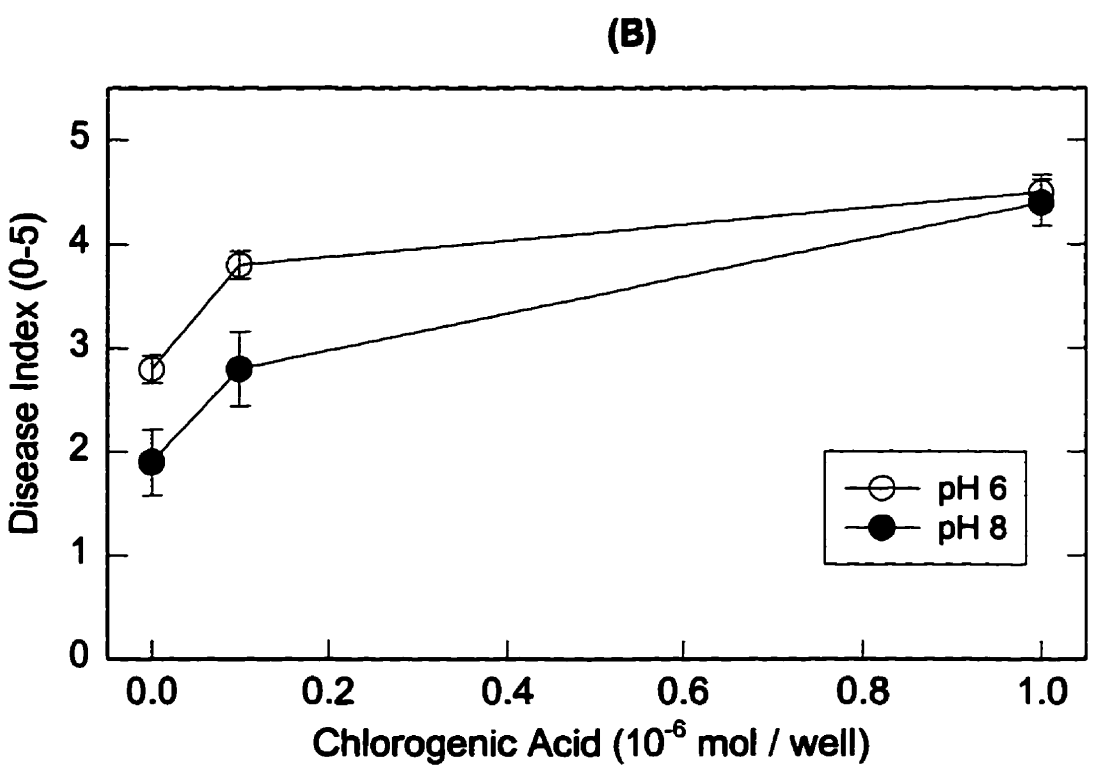
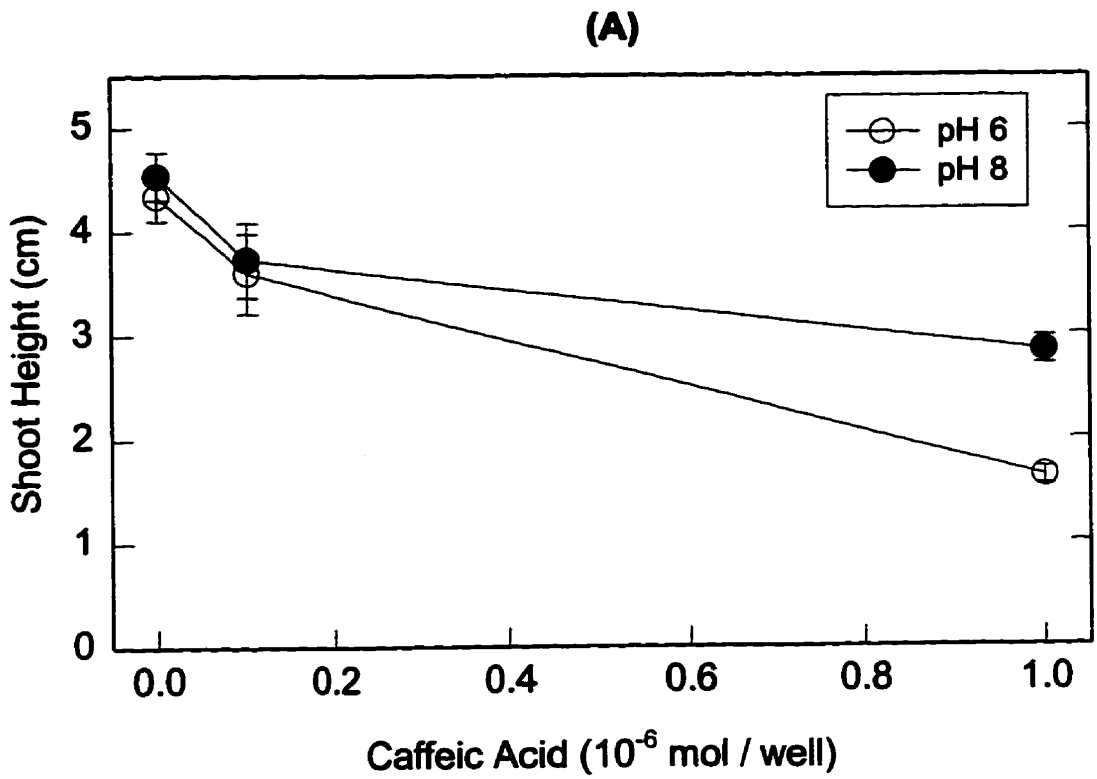

(B)
Disease Index (0-5)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Chlorogenic Acid (10^{-6} mol / well)
pH 6
pH 8

Figure 4.9: Effect of caffeic acid on shoot height of tomato seedlings grown in medium containing (A) 10^{-5} M iron and (B) no iron, at pH 6 and pH 8.

Quantities of 0.1 μmol and 1.0 μmol of caffeic acid in methanol were added to wells of 2 rows in each of 2 sterile microtube racks. Wells loaded with methanol served as controls. The wells of 1 rack were filled with plant nutrient medium containing 10^{-5} M iron and the other rack was filled with medium without iron (2.0 mL/well). Tomato seeds were germinated as described in the text. Five day old seedlings were transplanted into each of these wells. Microtube racks were kept in sterile clear plastic boxes and maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. Shoot heights were taken after 7 days. symbols represent mean values and error bars represent standard errors from 10 seedlings.


(A)
pH 6
pH 8
Shoot Height (cm)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Caffeic Acid (10^{-6} mol / well)

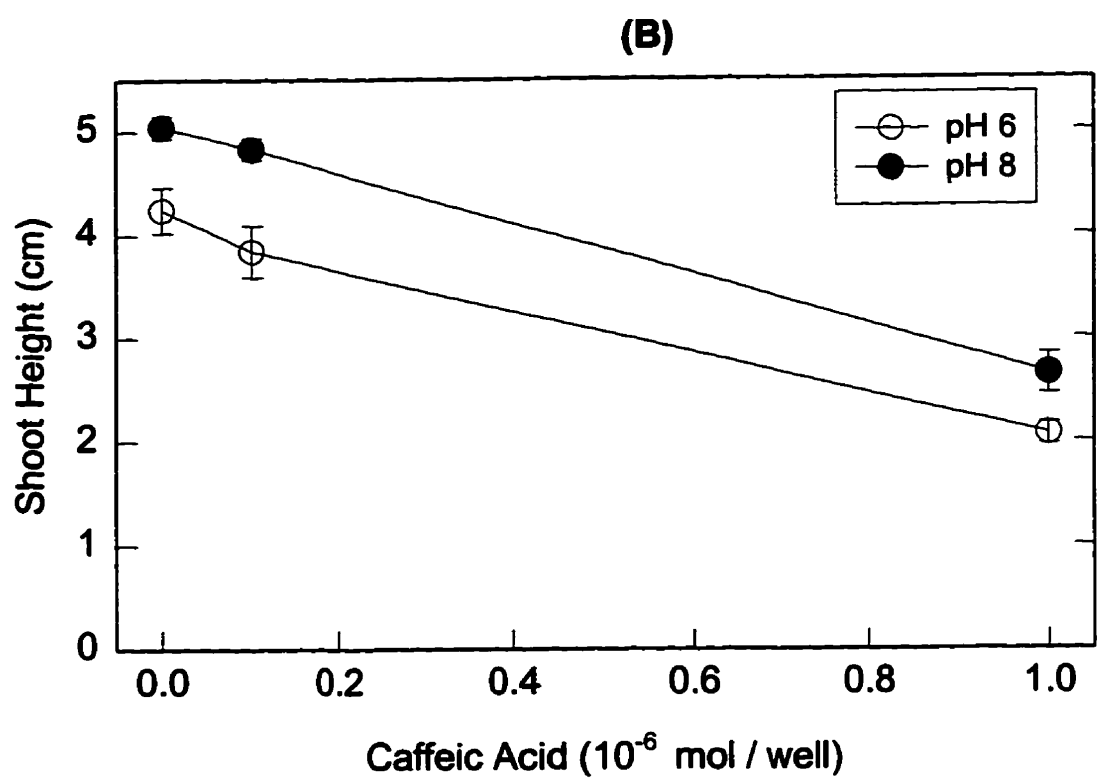
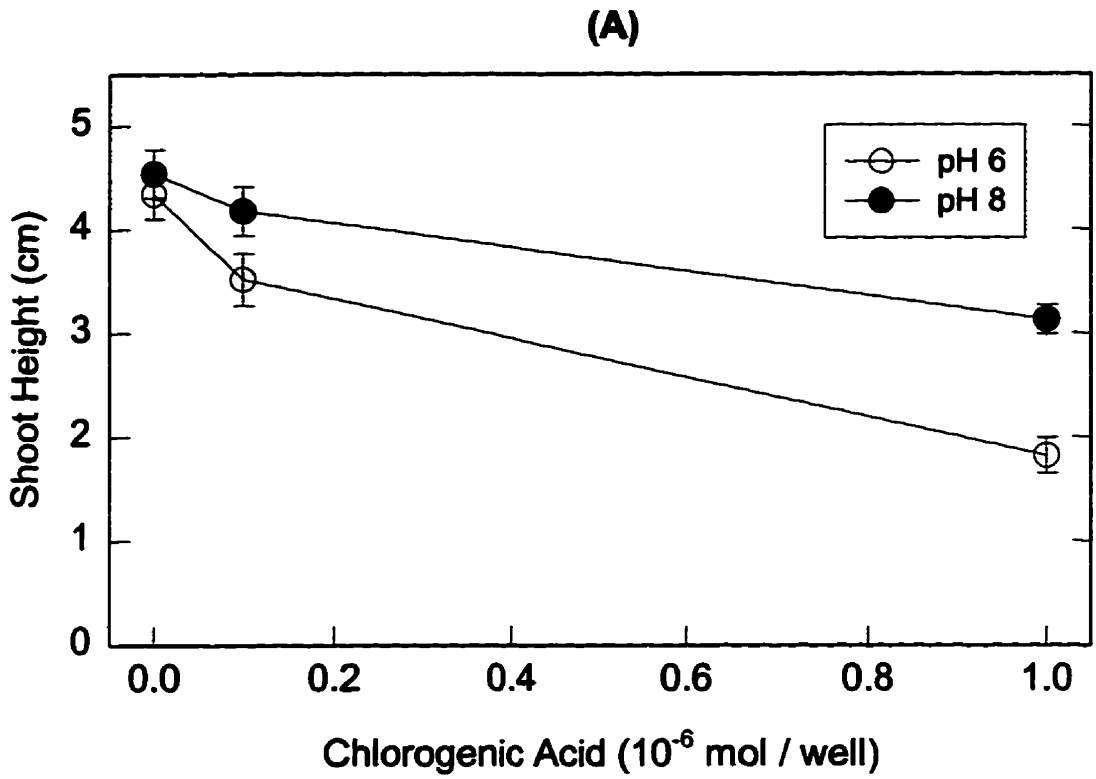
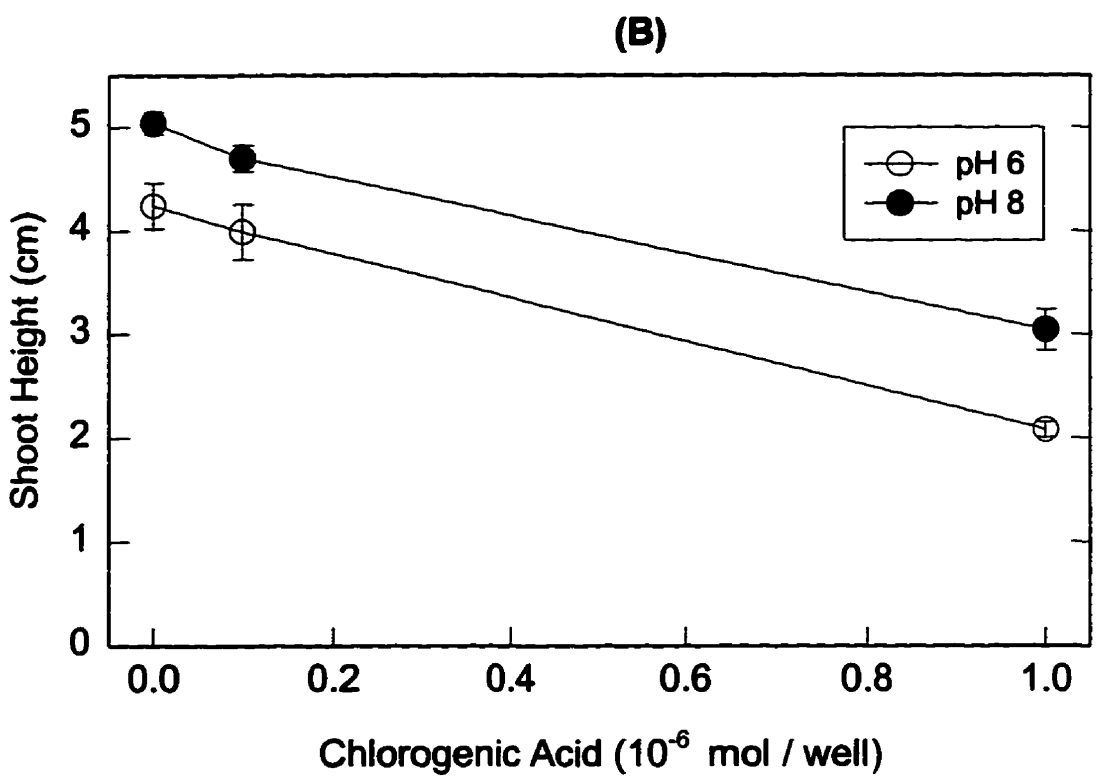

(B)
pH 6
pH 8
Shoot Height (cm)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
Caffeic Acid (10^{-6} mol / well)

Figure 4.10: Effect of chlorogenic acid on shoot height of tomato seedlings grown in medium containing (A) 10^{-5} M iron and (B) no iron, at pH 6 and pH 8.

Quantities of 0.1 µmol and 1.0 µmol of caffeic acid in methanol were added to wells of 2 rows in each of 2 sterile microtube racks. Wells loaded with methanol served as controls. The wells of 1 rack were filled with plant nutrient medium containing 10^{-5} M iron and the other rack was filled with medium without iron (2.2 mL/well). Tomato seeds were germinated as described in the text. Five day old seedlings were transplanted into each of these wells. Microtube racks were kept in sterile clear plastic boxes and maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. FCRR severity was assessed after 7 days using a disease index in Table 2.3. Symbols represent mean values and error bars represent standard errors from 10 seedlings.


(A)
Shoot Height (cm)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
pH 6
pH 8
Chlorogenic Acid (10^{-6} mol / well)


(B)
Shoot Height (cm)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
pH 6
pH 8
Chlorogenic Acid (10^{-6} mol / well)

resulted in significant ($p<0.05$) reductions in seedling shoot height (Figures 4.9 and 4.10). Caffeic acid and chlorogenic acid at 0.1 µmol/well did not significantly ($p>0.05$) affect the shoot height of seedlings in (+) iron medium, but in (-) iron medium the same concentration of both compounds significantly ($p<0.05$) reduced the shoot heights of tomato seedlings. At 1.0 µmol/well both compounds caused a brown discoloration and necrosis of the primary root tip.

4.3.5 Effect of Lettuce Root Extracts on FCRR Severity

Amendment of the tomato growth medium with the methanol extract of lettuce root tissue significantly ($p<0.05$) increased the severity of FCRR on the tomato seedlings regardless of the pH of the medium (Figure 4.11).

Comparison of control seedlings indicates that FCRR severity was not significantly ($p>0.05$) affected by the iron status of the medium at either pH 6 or pH 8. The severity of FCRR was greater at pH 6 than at pH 8 on both (-) iron and (+) iron but this effect was only significant ($p<0.05$) on (+) iron medium. No abnormal development or coloration of leaves or roots was observed on any of the uninoculated tomato seedlings treated with the lettuce root extract.

4.3.6 Effect of Lettuce and Tomato Companion Planting on FCRR Severity

None of the lettuce allelopathic treatments significantly ($p>0.05$) affected the severity of FCRR on tomato seedlings (Figure 4.12). In the FORL inoculated treatments the excised lettuce root tissue became grey in color while in the treatments involving

Figure 4.11: Effect of the methanol extract of lettuce root tissue on Fusarium crown and root rot (FCRR) severity on tomato seedlings grown in medium with 10^{-5} M iron and without added iron, at pH 6 and pH 8.

The methanol extract of lettuce root tissue was added to 4 rows of wells of a sterile microtube rack (1 mg of dried lettuce root extract / well). Four rows of wells were loaded with methanol as controls. The wells were filled with plant nutrient medium containing 10^{-5} M in iron or without iron at pH 6 and pH 8 (2.2 mL/well). Tomato seeds were germinated as described in the text. Five day old seedlings were transplanted into these wells. Each seedling was inoculated with 100 µL of a 10^6 *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) microconidia/mL suspension. Microtube racks were kept in sterile clear plastic boxes and maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. FCRR severity was assessed after 7 days using a disease index in Table 2.3. Symbols represent mean values and error bars represent standard errors from 10 seedlings.

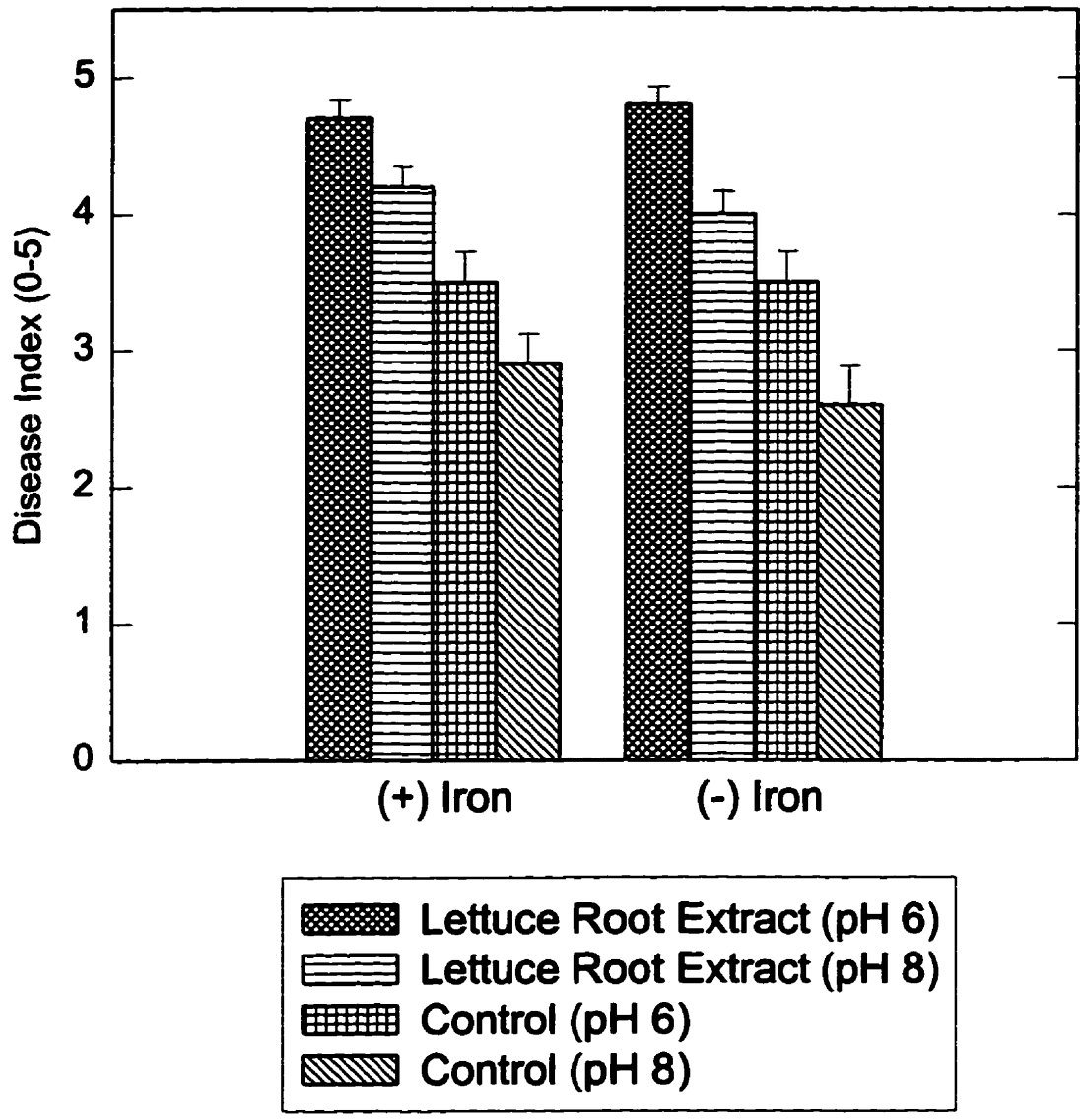
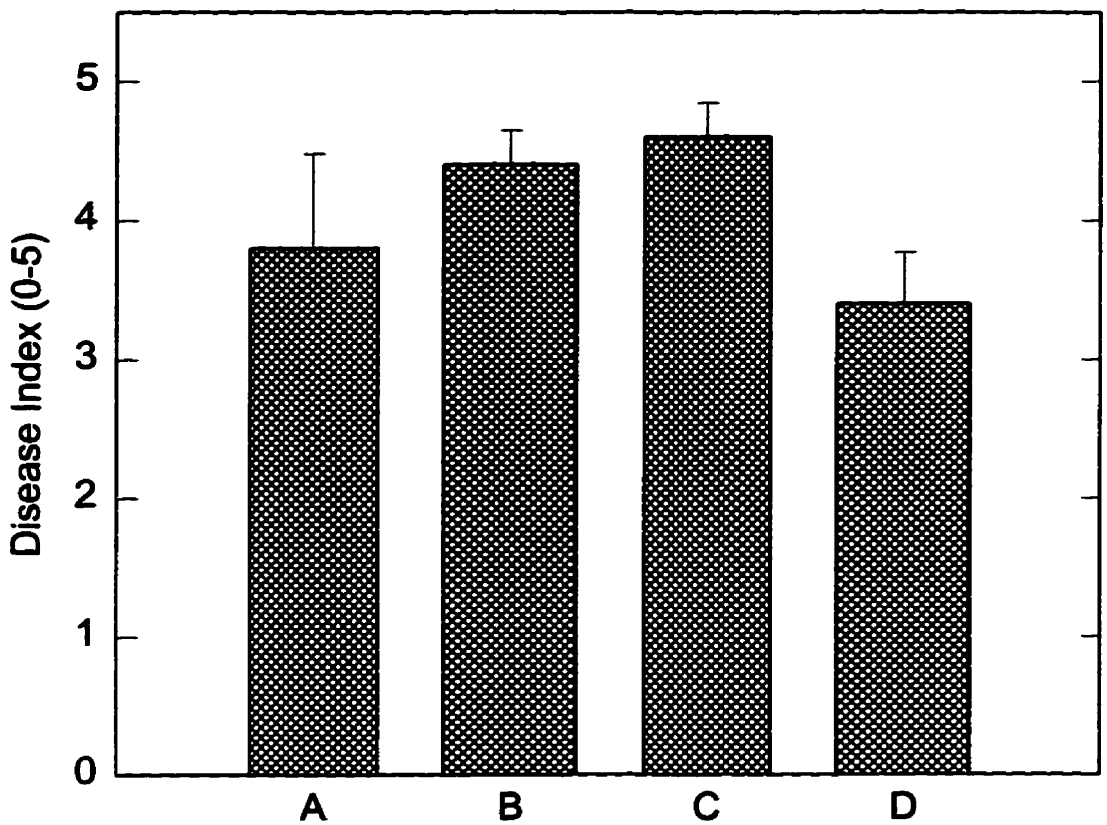

Disease Index (0-5)
0
1
2
3
4
5
(+) Iron
(-) Iron
Lettuce Root Extract (pH 6)
Lettuce Root Extract (pH 8)
Control (pH 6)
Control (pH 8)

Figure 4.12: Effect of lettuce allelopathic treatments on the Fusarium crown and root rot (FCRR) severity on tomato seedlings grown in plant nutrient medium at pH 6 containing 10 μM iron.

Tomato seedlings were grown in sterile plastic Petri plates (9cm diameter) with (A) no lettuce tissues, (B) excised lettuce root, (C) lettuce plant introduced at the time of FORL inoculation and (D) lettuce plant introduced 3 days prior to FORL inoculation. Petri plates were inoculated with 1 mL of a 10^6 *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) microconidia/mL suspension when tomato seedlings were 10 days old. Petri plates were maintained under a 16 hour (22° C) day and 8 hour (18° C) regime. FCRR severity was assessed after 14 days using a disease index in Table 2.3. Bars represent mean values and error bars represent standard errors from 5 seedlings.



Mean Tomato Seedling Disease Index
A - Lettuce Companion Plant (3 days prior to FORL inoculation)
B - Lettuce Companion Plant (concurrent with FORL inoculation)
C - Excised Lettuce Root (concurrent with FORL inoculation)
D - FORL Inoculated (Control)

living lettuce plants the entire lettuce root system was yellow in color compared to the roots of the lettuce plants in the uninoculated plates which were white. There were, however, no rot lesions or wilt symptoms on any of the living lettuce plants.
No damping-off or root rot symptoms or abnormal leaf or root development or change in color was observed on any the uninoculated tomato seedlings companion-planted with living lettuce plants or grown with an excised lettuce root system.

4.4 Discussion

A fundamental problem in testing phenolic compounds in bioassays is the low aqueous solubility of their non-conjugated forms (Sondheimer, 1964). Chlorogenic acid (Figure 3.1) has an aqueous solubility of only 16 mM and the solubility of caffeic acid is even lower (Sondheimer, 1964). The low solubility of non-conjugated phenolic compounds is overcome conventially by dissolving the phenolics in an organic solvent and then adding this solution to the aqueous bioassay medium. However, organic solvents may facilitate the transport of the test compound across the cell membrane or create artificially high solubilities of the test compounds in the bioassay medium. Therefore, it is essential that allelopathic bioassays mimic natural conditions as closely as possible in order to have practical significance (Inderjit and Dashini, 1995).

Dornbos Jr. and Spencer (1990) used organic solvents for the extraction of test compounds and then allowed the solvent to evaporate passively from the surface of the agar test medium prior to initiation of the bioassay, thereby eliminating their potential effect in the bioassay. For the reasons stated above a procedure based on Dornbos Jr. and

Spencer (1990) was used in all but one of the bioassays performed in this chapter. FORL Growth Bioassay 2 was performed using the conventional method of using a medium containing a known concentration of methanol. Results from FORL Growth Bioassay 2 would allow comparison to the results from an earlier microtube bioassay, FORL Growth Bioassay 1, which used a medium that was saturated with the test compounds but did not contain methanol.

Results from FORL Growth Bioassay 1 indicate that even without the presence of an organic solvent in the media sufficient concentrations of catechol, p-coumaric acid and cinnamic acid became solubilized to inhibit the growth of FORL mycelia. Under the same conditions the growth of FORL mycelium was unaffected by saturation of the medium with caffeic acid or chlorogenic acid. Chlorogenic acid was the only conjugated phenolic compound tested and hence the most soluble of the compounds tested.

The lack of an inhibitory effect on the growth of FORL mycelium by caffeic and chlorogenic acids on FORL found in FORL Growth Bioassay 1 could be a result of the relative abundance of chlorogenic acid and caffeoylglucose esters in tomato tissues (Fleuriet and Macheix, 1985). The development of tolerance and/or de-toxification mechanisms to host phenolic compounds is a common phenomenon in pathogenic plant fungi (Ravn *et al.*, 1994). This could also explain why catechol, which is not present in tomato (Carrasco *et al.*, 1978), and p-coumaric acid and cinnamic acid both of which occur in tomato at low to trace levels (Fleuriet and Macheix, 1985), were inhibitory to the growth of FORL mycelium.

The reactivity of phenolic compounds generally increases upon oxidation (Appel,

1993). Chlorogenic acid for example auto-oxidizes at pH values above 7.5 (Schaal and Johnson, 1955). However, in this study no differences were found in the inhibitory effect of any of the tested compounds between media at pH 6 and pH 8. This suggests that the auto-oxidation of these compounds did not occur under the conditions tested or that any increased inhibitory effect of these compounds at the higher pH level was not significant enough to be detected using the microtube bioassay.

The results of FORL Growth Bioassay 1 are dissimilar to those of Kasenberg (1991), who reported that both caffeic acid and chlorogenic acid inhibited the growth of FORL germ tubes. The difference in results may be due to differential toxicity of these compounds towards the different FORL physiological processes or the high concentrations (minimum of 10 mM) of compounds tested by Kasenberg (1991). Nevertheless, the lack of observed inhibition of FORL growth by caffeic acid prompted a second bioassay, FORL Growth Bioassay 2, using known concentrations of phenolic compounds solubilized in the medium and providing a more quantitative measure of FORL growth. Caffeic acid, which had no observed inhibition of FORL growth, and cinnamic acid, which was the most inhibitory compound in the first bioassay, were used in this second bioassay. Cinnamic acid was again found to be very inhibitory to the growth of FORL mycelium with 5 mM cinnamic acid causing a 90 % reduction in FORL growth. Caffeic acid was found to cause a 35 % reduction in the growth of FORL mycelium at 5 mM. However, methanol had to be added to the medium to attain this concentration of caffeic acid, which is unlikely to occur in the aqueous environment involved in the allelopathic control of FCRR. The use of caffeic acid at this concentration

to control FCRR, if feasible, is not advisable because even 1 mM of caffeic acid is phytotoxic to tomato plants (Mershie and Singh, 1988).

It was considered possible that compounds in lettuce root tissues from chemical classes other than phenols may be inhibitory towards the growth of FORL. Nagaoka *et al.*, (1995) recently reported the inhibition of the growth of FORL mycelium by unsaturated hydroxy fatty acids in extracts from a tomato rootstock (cross between tomato and *Lycopersicon hissutum* var. *glabratum*). Since in my study neither caffeic acid nor chlorogenic acid were found to be inhibitory to the growth FORL mycelium, various lettuce root tissue extracts were tested using the FORL Growth Bioassay 1 methodology. Different solvents, water at pH 6, water at pH 8, methanol, ethyl acetate, and hexane were used to extract different classes of compounds from lettuce root tissue. It was hoped that the relatively inhibitory effects of these extracts could be used as an initial step to elucidate the chemical nature of any compounds that are inhibitory to the growth FORL in lettuce root tissue. However, none of the lettuce root extracts reduced the growth of FORL mycelium. The reduction in the growth of FORL mycelium which was observed using water adjusted to pH 8 but not the lettuce extract prepared using water at pH 8 is likely an effect of pH on FORL growth, since acidic compounds would be extracted from dried lettuce root tissue and lower the pH of the extract.

In the FCRR Bioassay 1, the amount of phenolic compound tested is expressed in moles per well and not in molarity. However, assuming complete solubilization of the caffeic acid and chlorogenic acid the 1.0 µmol/well amendment would correspond to a concentration in the medium of 0.5 mM and 0.1 µmol/well to 0.05mM. Brammall (1986)

reported that 2.5 mM ferulic acid and 0.5 mM cinnamic acid caused lethal damage to the roots of tomato seedlings. Mershie and Singh (1988) reported that 1.0 mM caffeic acid and chlorogenic acid were phytotoxic to tomato plants. Kasenberg (1991), however, did not report any phytotoxic effect of 1.0 mM caffeic acid on the germination and early growth of tomato, but it is not clear if uninoculated controls were used in his study. In this study, amendment of the medium with 1.0 µmol/well (0.5 mM) of caffeic acid and chlorogenic acid increased the severity of FCRR on tomato seedlings. The observed increase in FCRR severity on FORL-inoculated tomato seedlings is likely due to the phytotoxicity of these amendments observed on uninoculated seedlings which was manifested in the discoloration and necrosis of the primary root tip.

The methanol extract of lettuce root tissue also increased the severity of FCRR on tomato seedlings. Based on the concentration of caffeic acid in lettuce root tissue calculated in chapter 3, the aliquot of lettuce root extract added to each well resulted in a caffeic acid concentration of approximately 5µM in the medium in each well. Each well would also have contained similar concentrations of chlorogenic acid and isochlorogenic acid, which were also present in the lettuce root extract. This level of lettuce root extract did not display any phytotoxic effects towards uninoculated tomato seedlings and may not have affected the resistance of tomato seedlings to FORL infection. The companion planting of tomato and lettuce seedlings had no effect on the severity of FCRR on FORL-inoculated tomato seedlings. Hartman and Fletcher (1991) reported that FCRR severity was actually increased in hydroponically grown tomato plants that were companion planted with lettuce until the production of fruit.

The combined results from the FORL growth bioassays and FCRR bioassays suggest that fungitoxicity of lettuce root phenolic compounds plays an insignificant role in the allelopathic control of FCRR by lettuce companion planting. Kasenberg (1991) reported that caffeic acid and chlorogenic acid inhibited the germination of FORL microconidia and the growth of FORL germ tubes and proposed that fungitoxicity of caffeic acid derivatives in lettuce roots is involved in the allelopathic control of FCRR. However, Kasenberg (1991) based this proposal on results from bioassays in which the minimal caffeic acid and chlorogenic acid concentration tested was 10 mM. In comparison with other studies testing the inhibitory effects of caffeic acid derivatives on fungal growth (Ravn *et al.*, 1989; Snook *et al.*, 1991), the concentration used by Kasenberg (1991) appears to be excessive and is unlikely to occur under natural conditions. The fact that caffeic acid and chlorogenic acid are the most abundant phenolic compounds in tomato roots (Spurr *et al.*, 1965) and that their presence in FORL-susceptible tomato seedling roots was confirmed in chapter 3 of my study further suggest that these compounds have low toxicity towards FORL.

The work of Hartman and Fletcher (1991) on mature tomato plants suggest that the lack of control of FCRR found in my study was not due to the use of tomato seedlings or the duration of exposure of tomato seedlings to lettuce root extracts or a lettuce companion plant. The reasons for the contradictory reports of control of FCRR by lettuce companion planting (Hartman and Fletcher (1991) and Jarvis (1989)) or by caffeic acid, Kasenberg (1991) and this study, are not known. The use of lettuce as a control agent for FORL originated with the observation that tomato plants grown in soil amended with

ground lettuce tissues (leaf and root) had less severe FCRR symptoms at the time of fruit harvest than plants grown in unamended soil (Jarvis and Thorpe, 1981). The original intent of Jarvis and Thorpe (1981) was to find plant residues which would selectively stimulate microorganisms that are antagonistic towards FORL.

It is possible that lettuce root compounds selectively stimulate microbes that are antagonistic to FORL, such as *Trichoderma harzianum* (Marois and Mitchell, 1981), and/or that induce resistance in tomato such as non-pathogenic *Fusarium* species (Brammall, 1986). Benhamou *et al.* (1994) reported that the growth of tomato seedlings on a medium amended with chitosan induced resistance to FORL in tomato seedlings, indicating that induced resistance is a mechanism of controlling FCRR. Further research is needed to assess the effect of lettuce-allelopathic treatments on the growth of soil microbes and on the defence physiology of tomato plants to determine the extent to which these mechanisms contribute to the allelopathic control of FCRR.

Chapter 5

Summary

This thesis is the first report of a direct relationship between iron concentration and the severity of FCRR on tomato seedlings. No reports could be found regarding the effect of iron concentration of the growth or pathogenicity of FORL but reports were found which indicate that high iron concentrations stimulate the growth (Woltz and Jones, 1971) and pathogenicity (Woltz and Jones, 1968) of *Fusarium oxysporum* f. sp. *lycopersici*, a wilt-causing pathogen of tomato related to FORL. Results in this thesis indicate that tomato seedlings grown at pH 8 have less severe FCRR symptoms than seedlings grown similarly at pH 6. This inverse relationship between FCRR severity and pH has previously been reported for tomato seedlings grown in soil (Woltz *et al.*, 1992). Jones *et al.* (1990) found that a high soil pH has no effect on the germination of FORL spores and concluded that pH induced alterations in the soil microflora are not responsible for the reduction in FCRR severity at high pH levels. The reduction of FCRR severity on tomato seedlings grown at elevated pH found in this thesis and by Woltz *et al.*, (1992) may be due to the reduction in the concentration of iron in solution that results from an increase in pH (Jugsujinda and Patrick Jr., 1977).

Kasenberg (1991) had previously reported a decrease in the severity of FCRR on tomato plants grown in soil amended with the synthetic iron chelator EDDHA. The importance of iron to the development of FCRR on tomato seedlings was confirmed in this thesis by the findings that the synthetic iron chelators, EDDHA and EDTA reduced

the severity of FCRR on tomato seedlings grown at pH 6 in otherwise axenic bioassays.

Results presented in this thesis indicate that amendment of the growth solution with synthetic iron chelators has potential for use as a FCRR control measure in commercial tomato transplant production. At the concentrations used, EDTA and EDDHA controlled FCRR on tomato seedlings equally, even though EDTA has lower affinity for iron than EDDHA (Lindsay, 1974). This result suggests that autoclaving at 121°C for 15 minutes may have disrupted the iron chelation ability of the EDDHA amendment in the nutrient solution. The mechanism of the disruption is not known, but Vanachter *et al.* (1988) found that sterilization of a nutrient solution using ozone destroyed the chelation ability of EDDHA but left the iron chelation ability of EDTA unaffected. The results presented here have potential practical significance in the hydroponic production of tomatoes because EDTA is significantly less costly than EDDHA (Jefferys and Wallace, 1968) but was found to have the same efficacy in reducing the severity of FCRR.

In the hydroponic production of tomato the pH of the nutrient solution is maintained within a pH range of 5.5 to 6.5 (Papadopoulos, 1991). In this range EDTA preferentially binds iron, thus allowing EDTA to limit iron availability to FORL. Scher and Baker (1982) found that EDDHA reduced the severity of *Fusarium* wilts on both radish and flax grown in soil. In contrast, EDTA was found to increase both diseases under the same conditions. However, the pH of the soil used was 7.3 and EDTA will preferentially bind cations other than iron at pH values of 6 and above, while EDDHA preferentially binds iron over the pH range of 4 to 10 (Lindsay, 1974). This means that

the pathogens did not compete for iron with EDTA and this would explain the lack of reduction in *Fusarium* wilt severity in soil amended with EDTA. The control of FCRR by EDTA found in this thesis illustrates the importance of considering parameters such as the redox potential and pH of the medium, the concentrations of chelators and the relative affinities of chelators for various metals when attempting to use chelators to restrict the availability of specific nutrients to pathogens.

Caffeic acid and the caffeic acid derivatives, chlorogenic acid and isochlorogenic acid were the only o-diphenol compounds detected in lettuce root tissue. Caffeic acid was detected in axenically grown lettuce root exudates in the concentration order of magnitude of 10^{-8} M. Trace levels of chlorogenic acid and isochlorogenic acid were also detected in lettuce root exudates. This is the first report of the detection of caffeic acid in lettuce root exudates. Isochlorogenic acid has previously been reported in lettuce roots (Cole, 1984) and chlorogenic acid and isochlorogenic acid have previously been reported in lettuce leaves (Ke and Saltveit Jr., 1988). Caffeic acid has previously been reported in the root exudates of other species (Barash *et al.*, 1993) including tomato (Olsen *et al* ., 1981).

Caffeic acid was chosen for use in FORL growth bioassay and FCRR severity bioassays because the bioactivity of naturally occurring caffeic acid derivatives such as chlorogenic acid and isochlorogenic acid, is attributed to the caffeic acid portion of these molecules (Snook *et al.*, 1991). Saturation of the medium with catechol, p-coumaric acid and t-cinnamic acid significantly reduced the growth of FORL mycelia. Cinnamic acid was the most inhibitory compound, with a concentration of 5 mM of t-cinnamic acid

causing a 90 % reduction in the growth of FORL mycelia. However, the growth of FORL mycelia was unaffected by saturation of the medium with caffeic acid or chlorogenic acid, the phenolics identified in lettuce roots and root exudates. The growth of FORL mycelium was unaffected by amendment of the growth medium with 1 mM caffeic acid, although 5 mM caffeic acid did cause a 35 % reduction in the growth of FORL mycelia. However, to attain this concentration of caffeic acid a methanol concentration in agar of approximately 5 % (v/v) was required. Besides, caffeic acid is phytotoxic towards tomato at much lower concentrations (Mershie and Singh, 1988).

Amendment of a hydroponic growth medium with 0.5 mM or 0.05 mM caffeic acid or chlorogenic acid, and the methanol extracts of lettuce root tissue increased the severity of FCRR on tomato seedlings. A concentration of 0.5 mM caffeic acid or 0.5 mM chlorogenic acid caused discoloration and necrosis of the primary root on tomato seedlings, even though Kasenberg (1991) reported no adverse effects of 1 mM caffeic acid on the germination of tomato seeds. The increased severity of FCRR on tomato seedlings exposed to caffeic acid and chlorogenic acid was attributed to the phytotoxicity of these compounds. Phytotoxicity of caffeic acid and chlorogenic acid towards tomato at a concentration of 1 mM has previously been reported by Mersie and Singh (1988). The companion planting of a lettuce plant and a tomato seedling had no effect on the severity of FCRR on the tomato seedling. Hartman and Fletcher (1991) reported that the companion planting of lettuce and tomato actually increased the severity of FCRR on the tomato plants. Results from this thesis indicate that the lettuce-mediated allelopathic protocols, which have been reported to control FCRR on mature tomato plants (Jarvis,

1989), cannot be used as control methods for FCRR on tomato seedlings.

The mechanisms for the allelopathic control of FCRR on mature tomato plants remain unknown and the effectiveness of the companion plant allelopathic protocol has been questioned (Hartman and Fletcher,1991). The growth substrate may have a significant effect on the efficacy of allelopathic control. Lettuce-mediated allelopathic control of FCRR on mature tomato plants is successful when sterilized soil (Jarvis and Thorpe, 1981) or organic hydroponic substrates such as sawdust are used (Jarvis, 1989), but not when inorganic hydroponic substrates such as rockwool are used (Hartman and Fletcher, 1991). Soil and hydroponic substrates will not only differ with regard to their binding of nutrients but may interact differently with phenolic compounds. Phenolic compounds are known to react with iron and manganese oxides in soils, resulting in the oxidation and polymerization of the phenolic compounds into large polyphenolic compounds (Lehmann *et al.*, 1987; Shindo and Huang, 1984). Large polyphenolic polymers such as tannins form much stronger complexes with iron than their constituent monomers and form insoluble complexes with iron that have been implicated in plant disease resistance (McDonald *et al.*, 1996; Mila *et al.*, 1996). Caffeic acid forms large polyphenolic complexes *via* both enzymatic (Cheynier and Moutounet, 1991) and auto-catalysed (Cilliers and Singelton, 1991) polymerization. The formation of polymers of caffeic acid derivatives released from lettuce tissues or as root exudates and the subsequent binding of iron by these polymers may explain the efficacy of allelopathic control in soil and organic hydroponic substrates. The fate of phenolic compounds in hydroponic growth substrates is not known and needs to be assessed.

The allelopathic control of FCRR may also operate *via* induced resistance in tomato. The mechanisms involved in induced resistance are not understood but chemicals which induce resistance often cause limited damage or necrosis to plant tissues, and a perturbation of normal metabolism is likely required for induced resistance (Fought and Kuc, 1996; Gaffney *et al.*, 1993; Lyon *et al.*, 1995). The phytotoxicity of lettuce o-diphenols might cause such a metabolic perturbation in tomato roots exposed to these compounds. The larger roots of older tomato plants may be less sensitive to caffeic acid. This would allow older plants to tolerate levels of caffeic acid derivatives that are phytotoxic to tomato seedlings. Exposure to lettuce residues or root exudates could cause the induction of wound repair metabolism in older tomato roots, which like resistance metabolism involves the incorporation of phenolic compounds into cell walls (Dixon and Paiva, 1995; Lamb *et al.*, 1989). This could increase tomato resistance to subsequent FORL infection. Phenolic acids such as caffeic acid also affect plant root growth and morphology, often causing the formation of adventitious roots (Baziramakenga *et al.*, 1994). This probably occurs through the alteration of peroxidase-mediated IAA destruction by these phenolic acids (Volpert et al., 1995). It is possible that exposure to lettuce exudates causes the formation of adventitious roots on tomato plants. The formation of adventitious roots lessens the symptoms of FCRR on tomato plants and can allow plants to escape FCRR (Jarvis, 1988). Studies would have to performed to determine whether lettuce root exudates alter tomato physiology in a manner which increases resistance to FORL.

Given that FCRR continues to be a serious problem in the production of tomato

transplants (Datnoff *et al.*, 1995) future work is required to develop effective protocols for the control of FCRR. Integrated disease management involving synthetic iron chelators, elevated pH of the growth medium (Jones et al., 1990), biological control agents (Datnoff et al., 1995; Lemanceau and Alabouvette, 1991; Sivan and Chet, 1993) and chemicals which induce FORL resistance (Benhanou *et al.*, 1994) may provide satisfactory control of FCRR. Research would be required to determine conditions that promote transplant growth and combinations of control treatments that minimize FCRR and adverse effects on tomato growth.

REFERENCES

Adzet T. and Puigmacia M., (1985) High-performance liquid chromatography of caffeoylquinic acid derivatives of *Cynara scolymus* L. leaves. J. Chromatogr., 348:47-453.

Appel H. M., (1993) Phenolics in ecological interactions: The importance of oxidation. J. Chem. Ecol., 19(7):1521-1552.

Baker C. and Orlandi E., (1995) Active oxygen in plant pathogenesis. Ann. Rev. Phytopathology, 33:299-321.

Barash I., Dori S., Mor H. and Manulis S., (1993) Role of Iron in Fungal Phytopathologies. In: *Iron Chelation in Plants and Soil Microorganisms.* Edited by L. L. Barton and B. C. Hemming. Academic Press Inc., San Diego, NewYork, Boston, London, Sydney, Tokyo and Toronto. pp. 251-267.

Bashan Y., Okon Y. and Henis Y., (1987) Peroxidase, polyphenoloxidase, and phenols in relation to resistance against *Pseudomonas syringae* pv. *tomato* in tomato plants. Can. J. Bot., 65:366-372.

Baziramakenga R., Simard R. R. and Leroux G. D., (1994) Effects of benzoic and cinnamic acids on growth, mineral composition, and chlorophyll content of soybean. J. Chem. Ecol., 20:2821-2833.

Beckman C. H., (1980) Defenses triggered by the invader - physical defenses. In: Plant Disease: An Advanced Treatise, Vol. 5, How Plants Defend Themselves. Edited by J. G. Horsfall and E. B. Cowling. Academic Press, New York London Toronto Sydney San Fransico. pp. 225-245.

Beckman C. H., Mueller W. C. and Mace M. E., (1974) The stabilization of artificial and natural cell wall membranes by phenolic infusion and its relation to wilt disease resistance. Phytopathology, 64:1214-1220.

Benhamou N., (1992) Ultrastructural and cytochemical aspects of chitosan on *Fusarium oxysporum* f. sp. *radicis-lycopersici*, agent of tomato crown and root rot. Phytopathology, 82:1185-1193.

Benhamou N., (1996) Elicitor-induced plant defense pathways. Trends in Plant Science, 1(7):233-240.

Benhamou N. and Lafontaine P., (1995) Ultrastructure and cytochemical characterization of elicitor-induced structural responses in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Planta, 197:89-102.

Benhamou N., Chamberland H. and Pauze F., (1990) Implications of pectin components in cell surface interactions between tomato root cells and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Plant Physiol., 92:995-1003.

Benhamou N., Grenier J. and Crispeels M., (1991) Accumulation of β-fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. Plant Physiol., 97:739-750.

Benhamou N., Lafontaine P. and Nicole M., (1994) Induction of systemic resistance to Fusarium crown and root rot in tomato plants by seed treatment with chitosan. Phytopathology, 84:1432-1444.

Bennett M., Gallagher M., Fagg J., Bestwick C., Paul T., Beale M. and Mansfield., (1996) The hypersensitive reaction, membrane damage and accumulation of autofluorescent phenolics in lettuce cells challenged by *Bremia lactucae*. The Plant Journal, 9(6):851-865.

Bernards M. A. and Ellis B. E., (1989) Phenylpropanoid metabloism in tomato cell cultures co-cultivated with *Verticilium albo-atrum*. J. Plant Physiol., 135:21-26.

Bernards M. A., Strack D., Wray V. and Ellis B. E., (1991) Caffeoyl glucosides in fungal challenged tomato suspension cultures. Phytochemistry, 30:497-499.

Bicchi C. P., Binello A. E., Pellegrino G. M. and Vanni A. C., (1995) Characterization of green and roasted coffees through the chlorogenic acid fraction by HPLC-UV and principal component analysis. J. Agric. Food Chem., 43:1549-1555.

Boyer R. F., Clarke H. M. and Sanchez S., (1989) Solubilization of ferrihydrite by plant phenloics: a model for rhizosphere processess. J. Plant Nutr., 12(5):581-592.

Brammall R., (1986) Host-parasite interactions in Fusarium crown and root rot disease of tomato. Ph. D. Thesis, University of Toronto, Ontario.

Brammall R. A. and McKeown A. W., (1989) An occurrence in Ontario of fusarium crown and root rot disease in field-grown processing tomatoes originating from multicelled tray transplants. Can. J. Plant Path., 11:75-77.

Brune M., Hallberg L. and Skanberg A-B., (1991) Determination of iron-binding phenolic groups in foods. J. Fd. Sci., 56:128-131.

Carrasco A., Boudet A. M. and Marigo G., (1978) Enhanced resistance of tomato plants to *Fusarium* by controlled stimulation of their natural phenolic production. Physiol. Plant Path., 12:225-232.

Chamberland H., Ouellette G. B., Pauze F. J. and Charest P. M., (1990) Immunocytochemical localization of tomato pectinesterase in root cells of tomato plants infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Can. J. Bot., 69:1265-1274.

Chaney R. L. and Bell P. F., (1987) Complexicity of iron nutrition: lessons for plant-soil interaction research. J. Plant Nutr., 10(9-16):963-994.

Charest P., Ouellette G. and Pauze F., (1984) Cytological observations of early infection process of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Can. J. Bot., 62:1232-1244.

Cheynier V. and Moutounet M., (1992) Oxidative reactions of caffeic acid model systems containing polyphenol oxidase. J. Agric. Food Chem., 40:2038-2044.

Cillers J. J. and Singleton V. L., (1991) Characterization of the products of nonenzymic autoxidative phenolic reactions in caffeic acid model system. J. Agric. Food Chem., 39:1298-1303.

Clifford M., (1986) Coffee bean dicaffeoylquinic acids. Phytochemistry, 25:1767-1769.

Cole R. A., (1984) Phenolic acids associated with resistance of lettuce cultivars to the lettuce root aphid. Ann. Appl. Biol., 105:129-145.

Datnoff L. E., Nemec S. and Pernezny K., (1995) Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biological Control 5:427-431.

Davis D., Waggoner P. and Dimond A., (1953) Conjugated phenols in the *Fusarium* wilt syndrome. Nature, 172:959-961.

Deiana S., Gessa C., Marchetti M., and Usai M., (1995) Phenolic acid redox properties: pH influence on iron (III) reduction by caffeic acid. Soil Sci. Soc. Am. J., 59:1301-1307.

Deverall B., (1961) Phenolase and pectic enzyme activity in the chocolate spot disease of beans. Nature, 189:311.

Dixon R. A. and Paiva N. L., (1995) Stress-induced phenylpropanoid metabolism. Plant Cell, 7:1085-1097.

Dornbos Jr. D. L. and Spencer G. F., (1990) Natural products phytotoxicity: A bioassay suitible for small quantities of slightly water-soluble compounds. J. Chem. Ecol., 16(2):339-352.

Dyer W. E., Henstrand J. M., Handa A. K. and Herrmann K. M., (1989) Wounding induces the first enzyme of the shikimate pathway in Solanaceae. Proc. Natl. Acad. Sci. USA., 86:7370-7773.

Elad Y. and Baker R., (1985) The role of competition for iron and carbon in suppression of chlamydospore germination of *Fusarium* spp. by *Pseudomonas* spp.. Phytopathology, 75:1053-1059.

Farkas G. L. and Kiraly Z., (1962) Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopath. Z., 44:105-150.

Fleuriet A. and Macheix J-J., (1985) Tissue compartmentation of phenylpropanoid metabolism in tomatoes during growth and maturation. Phytochemistry, 24(5):929-932.

Fought L. and Kuc J. A., (1996) Lack of specificity in plant extracts and chemicals as inducers of systemic resistance in cucumber plants to anthracnose. J. Phytopathol., 144:1-6.

Gaffney T., Friedrich L., Vernooij B., Negrotto B., Nye G., Ukness S., Ward E., Kessmann H. and Ryals J., (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754-756.

Graham T. L., (1991) A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. Plant Physiol., 95:584-593.

Hahlbrock K. and Schell D., (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol., 40:347-369.

Hartman J. R. and Fletcher J. T., (1991) Fusarium crown and root of tomatoes in the UK. Plant Pathol., 40:85-92.

Holden M. J, Luster D. G., Chaney R. L., Buckout T. J. and Robinsin C., (1991) Fe^{3+}-Chelate reductase activity of plasma membrane isolated from tomato *(Lycopersicon esculentum* Mill.) roots. Plant Physiol., 97:537-544.

Hunter R. E. (1974) Inactivation of pectic enzymes by polyphenols in cotton seedlings of different ages infected with *Rhizoctonia solani*. Physiol. Plant Pathol., 4:151-159.

Inderjit, (1996) Plant phenolics in allelopathy. Bot. Rev., 62:186-202.

Inderjit, and K. M. M. Dakshini. (1995) On laboratory bioassays in allelopathy. Bot. Rev., 61(1):28-44.

Jarvis W. R., (1988) Fusarium crown and root rot of tomatoes. Phytoprotection, 69:49-63.

Jarvis W. R., (1989) Allelopathic control of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. In: Vacular Wilt Diseases of Plants. NATO ASI Series H: Vol. 28. Edited by E.C. Tjamos and C. H. Beckman. Springer-Verlag, Berlin., pp. 479-486.

Jarvis W. R. and Shoemaker R. A., (1978) Taxonomic status of *Fusarium oxysporum* causing foot and root rot of tomato. Phytopathology, 68:1679-1680.

Jarvis W. R. and Thorpe H. J., (1981) Control of fusarium crown and root rot of tomato by soil ammendment with lettuce residues. Can. J. Plant Path., 3:159-162.

Jarvis W. R., Thorpe H. J. and MacNeil B. H., (1975) A foot and root rot disease of tomato caused by *Fusarium oxysporum*. Can. Plant Dis. Surv., 55:25-26.

Jefferys R. A. and Wallace A., (1968) Detection of iron ethylene di(o-hydroxyphenylacetate) in plant tissue. Agron. J., 60:613-616.

Jones J. P. and Woltz S. S., (1970) Fusarium wilt of tomato: Interaction of soil liming and micronutrient amendments on disease development. Phtopathology, 60:812-813.

Jones J. P., Woltz S. S. and Scott J. W., (1990) Factors affecting development of Fusarium crown and root rot. Proc. Fla. State Hort. Soc., 103:142-148.

Jones J. P., Jones J. B., Woltz S. S. and Scott J. W., (1993) Influence of soil pH, nitrogen source, and transplant drences on development of crown rot of tomato. Proc. Fla. State Hort. Soc., 106: 170-172.

Jugsujinda A. and Patrick Jr., W. H., (1977) Growth and nutrient uptake by rice in flooded soil under controlled aerobic-anaerobic and pH conditions. Agron. J., 69:705-710.

Kasenberg T. R., (1991) The allelopathic biocontrol of Fusarium crown and root rot of tomatoes. M. Sc. Thesis, The University of Western Ontario, Ontario.

Kasenberg T. R. and Traquair J. T., (1988) Effects of phenolics on growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici in vitro*. Can. J. Bot., 66:1174-1177.

Ke D. and Saltveit Jr., M., (1988) Plant hormone interaction and phenolic metabolism in the regulation of russet spotting in iceberg lettuce. Plant Physiol., 88:1136-1140.

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

Kloepper J. W., Leong J., Teintze M. and Schroth M., (1980) *Pseudomonas* siderophores: a mechanism explaining disease-surpressive soils. Curr. Microbiol., 4:317-320.

Kritzman G. and Chet I., (1980) The role phenols in the pathogenicity of *Botrytis allii*. Phytoparasitica 8(1):27-37.

Lamb C. J., Lawton M. A., Dron M. and Dixon R. A., (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell, 56:215-224.

Leatham G. F., King V. and Stahman M. A., (1980) *In vitro* protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. Phytopathology, 70:1134-1140.

Lee S. and Le Tourneau D. (1958) Chlorogenic acid content and *Verticillium* wilt resistance of potatoes. Phytopathology, 48:268-273.

Leeman M., den Ouden F. M., van Pelt J. A., Dirkx F.P.M., Steijl H., Bakker P. A. and Schippers B. (1996) Iron availability affects induction of systems resistance to Fusarium wilt of Radish by *Pseudomonas flourescens*. Phytopathology, 86:149-155.

Legrand M., (1983) Phenylpropanoid metabolism and its regulation in disease. In: *Biochemical Plant Pathology*. Edited by J. A. Callow. John Wiley & Sons, Chichester, New York, Brisbane, Toronto and Singapore. pp. 367-384.

Lehmann R. G., Cheng H. H. and Harsh J. B., (1987) Oxidation of phenolic acids by soil iron and manganese oxides. Soil Sci. Soc. Am. J., 51:352-356.

Lemanceau P. and Alabouvette C., (1991) Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. Crop Prot., 10:279-286.

Lindsay W. L., (1974) Role of chelation in micronutrient availability. In: *The Plant Root and its environment*. Edited by R. W. Carson. University Press Virginia, Charlottesville. pp. 507-524.

Loper J. E. and Buyer J. S., (1991) Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Int., 4:5-13.

Lyon G. D., Reglinski T. and Newton A.C., (1995) Novel disease compounds: the potential to "immunize" plants against infection. Plant Pathol., 44:407-427.

Mace M., Veech J. and Beckman C., (1972) Fusarium wilt susceptible and resistant tomato isolines: Histochemistry of vascular browning. Phytopathology, 62:651-654.

Madhosingh C., (1995) Relative wilt-inducing capacity of the culture filtrates of isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the tomato crown and root rot pathogen. J. Phytopathol., 143:193-198.

Manahan S. E., (1984) *Environmental Chemistry*. Brooks/Cole Publishing Company, Monterey, California. pp 57-81.

Maraite H. (1973) Changes in polyphenoloxidases and peroxidases in muskmelon (*Cucumis melo* L.) infected by *Fuarium oxysporum* f.sp. *melonis*. Physiol. Plant Pathol., 3:29-49.

Marois J. J. and Mitchell D. J., (1981) Effects of fungal communities on the pathogenic and saparophytic activities of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology, 71:1251-1256.

Marschner H., Romheld V. and Ossenberg-Neuhaus H., (1982) Rapid method for measuring changes in pH and reducing processes along roots of intact plants. Z. Pflanzenphysiol. Bd., 105:407-416.

Matern U. and Kneusel R. E., (1988) Phenolic compounds in plant disease resistance. Phytoparasitica, 16(2):153-170.

Matta A. and Dimond A. E., (1963) Symptoms of Fusarium wilt in relation to quantity of fungus and enzyme activity in tomato stems. Phytopathology, 53: 574-578.

Matta A., Gentile I. and Giai I., (1969) Accumulation of phenols in tomato plants infected by different forms of *Fusarium oxysporum*. Phytopathol., 59:512-513.

McDonald M., Mila I. and Scalbert A., (1996) Precipitation of metal ions by plant polyphenols: Optimal conditions and origin of precipitation. J. Agric. Food Chem., 44:599-606.

Menzies J. G. and Belanger R. R., (1996) Recent advances in cultural management of diseases of greenhouse crops. Can. J. Plant Plathol., 18:186:193.

Menzies J. G. and Jarvis W. R., (1994) Fusarium crown and root rot. In: *Diseases and Pests of Vegetable Crops in Canada.*. Edited by R. J. Howard, J. A. Garland and W. L. Seaman. pp. 346-347.

Menzies J. G., Koch C. and Seyward F., (1990) Additions to the host range of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Plant Dis., 74:569-572.

Mersie W. and Singh M., (1988) Effects of phenolic acids and ragweed Parthium (*Pathenium bysterophorus*) extracts on tomato (*Lycopersicon esculentum*) growth and nutrient and chlorophyll content. Weed Sci., 36:278-281.

Mihuta-Grimm L., Erb W. A. and Rowe R. C., (1990) Fusarium crown and root rot of tomato in greenhouse rock wool systems: Sources of inoculum and disease management with benomyl. Plant Dis., 74:996-1002.

Mila I., Scalbert A. and Expert D., (1996) Iron withholding by plant polyphenols and resistance to pathogens and rots. Phytochemistry, 42:1551-1555.

Morishita H., Iwahashi H., and Kido R., (1986) 3-o-caffeoyl-4-o-feruloylquinic acid from green robusta coffee beans. Phytochemistry, 25:2679-2680.

Nagaoka T., Ohra J., Yoshihara T. and Sakamura S., (1995) Fungitoxic compounds from the roots of tomato stock. Ann. Phytopathol. Soc. Jpn., 61:103-108.

Neilands J. B. and Leong S. A., (1986) Siderophores in relation to plant growth and disease. Ann Rev. Plant Physiol., 37:187-208.

Nicholson R. L. and Hammerschmidt R., (1992) Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol., 30:369-389.

Olsen R. A., Bennett J. H., Blume D. and Brown J. C., (1981) Chemical aspects of the Fe stress response mechanism in tomatoes. J. Plant Nutr., 3(6):905-921.

Ong B. Y. and Nagel C. W., (1978) High-pressure liquid chromatographic analysis of hydroxyconnamic acid-tartaric acid esters and their glucose esters in *Vitis vinifera*. J. Chromat., 157:345-355.

Papadopoulos A. P., (1991) Growing greenhouse tomatoes in soil and soilless media. Agriculture Canada Publication 1855/E.

Patil S. and Dimond A., (1967) Inhibition of *Verticillium* polygalacturonase by oxidation products of polyphenols. Phytopathology, 57:492-496.

Patrick Z. A., (1986) Allelopathic mechanisms and their exploitation for biological control. Can. J. Plant Path., 8:225-228.

Pierpoint W. S., (1969) o-Quinones formed in plant extracts: Their reactions with amino acids and peptides. Biochem. J., 112:609-616.

Pribil R., (1972) *Analytical Applications of EDTA and Related Compounds*. Pergamon Press, Oxford, New York, Toronto, Stdney and Braunschweig. pp. 27-31.

Ramirez-Martinez J. and Luh B., (1973) Phenolic compounds in frozen avocados. J. Sci. Fd. Agric., 24:219-225.

Ravn H., Andary C., Kovacs G. and Molgaard P., (1989) Caffeic acid esters as *In Vitro* inhibitors of plant pathogenic bacteria and fungi. Biochem. Syst. Ecol., 17(3):175-184.

Raymond K., Muller G, Matzanke B., (1984) Complexation of iron by siderophores a review of their solution and structural chemistry and biological function. In: Topics in Current Chemistry, Vol.123. Springer-Verlag, Berlin Heidelberg New York Tokyo. pp. 49-102

Rees S. and Harborne J., (1984) Flavonoids and other phenolics of *Cichorium* and related members of the Lactuceae (Compositae). Bot. J. Linnean Soc., 89:313-319.

Resh H. M., (1991) *Hydroponic Food Production*. Woodbridge Press Publishing Company, California, USA.

Retig N., (1974) Changes in peroxidase and polyphenoloxidase associated with natural and induced resistance of tomato to *Fusarium* wilt. Physiol. Plant Pathol., 4:145-150.

Retig N. and Chet I., (1974) Catechol-induced resistance of tomato plants to Fusarium wilt. Physiol. Pl. Path., 4:469-475.

Romheld V. and Marschner H., (1981) Iron deficiency stress induced morphological and physical changes in root tips of sunflower. Physiol. Plant., 53:354-360.

Romheld V. and Marschner H., (1983) Mechanism of iron uptake by peanut plants. Plant Physiol., 71:949-954.

Rowe R. C. and Farley J. D., (1981) Strategies for controlling fusarium crown and root rot in greenhouse tomatoes. Plant Dis., 65:107-112.

Sanchez L. E., Endo R. M. and Leary J. V., (1975) A rapid technique for identifying the clones of *Fusarium oxysporum* f. sp. *lycopersici* causing crown and root rot of tomato. Phytopathology, 65:726-727.

Schaal L. A. and Johnson G., (1955) The inhibitory effect of phenolic compounds on the growth of *Streptomyces scabies* as related to the mechanism of scab resistance. Phytopathology, 45: 626-628.

Scher F. M. and Baker R., (1982) Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogen. Phytopathology, 72:1567-1573.

Schwab A. P. and Lindsay W. L., (1989) A computer simulation of Fe(III) and Fe(II) complexation in nutrient solutions: II. Application to growing plants. Soil Sci. Soc. Am. J., 53:34-38.

Shido H. and Huang P. M., (1984) Catalytic effects of manganese(IV), iron (III), aluminum and silicon oxides on the formation of phenolic polymers. Soil Sci. Soc. Am . J., 48:927-934.

Siqueira J. O., Nair M. G., Hammerschmidt R. and Safir G. R., (1991) Significance of phenolic compounds in plant-soil-microbial systems. Crit. Rev. Plant Sci., 10(1):63-121.

Sivan A. and Chet I., (1993) Integrated control of fusarium crown and root rot of tomato with *Trichoderma harzianum* in combination with methyl bromide or soil solarization. Crop Prot., 12:380-386.

Snook M. E., Chortyk O. T. and Csinos A. S., (1991) Black shank disease fungus: inhibition of growth by tobacco root constituents and related compounds. In: *Naturally Occurring Pest Bioregulators*. ACS Symposium Series Vol. 449. Edited by P. A. Hedin. American Chemical Society, Washington, D.C..

Sondheimer E., (1964) Chlorogenic acids and related depsides. Bot. Rev., 30:667-712.

Spurr Jr. H. W., Hildebrandt A. C. and Riker A. J., (1965) The integral association of chlorogenic acid to crown gall tumor formation. Phytopathology, 55:1004-1008.

Sutherland M., (1991) The generation of oxygen radicals during host plant repsonses to infection. Physiol. Mol. Plant Pathol., 39:79-93.

Tang C. and Young C., (1982) Collection and identification of allelopathic compounds from the undisturbed root system of Biglata Limpograss (*Hemarthria altissima*). Plant Physiol., 69:155-160.

Vanachter A., Thys L., Van Wambeke E. and Van Assche C., (1988) Possible use of ozone for disinfestation of plant nutrient solutions. Acta Horticulturae, 221:295-302.

Volpert R., Osswald W. and Elstner E. F., (1995) Effects of cinnamic acid derivatives on indole acetic acid oxidation by peroxidase. Phytochemistry, 38:19-22.

Waites M., Reynolds S. and Frien J., (1978) The metabolism of chlorogenic acid in tuber discs of a resistant and a susceptible potato cultivar after inoculation with *Fusarium solani* var. *caeruleum*. Biochem. Soc. Trans., 6:441-442.

Welkie G. W. and Miller G. W., (19930 Plant iron uptake physiology by nonsiderophore systems. In: *Iron Chelation in Plants and Soil Microorganisms*. Edited by L. L. Barton and B. C. Hemming. Academic Press Inc., San Diego, NewYork, Boston, London, Sydney, Tokyo and Toronto. pp. 345-369.251-267.

Williams C. A., Goldstone F. and Greenham J., (1996) Flavonoids, cinnamic acids and coumarins from the different tissues and medicinal preparartions of *Taraxacum officinale*. Phytochemistry, 42(1):121-127.

Winkelmann G., (1992) Structures and functions of fungal siderophores containing hydroxamate and complexone type iron binding ligands. Mycol. Res., 96(7):529-534.

Winter M. and Herrmann K., (1986) Esters and glucosides of hydroxycinnamic acids in vegetables. J. Agric. Food Chem., 34:616-620.

Woltz S. S. and Jones J. P., (1968) Micronutrient effects on the *in vitro* growth and pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology, 58: 336-338.

Woltz S. S. and Jones J. P., (1971) Effect of varied iron, manganese and zinc nutrition on the *in vitro* growth of race 2 *Fusarium oxysporum* f. sp. *lycopersici* and upon the wilting of tomato cuttings held in filtrates from cultures of the fungus. Fla. State Hort. Soc. Proc., 84: 132-135.

Woltz S. S., Jones J. P. and Scott J. W., (1992) Sodium chloride, nitrogen source, and lime influence Fusarium crown and root rot severity in tomato. HortScience, 27(10):1087-1088.