Biochemical mechanisms for tolerance of citrus rootstocks against *Phytophthora nicotianae*

by

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

Citrus is the second largest fruit crop worldwide (Spiegel-Roy & Goldschmidt, 1996) and it is one of the foremost export fruit crops in southern Africa and in the rest of the world. According to statistics by the Citrus Growers Association in 2002, the South African industry consisted of approximately 59 000 ha of citrus planted, with 58 % of the total volume of production exported, 26 % processed and 16 % consumed locally (http://www.cga.co.za). South Africa is the third largest exporter of citrus after Spain and the United States of America. With an export period from April to October, South Africa's main competitors are Argentina, Chile and Australia, in addition to Israel, Spain, Egypt and the United States in the later part of the season. Capespan Group Holdings Ltd. (Bellville, Western Cape Province, South Africa), the main exporters of southern African citrus, reported an increase in South African fruit volumes from 46.1 million cartons in the year 2002, to 48.1 million cartons in 2003 (Capespan annual report 2003). Statistics by the National Department of Agriculture showed that the revenue generated by citrus fruit in 2003 increased by 25.8 % from 2002 to a total of R3635 million due to improved production (http://www.nda.agric.za).

Root rot of citrus is one of the most serious and economically important diseases of citrus worldwide (Timmer & Menge, 1988; Le roux *et al*., 1998). Although more than one soil-borne pathogen is associated with the citrus root rot complex, the most prominent is *Phytophthora* species (Timmer & Menge, 1988). *P. nicotianae* is the main cause of root rot in South Africa and *P. citrophthora*, which is associated with citrus collar rot and gummosis, is not so commonly found (Thompson *et al*., 1995). Maseko and Coutinho (2002) showed that

P. nicotianae Breda de Haan was the most frequently isolated species of *Phytophthora* in nurseries and orchards in the Limpopo and Mpumalanga Provinces of South Africa. They proposed that the subtropical climatic conditions of the regions that they sampled were more conducive for the growth of *P. nicotianae* and not for *P. citrophthora*.

Graham (1990) referred to rootstocks as tolerant rather than resistant, because fibrous roots of resistant rootstocks do become infected under artificial inoculations and in infested nursery and orchard soils. *P. nicotianae* causes decay of fibrous roots, especially on susceptible rootstocks (Agostini *et al*., 1991; Widmer *et al*., 1998). For the purpose of the present study, resistance and tolerance were used as synonyms. In general the term "resistance mechanisms" were used while rootstocks were referred to as tolerant rather than resistant.

P. nicotianae infects the root cortex causing it to become soft, discoloured and water-soaked, after which the cortex falls apart leaving only the white thread-like stele (Graham, 1995). Damage to fibrous roots in mature orchards causes tree decline and yield losses (Sandler *et al*., 1989). The tree is unable to maintain adequate water and mineral uptake and nutrient reserves in the roots are further depleted by the repeated fungal attacks, resulting in dieback and production losses. *P. nicotianae* also causes foot rot of the trunk and brown rot of the fruit (Timmer & Menge, 1988) but this is not so commonly observed in South Africa (Thompson *et al*., 1995).

The use of specific citrus rootstocks for commercial citrus production holds several advantages. They differ in their tolerance to soil salinity, cold injury and diseases such as *Phytophthora* root rot (Castle, 1987; Sulistyowati *et al*., 1990; Agostini *et al*., 1991; Graham, 1995; Spiegel-Roy & Goldschmidt, 1996). In South Africa, approximately 1600 rootstock

hybrids have been bred at the Institute of Tropical and Subtropical Crops, since 1993 (http://www.arc.agric.za/institutes/ itsc/main/citrus.htm). All of these rootstocks have different traits and this makes the choice of rootstock/scion combinations for specific production conditions difficult. Numerous methods have been used in the screening of citrus rootstocks for resistance against *Phytophthora* species (Carpenter & Furr, 1962; Cameron *et al*., 1972; Grimm & Hutchison, 1973; Afek *et al*., 1990), but all of them involve greenhouse seedling tests, which are quite time consuming. Although these existing techniques have their advantages, it is deemed necessary to develop reliable, high throughput screening techniques against *P. nicotianae* for rootstocks used under South African conditions.

There are several inconsistencies in the ranking of citrus rootstocks for resistance against root rot and gummosis. For instance, Graham (1990, 1995) ranked the rootstock Sour orange as susceptible based on its poor ability to regenerate roots when infected by *P. nicotianae*. Widmer *et al*. (1998) also described Sour orange as susceptible based on the same criteria. However, in 1990 Afek and Sztejnberg and Sulistyowati *et al*. showed that Sour orange is intermediately tolerant towards *P. citrophthora* infection based on lesion lengths and fresh weight determinations. In contrast, Timmer and Menge (1988) showed that Sour orange is highly resistant to infection by *Phytophthora*. Resistance or susceptibility towards gummosis is frequently incorrectly applied to root rot resistance as well. Furthermore, resistance of citrus rootstocks to one species of *Phytophthora* may not be similar to the response against a different species.

Graham (1995) observed a lower population of *P. nicotianae* in the rhizosphere of trifoliate orange and suggested that soil infestation could have been inhibited by a biochemical mechanism. Induced disease resistance response has been demonstrated in many plant species

against viruses, bacteria and against many phytopathogenic fungi (Kuc, 1995). These inducible responses following elicitor treatment, mechanical damage, or microbial attack include the synthesis of phytoalexins, reinforcement of cell walls by deposition of lignin, production of chitinase and glucanase lytic enzymes and accumulation of pathogenesis-related proteins (Lamb, 1989).

Secondary phenolic compounds or phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plant cells in response to pathogen infection or stress (Nicholson & Hammerschmidt, 1992; Kuc, 1995). Subba Rao and Strange (1994) proposed a number of criteria to determine whether a phytoalexin play a role in plant defence: (1) the compound must accumulate in response to infection; (2) the compound must be inhibitory to the invading organism; (3) the compound must accumulate to inhibitory concentrations in the vicinity of the parasite at the time the parasite ceases growing; (4) varying the rate of phytoalexin accumulation, it should cause a corresponding variation in resistance of the plant; (5) varying the sensitivity of the invading organism should cause a corresponding variation in its virulence. It is a very efficient way to combat pathogens, since all energy and carbon resources are directed to the early period of infection and the specific site of infection (Grayer & Kokubun, 2001).

Various types of phenolic compounds are produced by vascular plants (Julkunen-Tiitto, 1985). They can be found in almost any plant part for instance they can be cell wall components such as lignin, they can cover the external surface for instance waxes, or they can be found in vacuoles (Harborne, 1989). Phenolic compounds are rather diverse in their chemical nature and the most important chemical groups include the flavanoids, isoflavanoids, coumarins, diterpenes, stilbenes and free acids (Mansfield, 1985). Secondary

plant metabolites, especially phenolic-based compounds play an intricate role in human health due to their potent antioxidant and anticancer activity (Tanaka *et al*., 2003). In citrus, phenolic compounds are produced in response to pathogen attack and stress factors such as ultraviolet irradiation (Feldman & Hanks, 1968; Rodov *et al*., 1994; Dubery *et al*., 1999; Manthey *et al*., 2000). Their accumulation have an effect on fruit quality and human health, thus the production of phenolic compounds in citrus fruit is the focus of most investigations (Guadagni *et al*., 1973; Robbins, 1990; Vandercook *et al*., 1990).

Two antifungal compounds, xanthoxylin and scoparone were found in the bark of *Citrus limon* after infection with *P. citrophthora* (Hartmann & Nienhaus, 1974). Scoparone accumulation in the stem bark of citrus rootstocks was positively correlated with resistance of citrus to *P. citrophthora*, the causal pathogen of collar rot and gummosis (Afek *et al*., 1986; 1988; 1989; 1990; 1993; 1995). The role of scoparone accumulation in the roots of citrus rootstocks against *P. nicotianae*, the main cause of root rot in South Africa, has not been fully established. Aucamp *et al*. (2000) showed very low levels of scoparone accumulation in citrus roots, which did not correlate with resistance to *P. nicotianae* root rot. Similarly, Graham (1995) found very low levels of scoparone in the roots of susceptible Cleopatra mandarin in chlamydospore-infested soil and no scoparone was produced in tolerant Swingle citrumelo roots. Despite low concentrations, Sulistyowati *et al*. (1990) did correlate scoparone accumulation in roots of citrus with resistance towards *P. citrophthora*. Scoparone accumulation is therefore responsible for resistance against the collar rot fungus *P. citrophthora*, but it does not necessarily play a part in resistance against *P. nicotiana*e root rot.

Although the use of resistant rootstocks is of great value in the control of *Phytophthora* diseases, most of the time chemical control is still necessary. The systemic fungicide fosetyl-

aluminium (Aliette) has been effectively used in the control of *Phytophthora* and *Pythium* diseases (Farih *et al*., 1981; Sanders *et al*., 1983; Timmer & Castle, 1985; Cohen & Coffey, 1986). The precise mechanism of action of fosetyl-Al is not fully elucidated, but it has been shown to have a direct mode of action on the pathogen (Fenn & Coffey, 1984; 1985; 1989) as well as an indirect mode of action by inducing host defence responses (Guest, 1984; Afek & Sztejnberg, 1989; Nemestothy & Guest, 1990).

Fenn and Coffey (1984, 1985) demonstrated direct activity against *Phytophthora* in vitro and proposed that fosetyl-Al is degraded to phosphorous acid (H_3PO_3) in the plant, which is toxic to *Phytophthora* species. In contrast, Nemestothy and Guest (1990) showed that in tobacco plants fosetyl-Al enhances sesquiterpenoid phytoalexin accumulation, PAL activity, lignin deposition and ethylene biosynthesis, protecting it against *Phytophthora*. Afek & Sztejnberg (1989) also showed that fosetyl-Al has low direct antifungal activity against mycelial growth of the Oomycetes *in vitro*. They furthermore demonstrated that scoparone accumulated to higher levels in infected bark treated with fosetyl-Al or H_3PO_3 than in infected bark that was not treated. Thus at low levels, fosetyl-Al increases the host defence mechanisms and at higher levels it acts directly as a fungistat.

Aim of the study

To discover biochemical markers for resistance of citrus rootstocks to *P. nicotianae* root rot. In this study the focus was on resistance mechanisms in citrus roots, rather than in stems, because this is the primary pathway of infection for *P. nicotianae*. The objective was to determine the biochemical mechanisms involved in resistance of citrus rootstocks against *P. nicotianae* root rot. The qualitative and quantitative occurrence of inhibitory compounds, such as scoparone and total soluble phenolics, was studied in the roots of selected citrus rootstocks

ranging from susceptible to tolerant towards *P. nicotianae*. An additional objective was to develop a high-throughput screening method for resistance of citrus rootstocks towards *P. nicotianae* root rot.

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Chapter 2

ACCUMULATION OF TOTAL PHENOLICS IN *PHYTOPHTHORA NICOTIANAE* SUSCEPTIBLE AND TOLERANT CITRUS ROOTSTOCKS AND THE EFFECT OF FOSETYL-AL THEREON

ABSTRACT

The levels of total soluble phenolics were determined in citrus rootstocks tolerant and susceptible to *Phytophthora nicotianae* following inoculation with the pathogen. Extraction techniques were optimised by comparing different solvent systems in relation to the total amount of phenolics they extracted. Optimal extraction was achieved with 80% ethanol. Phenolic concentrations were higher in the roots of *Phytophthora*-tolerant Swingle, Macrophylla, Troyer and Sour orange rootstocks 21 days after inoculation, whereas only minor increases occurred in susceptible Rough lemon and Volkamer lemon rootstocks. Of the two intermediately tolerant rootstocks, Carrizo and C35, Carrizo produced an intermediate level of phenolics, but C35 showed only a low level. The baseline level before inoculation was 25 mg gallic acid equivalents/gram dry root material. The highest phenolic concentrations were recorded after 21 days in Troyer, rendering a maximum of 58.2 mg gallic acid equivalents/gram dry root material, in comparison to 48.1 mg/g in Rough lemon. Total phenolic concentrations correlated positively with tolerance to *P. nicotianae*, suggesting that total phenolics play a role in rootstock resistance. Treatment with the systemic fungicide fosetyl-Al further increased phenolic levels in most roots, with or without infection. Total

phenolic concentrations were 2 fold greater in inoculated rootstocks than in uninoculated rootstocks. Pre-treatment with fosetyl-Al stimulated a 2.2 fold increase in inoculated rootstocks relative to uninoculated rootstocks. This increase indicates that total phenolics play a role in the mode of action of this fungicide. Using fluorescence microscopy, the occurrence of phenolic compounds were visualized and were mainly located in the first layers of cortex cells just beneath the epidermis as well as in the vascular system. In conclusion, upon inoculation with *P. nicotianae*, tolerant rootstocks accumulated total phenolics to about 2.2 fold the baseline whereas total phenolics accumulated only 1.6 fold the baseline values in susceptible rootstocks.

INTRODUCTION

Phytophthora species cause severe diseases of citrus worldwide (Timmer & Menge, 1988; Graham, 1990). Rootstocks differ in their tolerance to soil salinity, cold injury and disease (Castle, 1987; Spiegel-Roy & Goldschmidt, 1996). Tolerance of some citrus rootstocks to *Phytophthora* has been amply demonstrated (Sulistyowati *et al*., 1990; Agostini *et al*., 1991; Graham, 1995) and it offers an excellent means of reducing losses due to *Phytophthora* root and collar rot.

Resistance responses are usually characterized by the early accumulation of secondary phenolic compounds that effectively isolates the pathogen at the point of infection (Nicholson & Hammerschmidt, 1992). Phenolic research in citrus is mainly focused on the production of phenolic compounds in fruit, and on the influence on fruit and juice quality (Guadagni *et al*., 1973; Robbins, 1990; Vandercook *et al*., 1990). The production of antifungal phenolic compounds has been demonstrated in citrus fruits (Rodov *et al*., 1994), peels (Dubery *et al*.,

1999), leaves (Manthey *et al*., 2000) and in citrus roots (Feldman & Hanks, 1968). In previous investigations, mostly by Afek and colleagues (1986; 1988; 1989; 1990; 1993; 1995), the phytoalexin scoparone has been implicated in the resistance mechanism of citrus against collar rot caused by *Phytophthora citrophthora*. They however did not study scoparone in citrus roots. Scoparone production in response to infection by *P. nicotianae* was studied in citrus roots by Aucamp *et al*. (2000) by means of capillary electrophoresis and thin layer chromatography (TLC), but scoparone levels did not show a strong correlation to root rot resistance.

The fungicide fosetyl-aluminium (aluminium tris-[o-ethyl phosphonate]) is known to be effective for control of some diseases caused by the Peronosporales (Farih *et al*., 1981; Sanders *et al*., 1983; Timmer & Castle, 1985; Cohen & Coffey, 1986). Fosetyl-Al exhibits a complex mode of action acting both directly on the pathogen (Fenn & Coffey, 1984; 1985; 1989) and indirectly by inducing host defence responses (Guest, 1984; Afek & Sztejnberg, 1989; Nemestothy & Guest, 1990). Despite numerous studies on the fungicide the exact mode of action of fosetyl-Al is not fully elucidated.

Fluorescence microscopy can be used to study phenolic compounds due to their autofluorescent properties, or they can be stained with phenol-specific reagents (Bayliss *et al*., 1997). It is a rapid and easy technique to examine host-pathogen interactions. In resistant and susceptible hosts, it can possibly demonstrate the difference in localization of fluorescence/phenolic accumulation, the distribution of the poly phenolics i.e. localized or systemically induced throughout healthy tissue and colour of fluorescence can give an indication of the specific group(s) of phenolics produced. Finally it can be used to investigate the time response of phenolic accumulation.

In this study, accumulation of total soluble phenolics in reaction to infection by *P. nicotianae* was investigated in citrus rootstocks tolerant and susceptible to the pathogen. The effect of fosetyl-Al treatment on total phenolic concentrations in the various rootstocks was also studied.

MATERIALS AND METHODS

Reagents

The Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (St Louis, MO, USA). Scoparone (6,7-dimethoxycoumarin) was purchased from Aldrich (Milwaukee, WI, 53216, USA). All solutions were prepared with distilled water that was deionised with a Milli-Q system (Millipore Corp. Bedford, MA. USA). Methanol, ethanol, chloroform and acetone were purchased from BDH Laboratory Supplies (Poole, England). All the salts and solvents were of analytical grade.

Plant and fungal materials

An isolate of *P. nicotianae* (freshly isolated from citrus roots) was cultured at 25 °C on PARPH (pimaracin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol) medium, selective for *Phytophthora* (Jeffers & Martin, 1986). Pathogen free seed was obtained from the citrus foundation block (Uitenhage, Eastern Cape Province, South Africa) and germinated in sterile vermiculite in a growth cabinet at 28 °C, and 80% humidity. After 14 days, when sufficient roots have developed, the seedlings were transplanted into a steam pasteurised bark/sand mixture (2:1 v/v) and grown under normal greenhouse conditions at 22-26 °C. Seedlings were watered three times a week with autoclaved water and fertilized once a month

with Polyfeed (Plaaskem (Pty) Ltd., Johannesburg, Gauteng, South Africa). Fertilization was done as a drench containing 2g/l Polyfeed (active ingredients: 263 g/kg N, 43 g/kg P, 134 g/kg K, 2.2 g/kg S, 1.65 g/kg Mg, 350 mg/kg Zn, 1000 mg/kg B, 70 mg/kg Mo, 750 mg/kg Fe, 300 mg/kg Mn, 75 mg/kg Cu). Rootstocks used were: two *Phytophthora*-susceptible citrus rootstocks Rough lemon (*Citrus jambhiri* Lush.) and Volkamer lemon (*C. volkameriana* Teng. & Pasq.), two intermediately susceptible rootstocks Carrizo citrange (*C. sinensis* X *Poncirus trifoliata*) and C35 citrange (*C. sinensis* X *P. trifoliata*) and four tolerant rootstocks Swingle citrumelo (*C. paradisi* X *P. trifoliata*), Troyer citrange (*C. sinensis* X *P. trifoliata*), Sour orange (*C. aurantium* L.) and Macrophylla (*C. macrophylla* Webster) (Burger, 2001). For ease of reading, only the cultivar names are henceforth referred to and full genera and species names (according to Saunt, 1990) can be found in the appendix (page 111).

Inoculation

In the first experiment, 6-month-old seedlings of two *Phytophthora*-susceptible citrus rootstocks (Rough lemon & Volkamer lemon) and two tolerant rootstocks (Troyer and Macrophylla) were compared in terms of total phenolic production in their roots, 1 to 21 days following inoculation with *P. nicotianae*. Seedlings were inoculated with *P. nicotianae* using the millet-seed inoculation method. One hundred ml sterile water was added to 200 g millet seed in high-density polyethylene bags and autoclaved for 20 minutes on two successive days. Each bag was inoculated with ten mycelial discs (6 mm in diameter) of a 14-day-old *P. nicotianae* culture on potato dextrose agar. Inoculated bags were incubated for four weeks at 25°C in the dark. A steam-pasteurised bark/sand mixture (2:1 v/v) was augmented with the millet seed at a ratio of 5 % v/v and sterile millet seed was used as control treatment. Fosetyl-Al (Aliette, Rhône-Poulenc Agrichem SA (Pty) Ltd, Onderstepoort, Gauteng Province, South Africa) treated and untreated plants were included for each rootstock with 4 replicate plants

for each treatment. Fosetyl-Al was applied as a foliar spray at the rate of 0.3 g/L. A concentration of 0.3 g/L was used since Afek and Sztejnberg (1989) demonstrated that accumulation of the phytoalexin scoparone was stimulated to its highest levels at this concentration. Plants were treated 7 days before inoculation, 1 day before inoculation and 13 days following inoculation. Plants were removed from the soil, their roots rinsed clean under running tap water and prepared for extraction 2, 7, 14 and 21 days following inoculation.

In the second experiment, 1-year-old seedlings of two *Phytophthora*-susceptible citrus rootstocks (Rough lemon & Volkamer lemon), two moderately susceptible rootstocks (Carrizo and C35) and four tolerant rootstocks (Swingle, Troyer, Sour orange and Macrophylla) were compared for total phenolic production in the roots at 21 days after inoculation with *P. nicotianae*. The same fosetyl-Al treatments were included as for the first experiment.

Disease assessment

Plants were harvested and their roots rinsed free from soil under running tap water. Disease severity was determined by rating each root system for visual root rot on a scale of 0 – 4 where 0 = 0% of root system rotted; 1 = 25%; 2 = 50%; 3 = 75% and 4 = 100% of root system rotted.

Extraction and quantification of total phenolic compounds

Five different solvent systems of increasing polarity (Snyder, 1974) were compared as to the total amount of phenolics they extracted. The solvents were methanol:chloroform (1:1), 100% ethanol, 80% ethanol in water, 80% methanol in water and methanol:acetone:water (7:7:6), where methanol:chloroform (1:1) was the least polar and methanol:acetone:water (7:7:6) the

most. An uninduced root sample of *Phytophthora*-tolerant Swingle rootstock was freeze-dried for 48 hours. The dried material was ground with a mortar and pestle to a fine powder. One millilitre of the respective solvents was added to a 0.05 g powdered root sample, vortexed for 30 seconds, shaken on an orbital shaker for 30 minutes, and centrifuged at 13 500 rpm (24 000 g) for 3 minutes. The extraction procedure was repeated 5 times and phenolic concentration determined after each extraction using the Folin-Ciocalteu reagent. An additional extraction procedure was carried out where the root sample was extracted twice for 1 hour followed by three 24-hour extractions.

In all subsequent experiments, one millilitre of 80% Ethanol was added to a 0.05 g powdered root sample, vortexed for 30 seconds, shaken on an orbital shaker for 1 hour, and centrifuged at 13 500 rpm (24 000 g) for 3 minutes. The extraction procedure was repeated twice and during the final extraction the samples were shaken for 24 hours instead of 1 hour. Supernatants were pooled and the organic solvent mixture was evaporated under a stream of nitrogen at room temperature. The residue was redissolved in 1 ml methanol.

The concentration of phenolic compounds was determined using the Folin-Ciocalteu reagent (Bray & Thorpe, 1954). The reaction mixture was scaled down to enable the use of 96 well ELISA-plates. A dilution series (10 – 1000 µg/ml methanol) were used to prepare standard curves for ferulic- and gallic acid for the quantification of phenolic content. The reaction mixture comprised: 170 µl distilled water + 5 µl standard or root extract sample + 50 µl 20% (*v/v*) Na_2CO_3 + 25 µl Folin-Ciocalteu reagent. After incubation at 40 °C for 30 minutes the absorbance was read at 690 nm using an ELISA reader (Multiskan Ascent V1.24354 – 50973 (Version 1.3.1)). Phenolic concentration is expressed as ferulic or gallic acid equivalents per gram dry root material.

Fluorescence microscopy

Two *Phytophthora*-susceptible citrus rootstocks (Rough lemon & Volkamer lemon), two moderately susceptible rootstocks (Carrizo and C35) and four tolerant rootstocks (Swingle, Troyer, Sour orange and Macrophylla) were subjected to fluorescence microscopy 21 days after inoculation with *P. nicotianae*. Root sections were stored in 3% Glutaraldehyde + phosphate buffer (pH 7). Transversal sections (10 – 20 μm) were cut with a microtome, mounted in sterile distilled water and observed under white light. Autofluorescence was examined under UV light using fluorescence microscopy (Zeiss, Mannheim, Germany) with standard fluorescent filter cubes with 365nm (filter set 487901) and 546 nm (filter set 487915) emissions. Images were captured with an AxioCam HR camera (Carl Zeiss Ltd, Mannheim, Germany).

Statistical analysis

Data was statistically analysed according to Duncan's multiple range test, using the SAS-system (SAS User's guide, 1999). A p-value of < 0.05 was considered as statistically significant.

RESULTS

Both the ferulic- and gallic acid standard curves demonstrated a linear relationship between 10 and 1000 μg/ml methanol with $R^2 = 0.9963$ and $R^2 = 0.9989$ respectively. Ferulic acid was initially used as reference during extraction optimisation, but it showed poor colour development with the Folin-Ciocalteu reagent in comparison to gallic acid. Gallic acid was therefore used in subsequent total phenolic studies. Extraction techniques were optimised by

comparing different solvent systems in relation to the total amount of phenolics they extracted.

Complete extraction was achieved for most of the solvents after four consecutive extractions (Figure 1). Optimal extraction was achieved with 80% ethanol followed by 80% methanol, methanol:acetone:water (7:7:6), 100% ethanol and methanol:chloroform (1:1), which correspond with the amphipathic nature (structure having both a hydrophobic and a hydrophilic part) of phenolic compounds. Ethanol 80% was more efficient when the root sample was extracted twice for one hour followed by a 24-hour extraction, in comparison to the 30-minute extractions. Complete extraction was therefore achieved after three extractions with 80% ethanol and it provided significantly higher levels of phenolic compounds in comparison to the other solvents (Figure 2). Methanol:chloroform (1:1) extracted similar levels of phenolic compounds in comparison to the other solvents, but exhibited high variability between repetitions of the same extraction.

Figure 3 depicts a time study of the levels of total soluble phenolic compounds in citrus rootstocks over a period of 21 days following inoculation by *P. nicotianae*. After 2 days there were no significant differences in phenolic content between the treatments or rootstocks. After 7 days, phenolic levels increased significantly in the infected treatments in comparison to the uninfected control treatments. After 14 days phenolic levels were almost 3 times higher in the infected treatments than in the controls. The tolerant rootstocks, Troyer and Macrophylla, showed the highest phenolic accumulation in comparison to the susceptible rootstocks, Volkamer lemon and Rough lemon. The highest levels were recorded after 21 days in tolerant Troyer, reaching a maximum of 58.2 mg gallic acid equivalents/gram dry root material, in

comparison to 48.1 mg/g in the susceptible Rough lemon. A plateau in phenolic concentration was observed for most of the treatments after 14 days.

For both Troyer and Macrophylla, total phenolic content was higher when the infected seedlings were treated with the fungicide fosetyl-Al during the time course study (Figure 3). Infected Rough lemon and Volkamer lemon seedlings however showed no significant increases in total soluble phenolic concentration when treated with fosetyl-Al.

In the second experiment the tolerant rootstocks Sour orange, Swingle and Macrophylla showed higher concentrations of phenolics in comparison to susceptible Rough lemon and Volkamer lemon rootstocks (Table 1). Of the two intermediately tolerant rootstocks, Carrizo and C35, Carrizo produced an intermediate level of phenolics, but C35 showed only a low level. Fosetyl-Al treatment further elevated total soluble phenolic content within the roots of citrus rootstocks (Table 2). The increments in mg gallic acid equivalents per gram dry weight from the uninfected control were 4.7 when treated with fosetyl-Al, 10.1 when infected and 12.3 when infected and treated with fosetyl-Al, indicating an additive effect.

Using fluorescence microscopy, the occurrence of phenolic compounds was visualized and it was mainly located within the cell walls (Figure 5). Fluorescence was present in both the untreated controls as well as in the roots infected by *P. nicotianae* of all the rootstocks, indicating small quantitative differences in colour distribution. A green fluorescence was observed under UV light with a blue excitation filter (365 nm) and under UV light with a green excitation filter (546 nm), a blue fluorescence was observed. The blue and green autofluorescence, indicative of phenolic compounds, was seen throughout the cortex, epidermis, the pericycle and xylem and phloem parenchyma, but high concentrations of

phenolic compounds were especially found in the first layers of cortex cells just beneath the epidermis (Figure 6) as well as in the vascular system (Figure 7). Some of the rootstocks in particularly Sour orange (Figures 8C & 8E) contained strong fluorescing vacuoles within their cortex, even in uninfected roots.

DISCUSSION

The Folin-Ciocalteu reagent is generally used for the quantification of total phenolic compounds from plant extracts (Bray & Thorpe, 1954; Swain & Hillis, 1959; Julkunen-Tiitto, 1985; Sauvesty *et al*., 1991; Cahill & McComb, 1992). In our Folin-Ciocalteu procedure the reaction mixture was scaled down to enable the use of 96-well ELISA-plates, making the procedure much quicker than the conventional test tube technique. This modification also made the procedure more cost-effective due to smaller reaction volumes and yet sensitive and reproducible for the determination of total phenolic concentration. It can therefore be used as a valuable tool especially when screening a large number of samples or during time-course studies of phenolic production.

Quantitative estimation of phenolic compounds in plant tissue is not always accurate, since some of them are unstable and degradable. The production of phenolics as well as their stability can be influenced by light, temperature, substrate conditions of the host, pH, oxidation and solvent properties during extraction (Julkunen-Tiitto, 1985; Sauvesty *et al*., 1992; Poorter & Villar, 1997). It is therefore necessary to ensure optimal growth conditions for the host, as well as to optimise extraction techniques for specific plant species or for different sources of plant material.

Different solvent systems were compared in relation to the total amount of phenolics they extracted. Extraction with 80% ethanol provided complete and significantly higher levels of total soluble phenolic compounds in comparison to the other solvents (Figure 1 & 2). Sauvesty *et al* (1992) found that ethanol provided the best extraction of phenolic acids. Furthermore, water does not always dissolve some of the phenolic compounds (Julkunen-Tiitto, 1985). The ethanol in an aqueous solution effectively breaks down hydrogen bonds between phenolics and insoluble cell wall components ensuring effective extraction (Sauvesty *et al*., 1992), probably due to their amphipathic nature.

In the time course study (Figure 3) it was demonstrated that the level of total soluble phenolic compounds increase in all citrus rootstocks once infected by *P. nicotianae*. This increase was greater in tolerant Troyer and Macrophylla rootstocks than in susceptible Rough lemon and Volkamer lemon rootstocks. A significant increase in total phenolic concentrations was observed after 7 days in the roots of citrus rootstocks, which reached a maximum after 14 days. In comparison, Cahill & McComb (1992) showed a rapid increase in total phenolic concentration in the roots of *Eucalyptus calophylla* within 24 hours following inoculation with *P. cinnamomi.* Phenolic concentrations reached a maximum after 3 days.

Uninfected roots of *E. calophylla* (resistant to *P. cinnamomi*) contained more than 3 X the amount of phenolic substances than those of *E. marginata* (susceptible). Inoculation with *P. cinnamomi* caused a rapid increase in total soluble phenolics in the roots of *E. calophylla*, whereas *E. marginata* showed only minor increases. Increases were up to 97 % above the control levels and the maximum concentration of total phenolics reached in resistant *E. calophylla* was approximately 3.6 mg *p*-coumaric acid equivalents/gram fresh weight. In contrast, in the present study total phenolic concentrations in tolerant Troyer roots increased

up to 58.2 mg gallic acid equivalents/gram dry root material, upon infection by *P. nicotianae*. The higher maximum total phenolic concentration reached in citrus roots is probably because extractions were done from dry root material, in comparison to fresh material used for *Eucalyptus* extractions. Gallic acid, which was used as reference compound in the citrus procedure, possibly has better colour development with the Folin-Ciocalteu reagent than *p*-coumaric acid used by Cahill *et al*. for *Eucalyptus*.

In the second experiment tolerant Sour orange and Swingle rootstocks showed higher concentrations of phenolics in comparison to susceptible Rough lemon and Volkamer lemon rootstocks (Table 1). Various studies have reported that a rapid increase in the production of phenolic compounds may restrict pathogen ingress and confer resistance to the host (Nicholson & Hammerschmidt, 1992). This accumulation may occur in two stages, firstly a rapid accumulation of phenols localized around the infection site and secondly the activation of specific defences such as the production of phytoalexins (Matern & Kneusel, 1988). Results of the present study suggest that upon infection by *P. nicotianae*, the greater accumulation of total soluble phenolic compounds confers tolerance to Swingle, Sour orange, Macrophylla and Troyer rootstocks. This provides evidence that the increase in phenolic concentrations in citrus roots plays a key role in tolerance of citrus against *P. nicotianae* root rot.

Total phenolic content was higher in both Troyer and Macrophylla roots when the infected seedlings were treated with fosetyl-Al, whereas Rough lemon and Volkamer lemon seedlings showed only small increases (Figure 3). Similarly Afek and Sztejnberg (1989) found that in susceptible niva rootstock (*C. reticulata* Blanco x *C. sinensis* (L.) Osbeck), scoparone production did not increase significantly after treatment with fosetyl-Al and inoculation with

P. citrophthora. They proposed that in niva the fosetyl-Al breakdown product, phosphorous acid (H_3PO_3), has a direct fungistatic effect on the pathogen without the added effect of scoparone. They concluded that fosetyl-Al concentrations have to be much higher in niva to halt pathogen growth than in the tolerant species. Similarly, this might apply to control of *P. nicotianae* root rot in Rough lemon and Volkamer lemon rootstocks.

In the second experiment, fosetyl-Al treatment further elevated total soluble phenolic content within the roots of citrus rootstocks (Table 2). It has been debated that fosetyl-Al has a direct mode of action (Fenn & Coffey, 1984; 1985; 1989) as apposed to an indirect mode of action against the Oomycetes (Guest, 1984; Nemestothy & Guest, 1990). Direct fungicidal activity can however only be observed *in vivo* when several times the normal application rate is used (Smillie *et al*., 1988; Fenn & Coffey, 1989). Indirect mode of action has been demonstrated such as the induction of phenolic compounds (Candela *et al*., 1995) and phytoalexins (Afek & Sztejnberg, 1989), lignin deposition and increased enzyme activity (Nemestothy & Guest, 1990). Our results show that with the application of fosetyl-Al, phenolic levels are increased in the presence or absence of infection (Table 2), suggesting an indirect mode of action.

Fluorescence microscopy revealed fluorescence or accumulation of phenolic compounds within the cell walls of all the rootstock categories (Figure 5 – 8). A green fluorescence was observed under UV light with a blue excitation filter (365 nm), which depicts the presence of flavonoid type compounds. Under UV light with a green excitation filter (546 nm) fluorescence appeared blue, which indicates the presence of hydroxy-cinnamic acid derivates. Structures of phenolic compounds that possibly accumulate in citrus roots can be seen in Figure 4.

A strong fluorescence was present in both the untreated controls as well as in the roots infected by *P. nicotianae*. Phenolic compounds are therefore constitutively present but it was difficult to observe distinct changes in fluorescence upon *P. nicotianae* infection. Dai and colleagues (1996) observed autofluorescence in cotton leaves within two hours following inoculation with *Xanthomonas campestris* pv. m*alvacearum*. In our studies, autofluorescence was examined after 21 days following inoculation with *P. nicotianae* and this can possibly explain why no significant differences were observed between the different rootstocks categories or treatments. Fluorescence microscopy can therefore not be used as a marker for rootstock resistance against *P. nicotianae*.

In conclusion, the results of the present study provide strong evidence that the increase in phenolic concentrations in citrus roots plays a key role in tolerance of citrus against *P. nicotianae* root rot. The ELISA plate Folin-Ciocalteu method used in this study has potential as a high throughput method for screening citrus rootstocks for resistance. Coinciding with its known ability to protect rootstocks against *P. nicotianae*, fosetyl-Al stimulated the increase in total soluble phenolic concentrations following inoculation with the pathogen. This provides evidence that elevation of phenolic levels is involved in the mechanism of action of fosetyl-Al in the control of *Phytophthora* root rot, therefore supporting an indirect antifungal mode of action.

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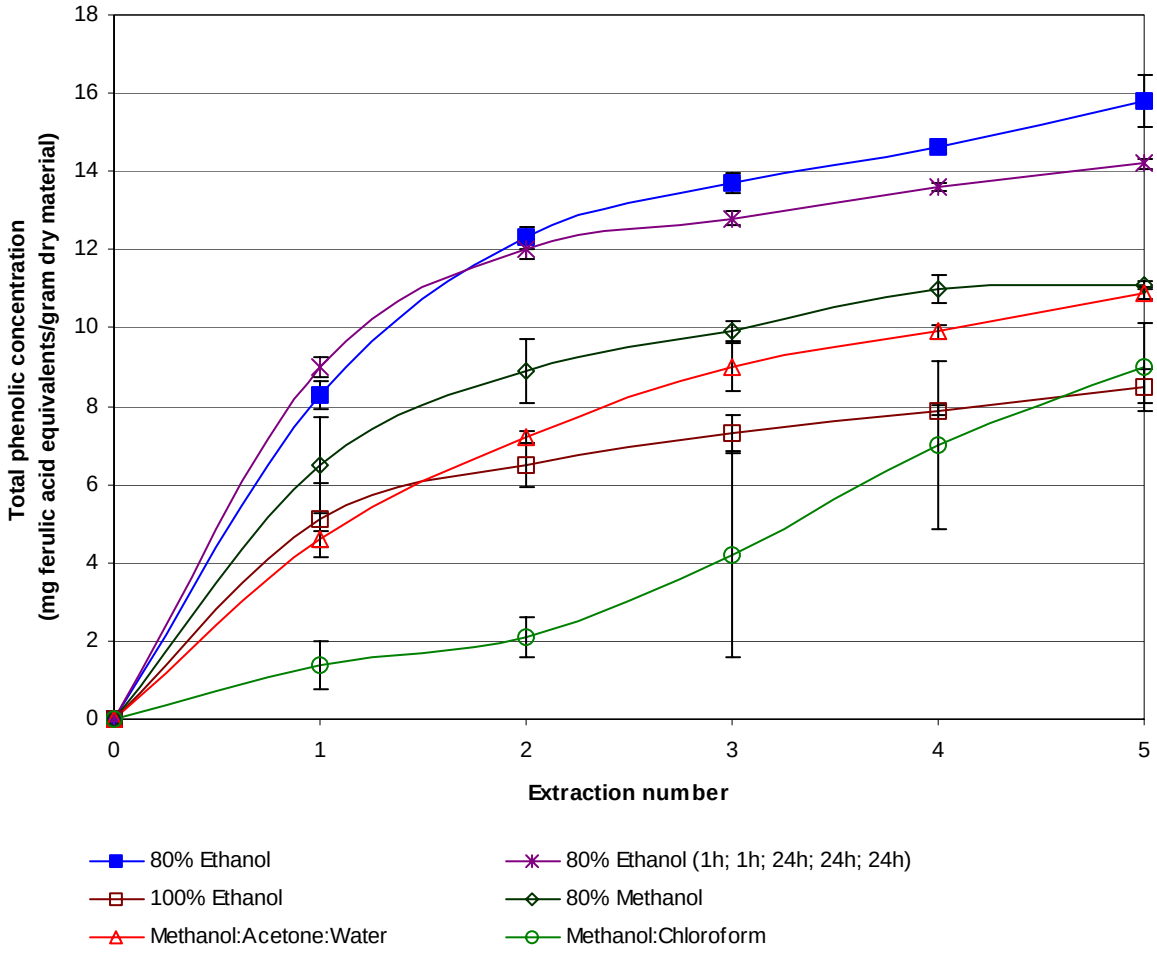

Figure 1. Solvent optimisation for total phenolic extraction from citrus roots: effect of different solvents on the amount of phenolics extracted from Swingle roots. All extractions were carried out for 30 minutes except for 80% ethanol where an additional procedure was followed by extracting the root sample twice for 1 hour followed by three 24-hour extractions.

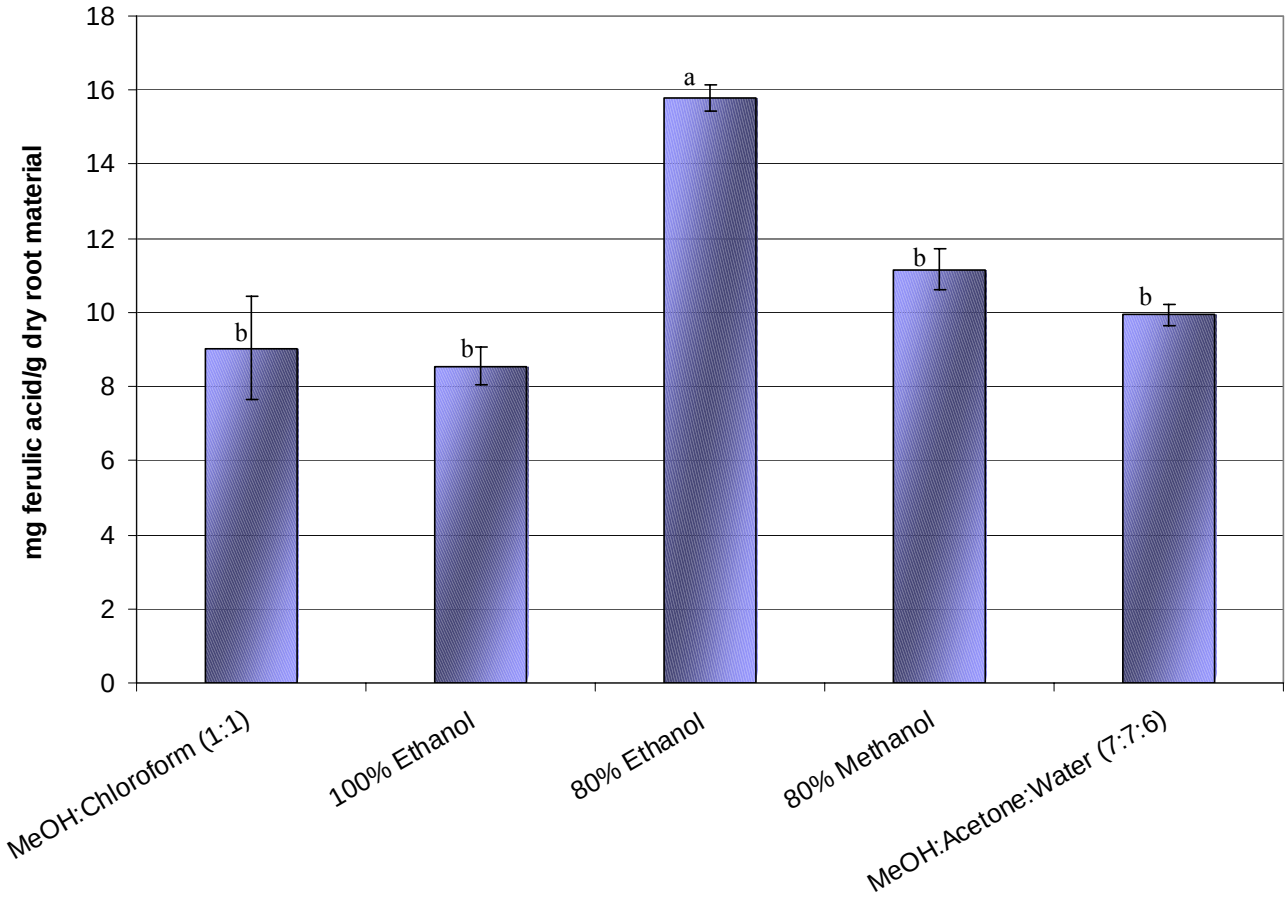

Figure 2. Effect of different solvents (with increasing polarity) on the amount of total phenolic compounds extracted from Swingle roots.

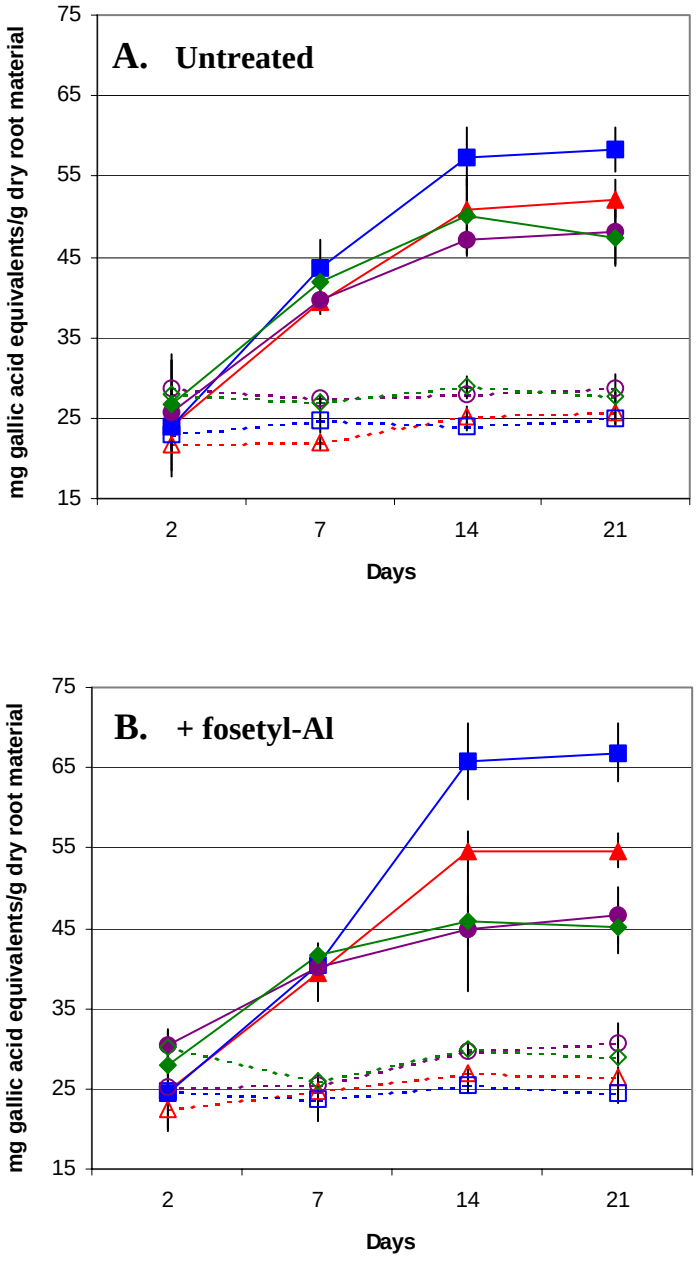

Figure 3. Total soluble phenolic concentration in (A) untreated and (B) fosetyl-Al treated roots of citrus rootstocks 1 to 21 days following inoculation with *Phytophthora nicotianae*. Macrophylla uninfected control (△) and infected (▲); Troyer uninfected control (□) and infected (■); Rough lemon uninfected control (○) and infected (●); Volkamer lemon uninfected control (◇) and infected (♦). Values are the means ± SD of four replicates.

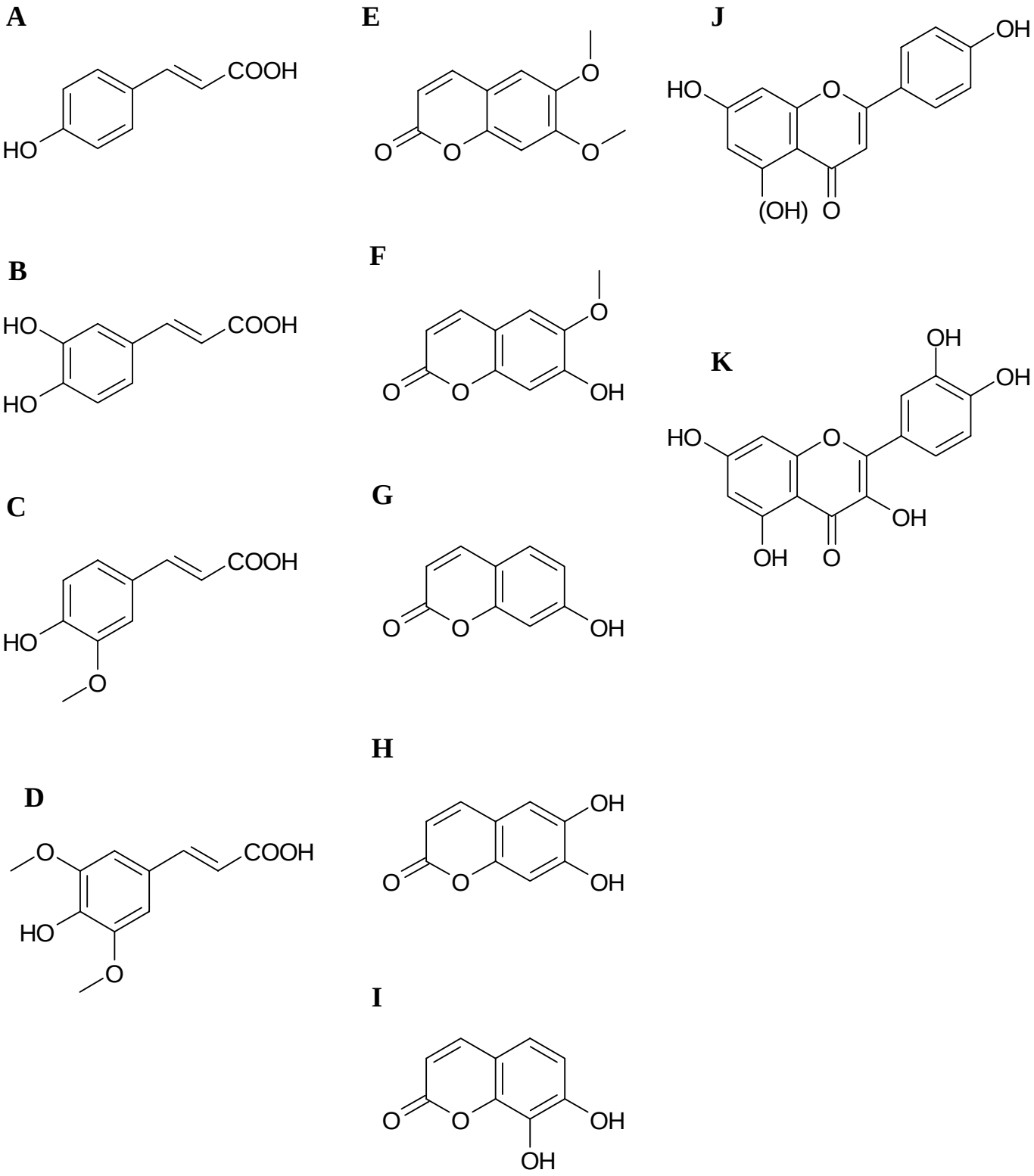

Figure 4. Structures of *p*-coumaric acid (A), caffeic acid (B), ferulic acid (C), sinapic acid (D), scoparone (E), scopoletin (F), umbelliferone (G), aesculetin (H), daphnetin (I), flavone (J) and quercetin (K).

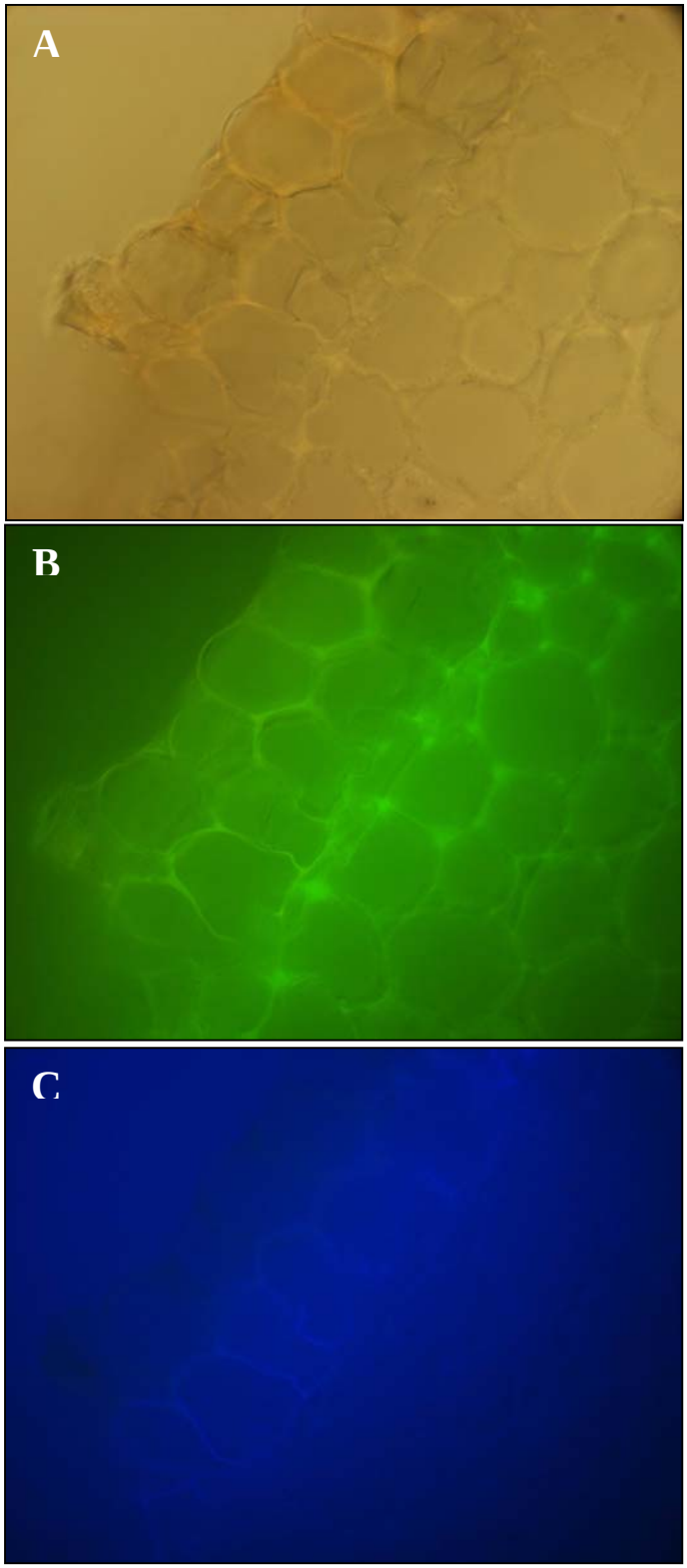

Figure 5. Transverse sections (x 1000 enlargement) of Macrophylla roots infected by *P. nicotianae* viewed under: **A**. normal light; **B**. fluorescence (365 nm) and **C**. fluorescence (546 nm). Fluorescence or phenolic accumulation was mainly located within the cell walls.

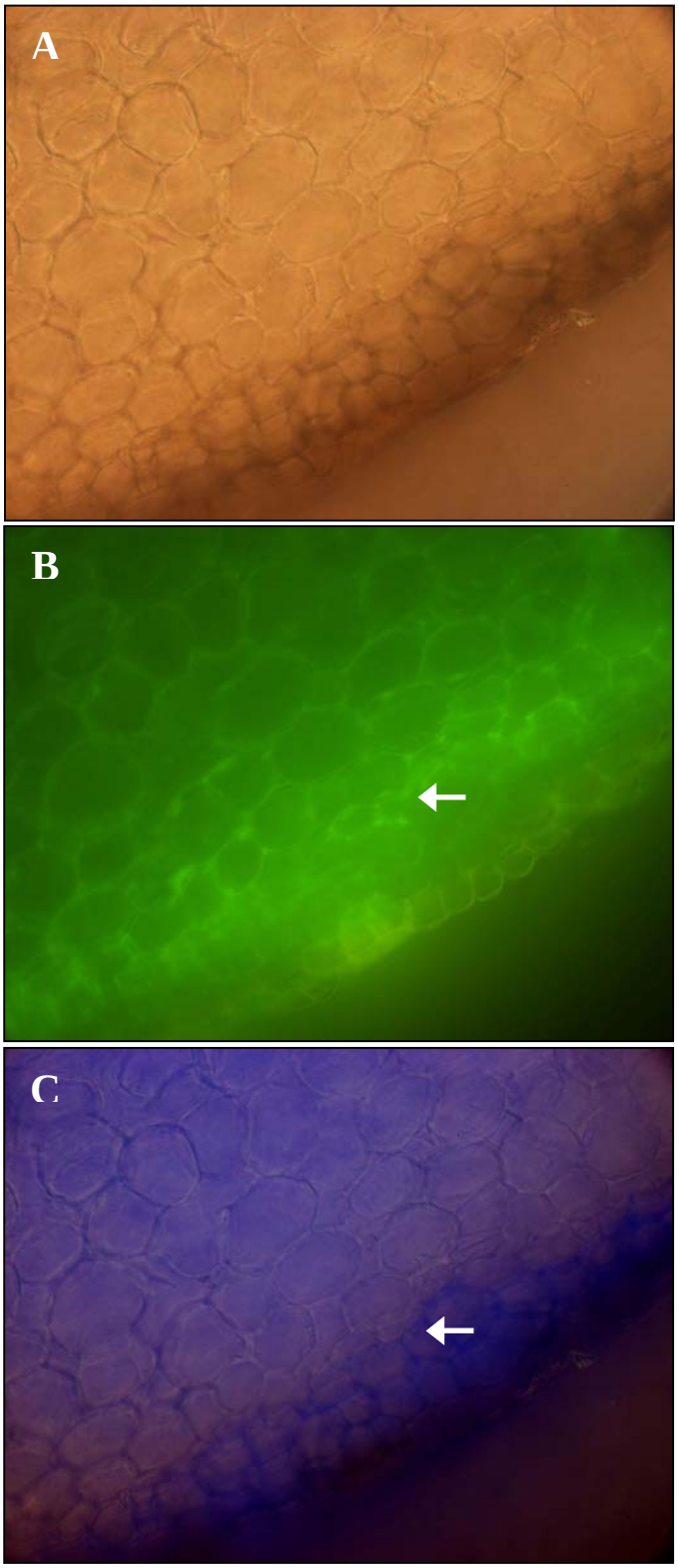

Figure 6. Transverse sections (x 200 enlargement) of uninfected Troyer roots viewed under: **A**. normal light; **B**. fluorescence (365 nm) and **C**. fluorescence (546 nm). Arrows indicate a strong fluorescence or high accumulation of phenolic compounds in the first layer of cortex cells just beneath the epidermis.

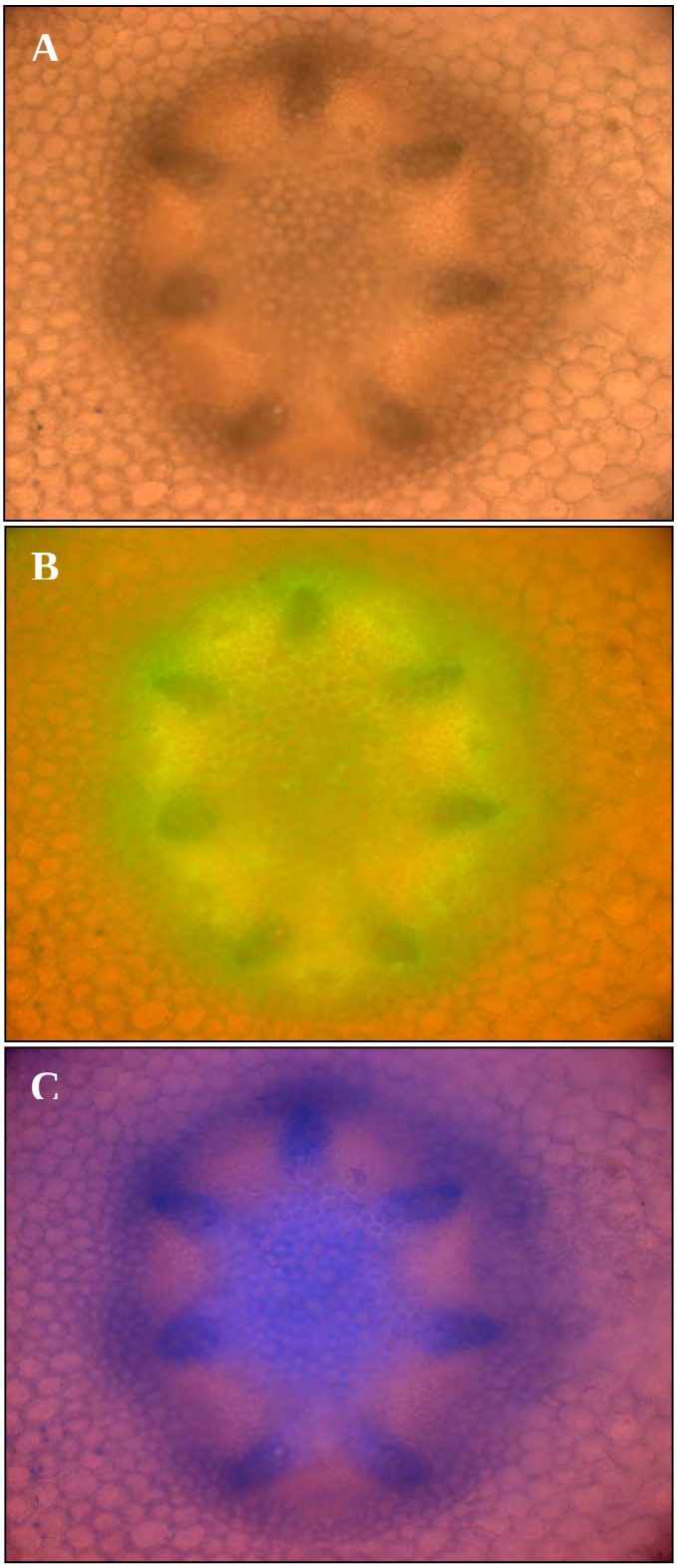

Figure 7. Transverse sections (x 400 enlargement) of Carrizo roots infected by *P. nicotianae* viewed under: **A**. normal light; **B**. fluorescence (365 nm) with normal light background and **C**. fluorescence (546 nm) with normal light background. A strong fluorescence or high accumulation of phenolic compounds is present within the vascular system.

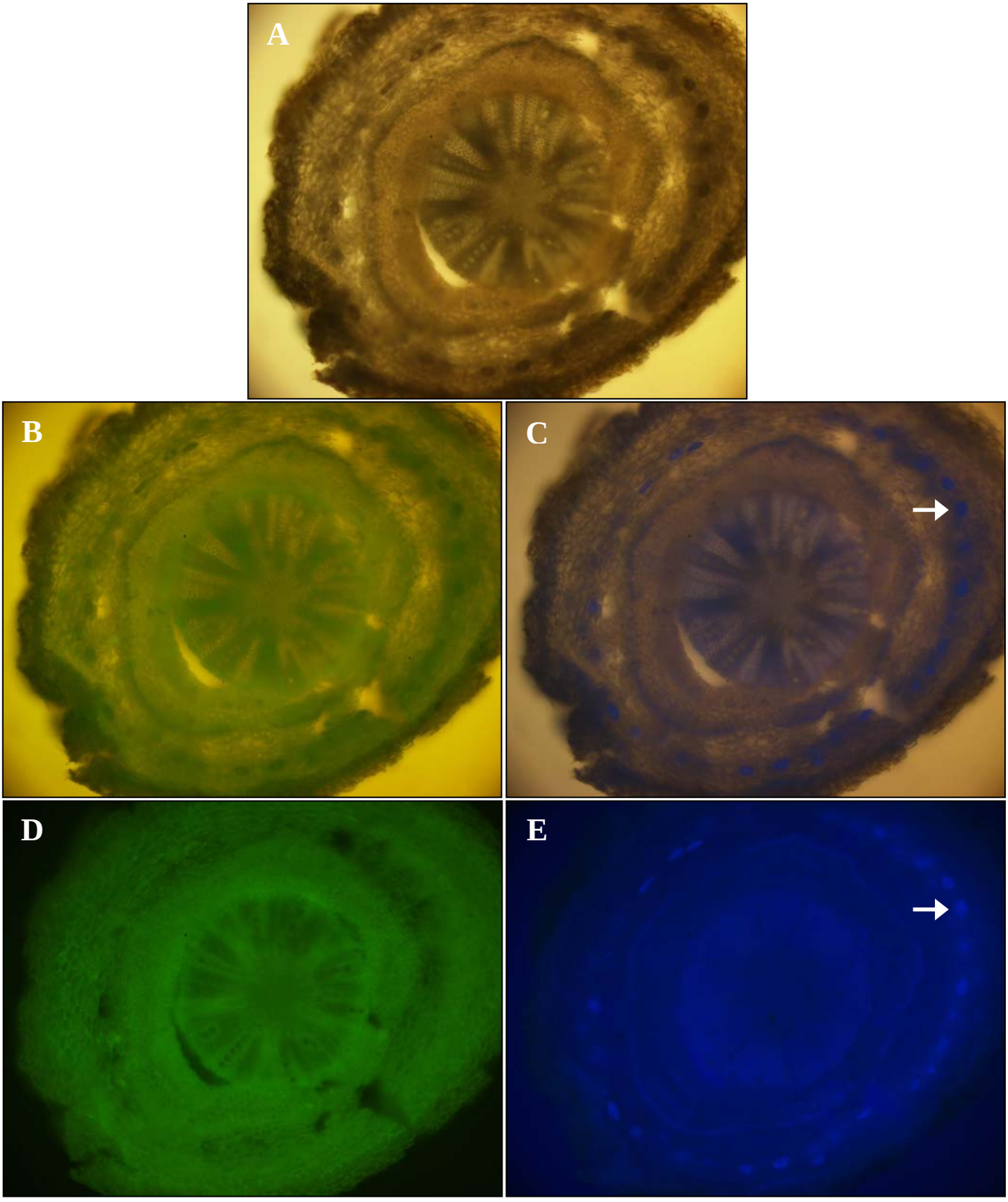

Figure 8. Transverse sections (x 100 enlargement) of uninfected Sour orange roots viewed under: **A**. normal light; **B**. fluorescence (365 nm) with normal light background; **C**. fluorescence (546 nm) with normal light background; **D**. fluorescence (365 nm) and **E**. fluorescence (546 nm). Arrows indicate the presence of strong fluorescing vacuoles within the cortex cells.

Table 1. Total soluble phenolic concentration in citrus rootstocks 21 days following inoculation with *P. nicotianae*

Rootstock	Phenolic concentration* (mg gallic acid equivalents / gram dry weight root material)	Root rot rating**
Sour orange (tolerant)	57.4 a	0.6 c
Swingle citrumelo (tolerant)	55.4 ab	0.6 c
Macrophylla (tolerant)	51.6 bc	0.9 bc
Troyer citrange (tolerant)	49.2 cd	0.6 c
Carrizo citrange (intermediate)	48.1 cd	0.9 bc
Rough lemon (susceptible)	45.9 de	1.2 ab
Volkamer lemon (susceptible)	43.3 ef	1.5 a
C35 citrange (intermediate)	39.5 f	1.3 a

* Mean of combined averages of four treatments with five replicates per treatment. Values followed by the same letter do not differ significantly according to Duncan's multiple range test (P=0.05).

** Disease severity was determined by rating each root system for visual root rot on a scale of 0 – 4 where 0 = 0% of root system rotted; 1 = 25%; 2 = 50%; 3 = 75% and 4 = 100% of root system rotted.

Table 2. Effect of *Phytophthora nicotianae* infection and fosetyl-Al treatment on total soluble phenolic accumulation in the roots of citrus rootstocks 21 days following inoculation

Treatment	Phenolic concentration* (mg gallic acid equivalents / gram dry weight root material)	Root rot rating**
Uninfected	42.0 c	0.0 c
Uninfected + fosetyl-Al	46.7 b	0.1 c
Infected	52.1 a	2.0 a
Infected + fosetyl-Al	54.3 a	1.6 b

* Mean of combined averages of eight rootstocks with five replicates per rootstock. Values followed by the same letter do not differ significantly according to Duncan's multiple range test (P=0.05).

** Disease severity was determined by rating each root system for visual root rot on a scale of 0 – 4 where 0 = 0% of root system rotted; 1 = 25%; 2 = 50%; 3 = 75% and 4 = 100% of root system rotted.

Chapter 3

DETERMINATION OF SCOPARONE IN CITRUS ROOTS BY MEANS OF THIN LAYER CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS*

ABSTRACT

Techniques for the detection of scoparone in citrus roots were evaluated with the aim of using scoparone as a marker of resistance, during the rapid screening of citrus rootstocks for resistance or tolerance against *Phytophthora nicotianae*. A new Micellar Electrokinetic chromatography (MEKC) method has been developed to determine the amount of the phytoalexin, scoparone, in citrus roots. This method was compared with the fluorescence thin layer chromatography (TLC) method in terms of its efficacy and suitability for scoparone detection. For MEKC, the separation and analysis was achieved with a running buffer of 100 mM SDS, 25 mM phosphate and 12% (*v/v*) methanol pH 6.2. Separation was performed at 12 kV with 25 °C and UV detection at 200 nm. A near complete recovery of scoparone was obtained with the extraction procedure. The detection limit with the MEKC method (2 µg/mL) was better than the TLC method (10 µg/mL). A rapid and complete extraction procedure for scoparone is described. This ensures reproducibility and reliable analysis of scoparone. MEKC with its high resolution and sensitivity is a suitable technique for the analysis of complex root extracts from citrus. The extraction procedure and high-resolution analytical method may enable further study of the induced compounds in citrus rootstocks.

*Aucamp, J.P., Kotze, S.S., Fourie, A., Labuschagne, N. & Apostolides, Z. 2000. Determination of Scoparone in citrus roots by Micellar Electrokinetic capillary chromatography. *Journal of High Resolution Chromatography* 23: 519-521.

INTRODUCTION

Two antifungal compounds were found in the bark of *Citrus limon* after infection with *P. citrophthora* (Hartmann & Nienhaus, 1974). One substance was identified as xanthoxylin, but no correlation between citrus resistance against *P. citrophthora* collar rot and accumulation of xanthoxylin could be demonstrated. The other substance was identified as scoparone and it was subsequently implicated as the compound involved in resistance of citrus to *P. citrophthora* (Afek *et al*., 1986; Afek & Sztejnberg, 1988; Afek & Sztejnberg, 1995).

Scoparone (6,7-dimethoxycoumarin) belongs to the coumarin group of phyto-chemicals. Structurally it is a planar molecule (Figure 1), and contains a benzopyrone nucleus that fluoresces under UV light of 365 nm.

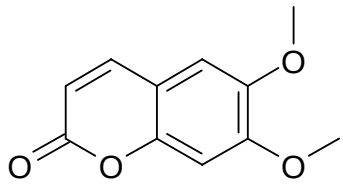

Figure 1. Chemical structure of scoparone.

Scoparone has been detected in the bark of citrus trunks, branches and fruit peels (Afek *et al*., 1986) as well as the roots of citrus plants inoculated with *P. citrophthora* (Sulistyowati *et al*., 1990). In previous studies scoparone levels in citrus plant material were determined with high

performance liquid chromatography, thin layer chromatography (Rodov *et al*., 1992; Kim *et al*., 1991) as well as spectrofluorimetry and radiolabelling (Afek & Sztejnberg, 1988).

Coumarin containing extracts from plants other than citrus have been analyzed by TLC (Mousa *et al*., 1997; Härmälä *et al*., 1992), HPLC (Mousa *et al*, 1997; Ganzera *et al*., 1997), GC (Arruda *et al*., 1993), Capillary zone electrophoresis (CZE) (Ochocka *et al*., 1995), and Micellar electrokinetic capillary chromatography (MEKC) (Ganzera *et al*., 1997).

Numerous methods have been used in the screening of citrus rootstocks for resistance against *Phytophthora* spp (Carpenter & Furr, 1962; Cameron *et al*., 1972; Grimm & Hutchison, 1973; Afek *et al*., 1990). In this study TLC and MEKC techniques were evaluated for the detection of scoparone in citrus roots. This was with the aim of developing a screening method that was faster and more effective than existing methods, for the determination of resistance to *P. nicotianae* root rot.

TLC is a mode of solid/liquid chromatography in which the sample is applied to a small spot at the origin of a thin sorbent layer supported on glass, plastic, or metal plate. The mobile phase (i.e. organic solvent) moves through the stationary phase (i.e. silica gel) by capillary action, sometimes assisted by gravity or pressure. Separation takes place with each of the compounds having the same total migration time, but different migration distances (Fried & Sherma, 1994).

None of the CZE or MEKC methods have been applied for the analysis of scoparone in any plant. MEKC makes use of micelle-forming surfactant solutions that give rise to separations that resemble reverse-phase liquid chromatography. The analyte e.g. scoparone associates

with anionic SDS-micelle. Its migration velocity is now slower than the electro-osmotic flow (EOF) of the buffer solutions within the capillary, which facilitates separation.

In this study MEKC was used as a new method for analysing scoparone in citrus roots in comparison to the fluorescence TLC method.

MATERIALS & METHODS

Reagents

Scoparone (6,7-dimethoxycoumarin) was purchased from Aldrich (Milwaukee, WI, 53216, USA). The SDS was analytical grade suitable for electrophoresis. All salts were of analytical grade. All solutions were prepared with distilled water that was deionised with a Milli-Q system (Millipore Corp. Bedford, MA. USA). Acetonitrile, methanol, chloroform, ethyl acetate, 2-propanol, toluene, and ρ-nitrophenol were purchased from BDH Laboratory Supplies (Poole, England). All the salts and solvents were of analytical grade.

Inoculation

An isolate of *P. nicotianae* (freshly isolated from citrus roots) was cultured at 25 °C on PARPH medium (Jeffers & Martin, 1986) selective for *Phytophthora*. Disease free 1-year-old seedlings of susceptible Rough lemon and Volckameriana, intermediately susceptible Carrizo and C35 and tolerant Swingle, were supplied by N Wenholdt of Casmar nursery (Mooinooi, North West Province, South Africa). To confirm the absence of pathogens, roots were randomly selected from seedlings of each rootstock, cut into segments of 5 mm in length, surface sterilised for 1 minute in 1.5 % sodium hypochloride solution and rinsed in sterile water. Ten root segments of each rootstock were plated out on potato-dextrose-agar (PDA) as

well as on PARPH medium (Jeffers & Martin, 1986) selective for *Phytophthora*. Plates were incubated at 25 °C in the dark and observed regularly over a two-week period for fungal growth. The seedlings were transplanted from the original bark mixture into a steam pasteurised bark/sand mixture (2:1) and grown under normal greenhouse conditions at 22-26 °C. Plants were watered three times a week with distilled water and fertilized once a month with Polyfeed (Plaaskem (Pty) Ltd., Johannesburg, Gauteng, South Africa). Fertilization was done as a drench containing 2g/l Polyfeed (active ingredients: 263 g/kg N, 43 g/kg P, 134 g/kg K, 2.2 g/kg S, 1.65 g/kg Mg, 350 mg/kg Zn, 1000 mg/kg B, 70 mg/kg Mo, 750 mg/kg Fe, 300 mg/kg Mn, 75 mg/kg Cu). Seedlings were inoculated with *P. nicotianae* using the millet-seed inoculation method (as described in chapter 1). The steam-pasteurised bark/sand mixture (2:1) was augmented with the millet seed at a ratio of 5 % *v/v* and sterile millet seed was used as control treatment.

Sample preparation

After 21 days the plants were removed from the growth substrate and their roots rinsed in tap water. The roots were dried overnight at 80 °C. The dried material was ground in liquid nitrogen with a mortar and pestle to a fine powder. A 0.1 g powdered root sample was extracted with 1 mL of a solvent mixture containing 2-propanol: ethyl acetate: chloroform (47.5:47.5:5). The sample was sonicated for 30 min in an ultrasonic water bath (Bransonic model 42) and then centrifuged at 14000 *g* for 5 min. The reaction procedure was repeated twice and the supernatants were pooled. The organic solvent mixture was evaporated *in vacuo*. The residue was redissolved in 1 mL acetonitrile, ultra-sonicated (Branson Sonifier/Cell disruptor B-30 with micro tip, 50% duty cycle setting 7, for 30 seconds) and centrifuged at 14000 *g* for 5 min to sediment all the undissolved matter. The acetonitrile supernatant was evaporated *in vacuo* and stored at 4 °C until required.

Thin-layer Chromatography

The stored samples were redissolved in acetonitrile to give a concentration of 1 g/mL root material. A volume of 5 µL was loaded onto a pre-coated Silica Gel 60 without fluorescent indicator obtained from Merck (Darmstadt, Germany).

The initial developing mixture toluene: ethyl acetate (1:1) used by Afek & Sztejnberg (1993), was further optimised to ensure sufficient separation for root extracts. Other toluene:ethyl acetate ratios evaluated were: (1: 0.95), (1: 0.9), (1: 0.85), (1: 0.8), (1: 0.75), (1: 0.7), (0.95: 1), and (0.9: 1). Another TLC-developing mixture that is used during chromatography of coumarin drugs (Wagner *et al*., 1983) was also evaluated. Toluene (50 ml) and ether (50 ml) were shaken for 5 minutes with 50 ml of 10% acetic acid in a separating funnel. The lower phase was discarded, and the toluene-ether mixture was used for TLC.

TLC plates were examined under UV light of 365 nm. The spots were quantified with image analysing software (NIH image analyzer, NIH 1.61/ppc). The density of the spots of the scoparone standards (10 – 200 µg/mL) were used to set up a standard- or calibration curve, which was then used to determine the concentrations of the samples.

Capillary Electrophoresis

The initial conditions from which the method was optimised was 100 mM SDS, 25 mM phosphate, 10% (*v/v*) methanol, pH 7.0. It was found that a decrease in the pH increased the resolution between scoparone and adjacent peaks. The pH was varied between 6.2 and 7.0. A pH of 6.2 was considered optimal for the analysis. It is not practical to do analysis at a lower pH since EOF decreases with decrease in pH. The methanol concentration was varied between 6 and 12% (*v/v*). The resolution of scoparone and adjacent peaks increased with an

increase in methanol concentration. A 12% (*v/v*) methanol concentration resolved scoparone well enough, and therefore no higher methanol concentrations were tested.

The refrigerated material was redissolved in the capillary electrophoresis running buffer containing 100 mM SDS, 25 mM phosphate and 12% (*v/v*) methanol pH 6.2, fortified with 30% (*v/v*) acetonitrile and 0.1 mg/mL ρ-nitrophenol (pNP) as a internal standard. The final concentration of dry root material in the running buffer was 0.5 g/mL. Analysis was performed on a Beckman PACE 2100 (Beckman Instruments, Fullerton, CA, USA). An uncoated fused silica column with an i.d. of 50 µm and a total length of 49 cm was used. The effective separation length of the column was 42 cm. Samples and standards were pneumatically injected (0.5 psi) for 2 s. The operating temperature was 25 °C. The resulting electropherograms were monitored at 200 nm.

A 100 mM SDS, 25 mM sodium phosphate buffer at pH 6.2 with 12% (*v/v*) methanol was used for the MEKC method. The separations were done with a voltage of 12 kV. Each day the capillary was rinsed with 0.1 M HCl for 10 min, H_2O for 5 min, 1 M NaOH for 5 min, H_2O for 5 min, 0.1 M NaOH for 10 min, and finally H_2O for 10 min. Between runs the capillary was rinsed with H_2O for 1 min, 0.1 M HCl for 2.5 min, H_2O for 1 min, 0.1 M NaOH for 2 min, and H_2O for 2 min. Five equilibration runs were done with standards before samples were analyzed. The capillary was stored in distilled water overnight.

Recovery Study

The extraction efficiency was determined by measuring the amount of scoparone in a root sample. Samples from the same origin were spiked with scoparone at 5, 10, 20, and 40 µg/g

dry root material before extraction. The scoparone levels in the spiked samples were then quantified and compared with the theoretical value calculated for complete extraction.

RESULTS

The initial toluene: ethyl acetate (1:1) mixture provided fairly good separation of scoparone from the other unidentified compounds on the TLC plates, but quantification with computer analysing software was still difficult. Very low levels of scoparone in the samples made analysis even more difficult, thus better separation of the compounds was necessary. Chromatographic separation was slightly better using a toluene: ethyl acetate ratio of 1: 0.75 as mobile phase. None of the other ratios gave better separation.

The acetic acid-saturated toluene:ether (1:1) mixture worked very well and allowed better separation of scoparone from the other compounds on the silica plate. The only drawback of this solvent is that it must be freshly prepared every time before use.

The TLC method (Figure 2) was useful for screening large number of samples. The calibration curve for scoparone with the TLC method showed a linear relationship between 10 and 200 μg scoparone/mL acetonitrile ($r = 0.99$) or 10-200 μg/g dry plant material. The limit of detection was 10 μg scoparone/mL. After a fungal challenge, the scoparone levels increased several fold in citrus root material. Increases in scoparone levels of 10 μg/g or more in root material were easily detectable with TLC. TLC is considered the ideal analytical tool for time course studies of scoparone induction patterns in citrus plants.

Under the analytical conditions described for MEKC, scoparone was completely separated from co-extracted compounds (Figure 3.A and B). The scoparone peak area and shape were constant over a wide range of electrophoretic conditions suggesting that the peak was homogeneous. The amount of scoparone in the induced sample determined by the MEKC and TLC methods were similar. This indicates that no other compounds co-migrate with scoparone. Another advantage of this MEKC method is that the entire electrophoresis process is relatively rapid, having a 15-minute analysis time.

In the root extracts the scoparone was identified by means of spiking. The root extracts contained several resolved unidentified compounds. These compounds were found to influence the migration time between runs significantly by adhering to the column wall. Rinsing with 0.1 M HCl removed these unwanted compounds from the column walls and ensured repeatable electro-osmotic flow and migration times.

The MEKC method is more selective and sensitive than the TLC method and can detect scoparone level increases of 4 μg/g root material very accurately. The limit of the detection for scoparone was experimentally determined as 2 μg/mL or 4 μg/g dry root material. The calibration curves showed a linear relationship between 2 and 60 μg/mL for scoparone (r = 0.995) and 20 and 160 μg/mL for ρ-nitrophenol (r = 0.995). The scoparone level for the uninduced sample (Figure 3.A) was determined as 5.9 μg/g root material and for the induced sample (Figure 3.B) as 23.4 μg/g root material. The repeatability of the migration time in the analytical method was tested and data for intra-day (n = 5) runs and inter-day runs (n = 5) are shown in Table 1.

The MEKC method is ideal for analysing samples from different cultivars exhibiting similar induction patterns and scoparone levels. At uninduced levels, scoparone is a minor secondary metabolite in citrus plant material with levels of less than 10 µg/g dry plant material. Upon induction of plant defence mechanisms, the scoparone reaches levels of up to 200 µg/g dry plant material in the bark and fruit peels of citrus plants (Afek *et al*., 1986; Afek & Sztejnberg, 1988).

The low levels of scoparone production in samples make complete extraction essential for reproducibility. It was found that 2-propanol extracted scoparone better that methanol or ethanol. The solvent mixture 2-propanol: ethyl acetate: chloroform (47.5:47.5:5) as initial extraction solvent achieved a complete extraction of scoparone from the powdered roots. The acetonitrile alone did not achieve a complete extraction of scoparone from powdered plant material.

The solvent mixture was removed *in vacuo* and the residue was then redissolved in acetonitrile. The more polar acetonitrile dissolves the scoparone while other non-polar molecules initially extracted with the solvent mixture remain insoluble. The stored samples were redissolved in the modified running buffer containing 30% (*v/v*) acetonitrile. The organic solvent and hydrophobic SDS selectively dissolves the scoparone while some of the background molecules remain insoluble. The recovery of scoparone by this extraction procedure is close to 100% as shown in Table 2.

DISCUSSION

Using MEKC, the scoparone level for the uninduced sample (Figure 3.A) was determined as 5.9 µg/g dry root material and for the induced sample (Figure 3.B) as 23.4 µg/g root material. Only a 4-fold increase in the scoparone level in induced roots was observed in this study, whereas Afek & Sztejnberg (1988) showed a 9-fold increase in the bark of tolerant Macrophylla upon infection by *P. citrophthora*. Differences in citrus cultivars and *Phytophthora* species, as well as induction and sample collection methodologies may explain why the induction level obtained is different from those found in literature.

TLC revealed distinct differences between phenolic profiles of susceptible and tolerant rootstocks (Figure 2), but very low levels of scoparone were present within the roots pre- or post treatment. There were quite a number of other unidentified fluorescent compounds. A distinct yellow fluorescent compound, with R_F = 0.82, was detected in tolerant swingle that was absent in susceptible rough lemon rootstocks (Figure 2, lane 15 & 16). The identity and role of these other unknown fluorescent compounds in rootstock resistance against *P. nicotianae* is yet to be determined.

Graham (1995) showed that upon *P. nicotianae* infection, scoparone accumulated to very low levels in the roots of susceptible Cleopatra mandarin and was not produced at all in tolerant Swingle roots. In our study, no correlation between citrus resistance and accumulation of scoparone could be demonstrated. Preliminary results therefore show that scoparone, which dictates resistance against *P. citrophthora* stem canker, is not as such involved in the mechanism of resistance in the roots. A more thorough investigation of scoparone

accumulation is however needed, in order to determine its role in the resistance mechanism active within the roots against *P. nicotianae*.

MEKC with its high resolution and sensitivity is a suitable technique for the analysis of complex root extracts from citrus. The MEKC analytical method complements the use of TLC and spectrofluorimetry in studies that investigate the production of scoparone in plants. This method can be applied in screening new citrus rootstocks for *P. nicotianae* and *P. citrophthora* resistance. A rapid and complete extraction procedure for scoparone is also described. This ensures reproducibility and reliable analysis of scoparone. The extraction procedure and high-resolution analytical method may enable further study of the induced coumarins in plants.

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Table 1: Summary of the intra- and inter-day variation on the migration times (minutes).

Compound	Intra-Day Variation (n = 5)			Inter-Day Variation (n = 5)		
	Mean	S.D.	%RSD	Mean	S.D.	%RSD
pNP	8.9	0.09	1.02	8.71	0.28	3.17
Scoparone	11.54	0.15	1.31	11.25	0.4	3.55

Table 2: Summary of the data obtained from the recovery study.

Amount spiked (ug/mL)	Amount detected (ug/mL)	Recovery (%)	RSD (%)
0	4.542	—	—
5	9.62	101.64	5.44 (3)
10	13.97	99.16	7.11 (3)
20	25.1	102.77	4.92 (3)
40	46.27	100.14	5.11 (3)

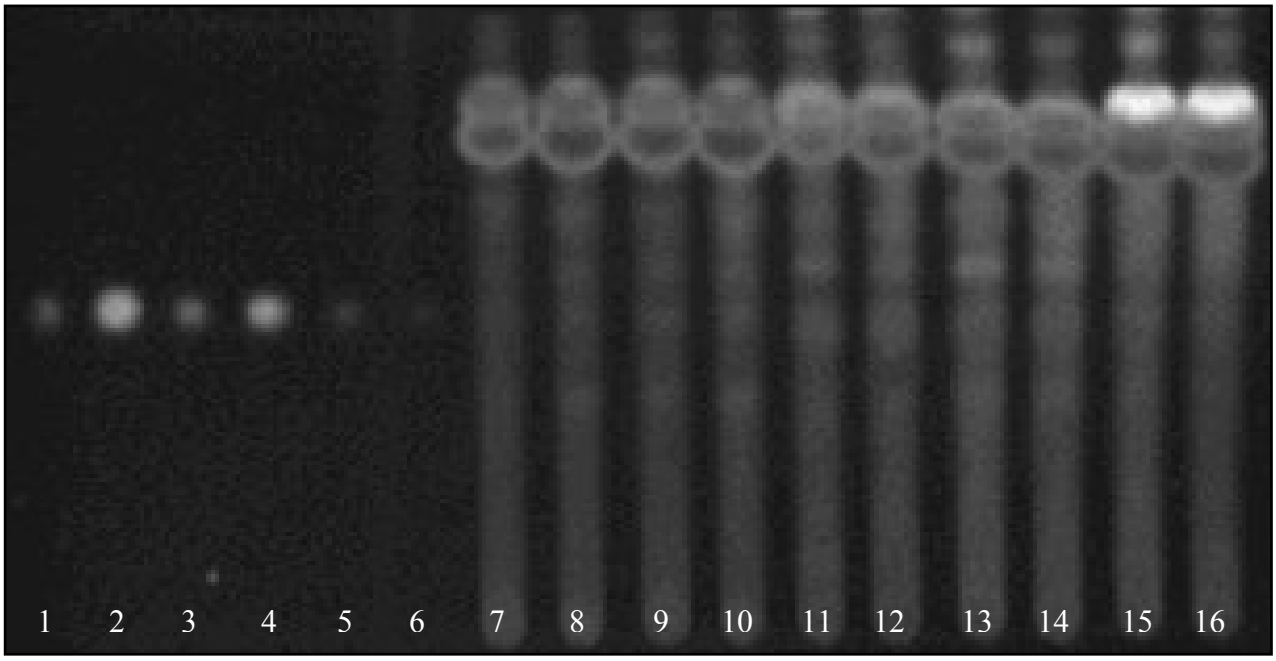

Figure 2. TLC analysis: Lane 1-6, scoparone standards 10-200 µg/mL. Lane 7-16, induced citrus root extracts showing very low scoparone production. Lane 7 & 8 = root extracts from Rough lemon rootstock; lane 9 & 10 = Volckameriana; lane 11 & 12 = C35; lane 13 & 14 = Carrizo; lane 15 & 16 = Swingle.

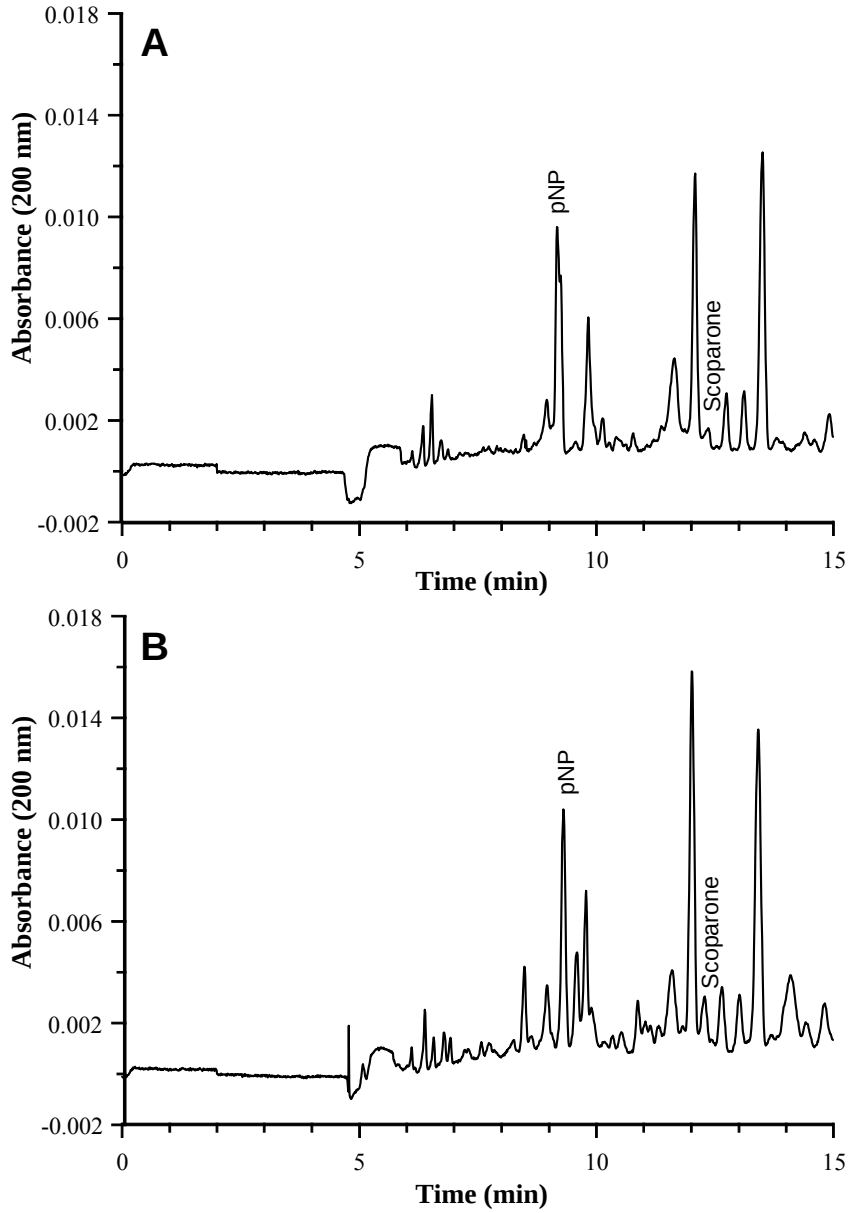

Figure 3. **A**: Electropherogram of an uninduced root extract. **B**: Electropherogram of an induced root extract. Both extracts are prepared at a concentration of 0.5 g/mL and analysed with conditions as stipulated under Materials & Methods.

Chapter 4

ROLE OF THE PHYTOALEXIN SCOPARONE IN RESISTANCE OF CITRUS ROOTSTOCKS AGAINST *PHYTOPHTHORA NICOTIANAE*

ABSTRACT

The *in vitro* dose-response of *Phytophthora nicotianae* to scoparone, fosetyl-Al (Aliette), and furalaxyl (Fongarid) was determined by means of the standard agar amendment method. Both scoparone and fosetyl-Al inhibited the mycelial growth *in vitro* with ED_{50} values of 37.76 and 75.03 μg/ml (ppm) respectively. Furalaxyl was used as a reference, giving 100% inhibition of growth at 1 ppm. In a time course study, TLC was used to examine scoparone accumulation in infected roots of Rough lemon, Volckameriana, Carrizo, C35 and Swingle rootstocks, over a period of 29 days following inoculation with *P. nicotianae*. Scoparone was first detected 8 days after inoculation. It reached a maximum at 12 days, after which accumulation declined. Static aqua cultures, consisting of small (250 ml) plastic containers, were used to evaluate the use of zoospore suspensions of *P. nicotianae* as an alternative infection method for citrus rootstocks. Rootstock used were Rough lemon, Swingle, Troyer, Carrizo, Volckameriana, Sour orange, C35, and Macrophylla. Uniform infection was achieved and Swingle, Macrophylla, and Sour orange proved to be the most tolerant towards *P. nicotianae* in the aqua culture system. A larger hydroponic system (10L containers) was used to examine the production of scoparone in the roots of citrus rootstocks, upon *P. nicotianae* infection. Rootstocks used were Rough lemon, Carrizo, Troyer and Swingle. TLC-analysis of citrus root

extracts revealed quite a number of unidentified fluorescent compounds. A distinct yellow fluorescent compound (R_F = 0.82) was detected in tolerant swingle that was absent in susceptible rough lemon rootstocks. None of the different rootstocks however showed any induction of scoparone synthesis. A novel technique was developed for the detection of fluorescent compounds that has been excreted from citrus roots upon infection by *P. nicotianae*. Zoospore suspensions of *P. nicotianae* were used to inoculate detached root pieces of Rough lemon, Swingle, Troyer, Carrizo, Volckameriana, Sour orange, C35, and Macrophylla rootstocks. Following extraction, TLC revealed up to seven different fluorescent compounds that were excreted. No scoparone was detected in these extracts for any of the rootstocks. From these results it is concluded that scoparone, which dictates resistance against *P. citrophthora* stem canker, is not as such involved in the mechanism of resistance in the roots against *P. nicotianae*. Further studies are therefore necessary to determine the structure, identity and possible antifungal activity of the other unknown compounds from citrus root extracts.

INTRODUCTION

Afek *et al*. (1986; 1988; 1989; 1995) found that scoparone accumulation is induced in the stems of citrus seedlings resistant and susceptible to *P. citrophthora* the causal pathogen of collar rot or gummosis of citrus. The concentration is however higher and increases more rapidly in the resistant species within 24 hours after inoculation (Figure 1). Following inoculation with *P. citrophthora*, maximum concentrations are reached after four days after which it diminishes towards the eighth day. Afek & Sztejnberg (1988) demonstrated direct antifungal activity of scoparone *in vitro*. Scoparone inhibited the mycelial growth of

Phytophthora citrophthora, Verticillium dahliae, Hendersonula toruloidea, Bortryodiplodia theobromae, Colletotrichum gloeosporioides, Penicillium digitatum, and *Penicillium italicum.*

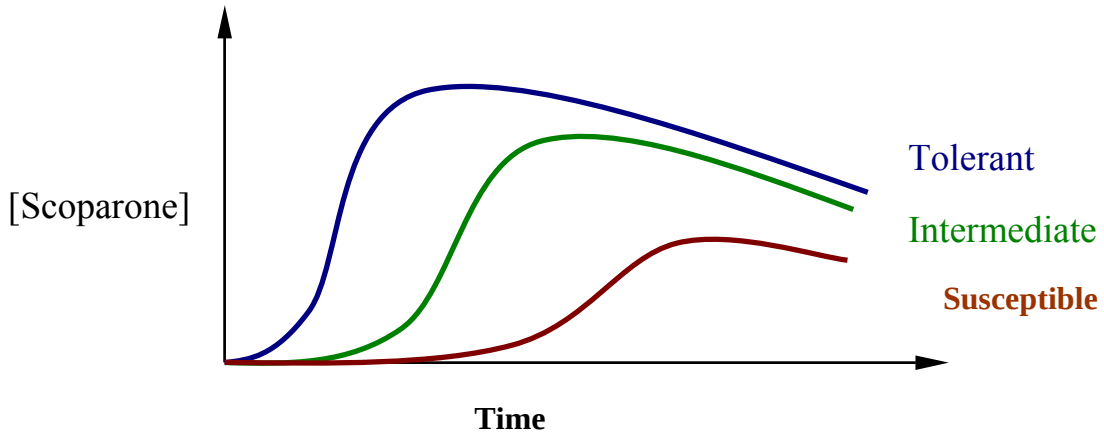

Figure 1. Accumulation of scoparone in the bark of resistant-, intermediately susceptible-, and susceptible citrus rootstocks. In the tolerant citrus species the onset of scoparone production is much earlier, the rate of accumulation faster and the final concentration much higher than in the susceptible rootstocks. Representation based on results of Afek & Sztejnberg (1988; 1989).

Sulistyowati *et al*. (1990) positively correlated the accumulation of scoparone, in the stem bark and roots of citrus, with resistance towards *P. citrophthora*. Scoparone synthesis was the highest in the completely necrotic, central zone of the stem lesions caused by *P. citrophthora,* and lower concentrations occurred in tissue along the margins of infection sites. In the roots they could only detect scoparone in the heavily invaded zones, and the concentration was a factor of about 5 less than in the stem lesions. They concluded that scoparone production occurs in cells prior to their death, and it only occurs in locations were it could inhibit fungal growth.

Little success has been achieved with the use of protective fungicides as control measure for *Phytophthora* diseases, but considerable progress has been made with the use of systemic therapeutic fungicides such as fosetyl-Al, metalaxyl and furalaxyl (Figure 2) (Farih *et al*., 1981). Fosetyl-Al has low direct antifungal activity against mycelial growth of the Oomycetes *in vitro* (Afek & Sztejnberg, 1989). It has been proposed that fosetyl-Al is degraded to H_3PO_3 (phosphorous acid) in plant tissue, or that it may act indirectly by triggering a host resistance response (Fenn and Coffey, 1984). Afek & Sztejnberg (1989) showed that scoparone accumulated to higher levels in infected bark treated with fosetyl-Al or H_3PO_3 than in infected bark that was not treated. Thus at low levels fosetyl-Al increases the host defence mechanisms, and at higher levels it acts directly as a fungistat. Acibenzolar-S-methyl (Bion; Actigard) (Figure 2) is another chemical compound that is believed to induce natural plant defence mechanisms. It is a derivative of salicylic acid and acts as a systemic "plant activator", enhancing resistance in the host plant against subsequent infection by pathogenic microorganisms (Prats et al., 2002; Baysal *et al*., 2003).

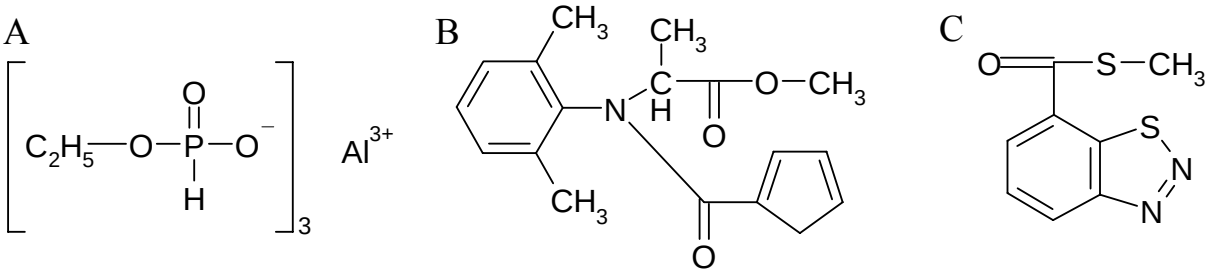

Figure 2. Chemical structures of fosetyl-Al (A), furalaxyl (B) and acibenzolar-S-methyl (C).

Phytoalexin studies are mainly conducted in specific plant cells and little work is focussed on the excretion of phytoalexins from plant tissue. El Modafar and colleagues (1995) studied the biochemical and histological responses of plane tree (*Platanus* spp.) after infection by *Ceratocystis fimbriata* f. sp. *platani*. Infection led to the biosynthesis of two hydroxycoumarin

phytoalexins, scopoletin and umbelliferone and this was correlated with fungitoxic activity. In their study, phytoalexins were excreted from *Platanus* leaves into tiny droplets of conidial suspension of *Ceratocystis fimbriata* f. sp. *platani*. In the present study, the possible excretion of phytoalexins from citrus roots upon *P. nicotianae* infection was also investigated.

In previous studies, scoparone accumulation in stem bark was positively correlated with resistance of citrus rootstocks to *P. citrophthora*, the causal pathogen of collar rot and gummosis. *P. nicotianae* is the main cause of root rot in South Africa (Thompson *et al*., 1995), therefore in the present study the focus was moved from citrus stems to the roots, which is the primary pathway of infection for *P. nicotianae*. Scoparone accumulation in the roots of citrus rootstocks was examined over a period of time, in rootstocks with varying levels of resistance towards *P. nicotianae*. The direct antifungal activity of scoparone was determined against *P. nicotianae in vitro*. Chemical elicitation of scoparone production was evaluated by treatment with fosetyl-Al and acibenzolar-S-methyl.

MATERIALS & METHODS

Reagents

Iso-buthyl-methyl-ketone (IBMK) was purchased from Merck (Darmstadt, Germany). Scoparone (6,7-dimethoxycoumarin) was purchased from Aldrich (Milwaukee, WI, 53216, USA). Pre-coated Silica Gel 60 plates without fluorescent indicator were obtained from Merck (Darmstadt, Germany). All solutions were prepared with distilled water that was deionised with a Milli-Q system (Millipore Corp. Bedford, MA. USA). Acetonitrile, methanol, chloroform, ethyl acetate, 2-propanol and toluene were purchased from BDH Laboratory Supplies (Poole, England). All the salts and solvents were of analytical grade.

Bioassay

Scoparone, fosetyl-Al (Aliette, Rhône-Poulenc Agrichem SA (Pty) Ltd, Onderstepoort, Gauteng Province, South Africa) and furalaxyl (Fongarid, Novartis SA (Pty) Ltd, Kempton Park, Gauteng Province, South Africa) were assayed for inhibition of *P. nicotianae in vitro*. It was done according to the standard agar amendment method where increasing concentrations of the specific compound were added to cooled molten potato dextrose agar (PDA) immediately before pouring into 90 mm petri dishes. A concentration series of 1, 10, 25, 50, and 100 parts per million of the active ingredient for each of the products was prepared with 10 replicates for each concentration. The control treatment contained no fungicide. A 3-mm diameter disk from an actively growing 6-day old colony of *P. nicotianae* on PDA was placed fungal side downwards in the centre of each plate. The average radial growth of each culture was recorded after 6 days by taking four radial measurements. The percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Average radial growth of the control} - \text{Average radial growth of treatment}}{\text{Average radial growth of the control}} \times \frac{100}{1}$$

For the *in vitro* testing of scoparone, the standard agar amendment method did not provide adequate results. A liquid culture comprising yeast-extract (YE) solution (1 g yeast-extract + 10 g glucose in 1L water) was used instead of the solid agar method. Scoparone was added to the YE broth in increasing concentrations. The YE broth was inoculated with 3 mycelial discs (6 mm in diameter) of a 6-day-old *P. nicotianae* culture on PDA. Six days following inoculation the mycelium was harvested by filtration, dried at room temperature for 24 hours, and weighed.

Disease status of citrus rootstocks

To confirm the absence of pathogens, roots were randomly selected from seedlings of each rootstock, cut into segments of 5 mm in length, surface sterilised for 1 minute in 1.5 % sodium hypochloride solution and rinsed in sterile water. Ten root segments of each rootstock were plated out on potato-dextrose-agar (PDA) as well as on PARPH medium (Jeffers & Martin, 1986) selective for *Phytophthora*. Plates were incubated at 25 °C in the dark and observed regularly over a two-week period for fungal growth.

Time course study of scoparone production in citrus roots in *P. nicotianae* infested soil

P. nicotianae (freshly isolated from citrus roots) was cultured at 25 °C on PARPH medium (Jeffers & Martin, 1986) selective for *Phytophthora*. Disease free 4-month old seedlings of *Phytophthora*-susceptible rootstocks Rough lemon and Volckameriana, two moderately susceptible rootstocks Carrizo and C35 and one tolerant rootstock Swingle, were supplied by N Wenholdt of Casmar nursery (Mooinooi, North West Province, South Africa). Seedlings were transplanted from the original bark mixture into 1.5 L plastic pots in a steam-pasteurised sand/peat/soil-mixture (1:1:1 *v/v/v*) and grown under normal greenhouse conditions at 22-26 °C. Seedlings were watered three times a week with autoclaved water and fertilized once a month with Polyfeed (Plaaskem (Pty) Ltd., Johannesburg, Gauteng, South Africa). Fertilization was done as a drench containing 2g/l Polyfeed (active ingredients: 263 g/kg N, 43 g/kg P, 134 g/kg K, 2.2 g/kg S, 1.65 g/kg Mg, 350 mg/kg Zn, 1000 mg/kg B, 70 mg/kg Mo, 750 mg/kg Fe, 300 mg/kg Mn, 75 mg/kg Cu).

Inoculation of the seedlings was done using the millet-seed inoculation method (as described in chapter 1). The steam-pasteurised sand/peat/soil-mixture (1:1:1 *v/v/v*) was augmented with the colonised millet seed at a ratio of 5 % *v/v* and sterile millet seed was used as control

treatment. The plants were removed and their roots rinsed in tap water every second day following initial inoculation, up to 29 days. Each treatment consisted of four replicates. Extraction of the roots and scoparone quantification was performed as described hereafter.

Determination of scoparone production in citrus roots in hydroponic systems

In the first experiment, static aqua cultures were used to evaluate the use of zoospore suspensions of *P. nicotianae* as an alternative infection method for citrus rootstocks. Scoparone production was not investigated during this experiment, but extraction and quantification was however done in the following two hydroponic experiments. Eight rootstocks were used namely: Rough lemon, Swingle, Troyer, Carrizo, Volckameriana, Sour orange, C35, and Macrophylla. Seeds, obtained from the citrus foundation block (Uitenhage, Eastern Cape Province, South Africa), were germinated in sterile vermiculite in a growth cabinet at 28 °C, and 80% humidity. After 14 days, when sufficient roots have developed, the seedlings were transplanted into 250 ml plastic containers filled with half strength Hoaglands nr 1 solution (KH_2PO_4, KNO_3, $MgSO_4$, $Ca(NO_3)_2$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, H_3BO_3) , modified from Janse van Rensburg (1998). Seedlings were infected by adding 5 mL of a zoospore suspension of *P. nicotianae* to the nutrient solution. Each treatment consisted of five replicates. Seedlings were growth in a growth cabinet at 25 °C for 14 days after which the plants were harvested and disease assessment conducted as described hereafter.

In order to ensure efficient zoospore production different growth media and methods for the induction of zoospore release were evaluated. Media included V8 juice agar, cornmeal agar, water agar and PARPH, which was all supplemented with β-sitosterol. The best sporangia production was achieved using cornmeal agar supplemented with β-sitosterol. As previously

reported (Kim & Hwang, 1992), exposure of the isolates to fluorescent light for 48 hours did not have a significant enhancing effect on sporangia production. In the first method for zoospore release, mycelial mats were gently brushed with a hockey stick in a small amount of autoclaved water. It was chilled at 4 °C for 30-60 minutes, after which it was returned to room temperature (Kim & Hwang, 1992). Secondly, cultures were homogenised for 10 seconds in a warring blender and diluted in 1L sterile water. Two Petri plate cultures of *P. nicotianae* per 1L of sterile water were used. Zoospore release was induced by chilling the culture suspension for 60 minutes at 10 °C (Botha *et al*., 1989). This method gave the best zoospore production.

In a second experiment, scoparone production was monitored in citrus roots that were treated with fosetyl-Al (Aliette) or with acibenzolar-S-methyl (Novartis SA (Pty) Ltd, Kempton Park, Gauteng Province, South Africa) in susceptible and tolerant rootstocks. Disease free 4-month old rootstocks were obtained from N Wenholdt of Casmar nursery (Mooinooi, North West Province, South Africa). Rootstocks used were Rough lemon, Carrizo, Troyer and Swingle. Seedlings were removed from the original bark mixture, their roots carefully rinsed in tap water and transferred to 10L hydroponic containers containing half strength Hoaglands nr 1 solution. Each container was fitted with a lid containing 4 holes. Four seedlings were placed in each container with their roots immersed in the nutrient solution and their stems supported with strips of foam rubber. Plants were grown in a greenhouse at 22-26 °C on a rotating table, which ensured uniform growing conditions for all rootstocks. The solution was aerated for 5 minutes every hour, by means of air hoses that were connected to a compressor.

Plants were treated with fosetyl-Al (500 µg/ml or ppm) or with acibenzolar-S-methyl (300 µg/ml), which were applied to the nutrient solution in the 10L containers 3 days prior to

infection. The treatments for each of the four rootstocks consisted of an uninfected control, uninfected + acibenzolar-S-methyl, uninfected + fosetyl-Al, infected control, infected + acibenzolar-S-methyl, and infected + fosetyl-Al. The nutrient solution was replaced with fresh solution just before inoculation. Four replicates for each treatment were used. The preparation of zoospore suspensions was done as previously described. Two hundred ml of zoospore suspension was added to the 10L pots for the infected treatments. After 6 days the plants were removed and their roots rinsed in tap water. Scoparone extractions from the roots and TLC analysis were performed as described hereafter.

A third experiment was performed, since the disease pressure in the previous experiment proved to be too high. The procedure was similar to that of experiment two, but a lower inoculum concentration as well as a higher fosetyl-Al concentration was used. Three seedlings of healthy, 6-month old *Phytophthora*-susceptible Rough lemon and tolerant Swingle rootstocks were placed in each container with their roots immersed in the nutrient solution. Plants were treated with fosetyl-Al [3000 µg/ml (ppm)] applied as a foliar spray at 7 days prior to infection, 1 day prior to infection, and 7 days following infection. The treatments for each of the two rootstocks consisted of: an uninfected control, uninfected + fosetyl-Al, infected control, and infected + fosetyl-Al. The preparation of zoospore suspensions was done as previously described. A lower inoculum concentration was used where only one petri dish culture of *P. nicotianae* was homogenised in a blender and diluted in 1L of sterile water. One hundred ml of this suspension was added to the 10L pots for the infected treatments. Time intervals of sampling for scoparone extraction following infection were as follows: 1 day, 4 days, 8 days, and 12 days following inoculation by *P. nicotianae*. Scoparone extractions from the roots and TLC analysis were performed as described hereafter.

Determination of scoparone excreted from citrus roots

Citrus rootstocks used during this experiment were: Rough lemon, Swingle, Troyer, Carrizo, Volckameriana, Sour orange, C35 and Macrophylla rootstocks. Seeds of the above rootstocks were germinated in sterile vermiculite in a growth cabinet at 28 °C, and 80% humidity. After 14 days, when sufficient roots have developed, the seedlings were transplanted into a steam pasteurised sand/peat/soil mixture (1:1:1 *v/v/v*). When the roots reached a thickness of approximately 2 mm, 2.5 cm root pieces were excised from each of the different rootstocks and placed in a 1.5 mL eppendorf tube, containing 1 ml of a zoospore suspension of *P. nicotianae* in sterile distilled water. As a negative control, sterile distilled water was used.

The eppendorf tubes with root pieces were incubated in the dark at 25 °C for 4 days. The tubes were periodically viewed under UV light (365 nm) to monitor the excretion of fluorescent compounds. After 4 days the root pieces were removed, the inoculum suspension sucked up into a syringe, and filtered through a 0.22 µm filter (Millipore). The suspension was subsequently transferred into 5 mL glass vials and extracted with 1 mL of IBMK. The vial was shaken for 2 minutes after which it was allowed to separate into an organic phase and an aqueous phase. The organic phase was removed, transferred to eppendorf tubes and the IBMK evaporated under N_2 gas. The residue was redissolved in 100 µL acetonitrile, ultra-sonicated (Branson Sonifier/Cell disruptor B-30 with micro tip, 50% duty cycle setting 7, for 30 seconds) and centrifuged to sediment the undissolved matter. The acetonitrile was evaporated under N_2 gas, the residue resuspended in 30 µL acetonitrile and subjected to TLC analysis.

Extraction and quantification of scoparone

Citrus roots were dried overnight at 80 °C. The dried material was ground in liquid nitrogen with a mortar and pestle to a fine powder. A 0.1 g powdered root sample was extracted with 1 mL of a solvent mixture containing 2-propanol: ethyl acetate: chloroform (47.5:47.5:5). The sample was sonicated for 30 min in an ultrasonic water bath (Bransonic 42) and then centrifuged at 14000 *g* for 5 min. The reaction procedure was repeated twice and the supernatants were pooled. The organic solvent mixture was evaporated under a stream of nitrogen at room temperature. The residue from the initial extraction step was redissolved in acetonitrile to give a concentration of 1 g root material/ml. It was then ultra-sonicated (Branson Sonifier/Cell disruptor B-30) and centrifuged to sediment all the undissolved matter. A volume of 5 µL was loaded onto pre-coated Silica Gel 60 TLC plates without fluorescent indicator (Merck, Darmstadt, Germany). The plates were developed with toluene:ethyl acetate (1:1). Scoparone fluoresces under UV light of 365 nm. The fluorescent spots were quantified with image analysing software (NIH image analyzer, NIH 1.61/ppc) (Aucamp *et al*., 2000).

Quantification of unknown yellow fluorescent compound (U82)

Uninfected roots of citrus rootstocks were subjected to TLC analysis (as described before), in order to quantify U82 by measuring the length of the spot on the TLC chromatograms.

Disease assessment

Plants were harvested and their roots rinsed free from soil under tap water. Disease severity was determined by rating each root system for visual root rot on a scale of 0 – 4 where 0 = 0% of root system rotted; 1 = 25%; 2 = 50%; 3 = 75% and 4 = 100% of root system rotted.

Statistical analysis

Data was statistically analysed according to Duncan's multiple range test, using the SAS-system (SAS User's guide, 1999). A p-value of < 0.05 was considered as statistically significant.

RESULTS

Both scoparone (Figure 3) and fosetyl-Al (Figure 4) inhibited the mycelial growth of *P. nicotianae in vitro*, with ED_{50} values of 37.76 and 75.03 µg/ml (ppm) respectively. Furalaxyl, which was used as a reference, rendered 100% inhibition of growth at 1 ppm (Figure 5).

In the time course study where scoparone production was examined 1 to 29 days following inoculation with *P. nicotianae*, scoparone was first detected by TLC analysis on the 8th day following inoculation (Figure 6). In Volckameriana and Rough lemon rootstocks it reached a maximum at 12 days, after which accumulation declined. Although there were no significant differences in scoparone concentration between the various rootstocks, C35 and Carrizo maintained higher levels of scoparone than the other rootstocks from 20 days onwards. Swingle was the most tolerant rootstock against *P. nicotianae* showing very little root rot symptoms, in comparison to Rough lemon and Volckameriana. Surprisingly C35 and Carrizo, which are classified as moderately susceptible, showed quite severe root rot (data not shown).

During the elicitation of scoparone production in a hydroponic system, the zoospore inoculation method in the hydroponic system rendered a much higher disease severity (root rot) than the millet seed inoculation method. Uniform infection was achieved with all citrus rootstocks tested in the small (250 ml) aqua cultures (Figure 7). Macrophylla, Sour orange,

and Swingle proved to be the most tolerant towards *P. nicotianae*, showing very little root rot symptoms (Figure 8). *Phytophthora*-susceptible Rough lemon and Volckameriana, intermediately susceptible Carrizo and C35, as well as *Phytophthora*-tolerant Troyer rootstocks showed quite severe root rot symptoms in the hydroponic system.

In the second hydroponic experiment where 10L containers were used, no significant differences in resistance were observed between the different rootstocks (Figure 9). The concentration of *P. nicotianae* inoculum thus proved to be too high, since the roots of all rootstocks showed extensive root rot. None of the different rootstocks or treatments showed any induction of scoparone synthesis. In the third experiment, even though a lower concentration of *P. nicotianae* inoculum was used, Swingle, which is known to be tolerant towards *P. nicotianae* was severely affected (Figure 10). The application of fosetyl-Al did reduce root rot severity in infected Swingle seedlings, but not in infected Rough lemon seedlings. None of the rootstocks or treatments showed any induction of scoparone synthesis (Figure 11 & 12).

TLC-analysis of citrus root extracts revealed quite a number of other unidentified fluorescent compounds (Figure 12, A - I). A distinct yellow fluorescent compound, with R_F = 0.82, was detected in tolerant Swingle that was absent in susceptible Rough lemon rootstocks (Figure 12, C). This yellow fluorescing compound was provisionally labelled Unknown 82 or U82. Tolerant Sour orange and Swingle rootstocks produced considerable amounts of U82 followed by moderate concentrations in Troyer, Macrophylla, Carrizo and C35, whereas it was absent in susceptible Volckameriana and Rough lemon rootstocks (Table 1).

In the detached root technique for the determination of excreted phenolic compounds, a faint fluorescence was observed in the inoculated eppendorf tubes at 6 hours onwards, after which it rapidly increased. TLC analysis of excreted fluorescent compounds from the roots of different citrus rootstocks can be seen in Figures 13 - 17. When IBMK is added during the extraction procedure, it dissolves the excreted fluorescent compounds and they should therefore all be in the organic phase. To determine if IBMK gives complete extraction, the aqueous phase from uninfected and infected roots was also subjected to TLC analysis (Figure 13). No fluorescent spots were observed, indicating that all compounds were fully extracted by IBMK. The organic phase from the uninfected roots was analyzed for each rootstock to determine if any of the compounds were present before inoculation with *P. nicotianae*. Only Sour orange (Figure 16), Volckameriana (Figure 16), and C35 (Figure 17) showed extremely faint bands before infection.

TLC revealed a number of unknown fluorescent compounds that were excreted upon infection by *P. nicotianae* (Figures 13 – 17). Again no scoparone was produced by any of the rootstocks evaluated. There were unknown compounds excreted with R_F values (R_F = 0.37) close to that of scoparone (R_F = 0.34), but the colour of fluorescence and R_F values were more similar to that of umbelliferone (R_F = 0.36). None of the rootstocks excreted these compounds in measurable amounts before infection, but they were rapidly excreted in great quantities once infected with *P. nicotianae*.

DISCUSSION

Direct fungicidal activity of scoparone, fosetyl-Al (Aliette), and furalaxyl (Fongarid) were confirmed against *P. nicotianae* by means of *in vitro* testing. The standard agar amendment

method was suitable for the *in vitro* testing of fosetyl-Al (Figure 4). Although scoparone did not reduce colony diameter, it caused a visible decrease in the density of mycelial growth (Figure 3). It was therefore necessary to test the effect of scoparone on *P. nicotianae* mycelial growth in a liquid yeast-extract/glucose-broth. Scoparone rendered an ED_{50} value of 37.76 µg/ml (ppm) whereas fosetyl-Al gave an ED_{50} value of 75.03 µg/ml. Furalaxyl was used as a reference, giving 100% inhibition of growth at 1 ppm (Figure 5).

In the time course study, scoparone production was measured in citrus roots over a period of 29 days in soil inoculated with *P. nicotianae*. Swingle was the most tolerant towards *P. nicotianae* in comparison to Rough lemon and Volckameriana that were fairly susceptible and Carrizo and C35 that showed severe root rot. The millet-seed inoculation method however did not provide adequate or uniform infection throughout the root systems. The amount of soil moisture influences the severity of *Phytophthora* diseases, since motile zoospores are the primary way of infection (Broadbent & Gollnow, 1992). Variable levels of soil moisture, as a result of the seedling watering procedure and a low inoculum pressure produced by the millet-seed inoculation method, might therefore explain the inconsistent observed disease severity.

Scoparone accumulation was induced in the roots of all of the different rootstocks regardless their degree of susceptibility or tolerance towards this pathogen (Figure 6). In contrast to the work of Afek & Sztejnberg (1988) who observed scoparone production within 24-hours and maximum concentrations after 4 days in stem bark, scoparone could first be detected 8 days after inoculation, and concentrations reached a maximum after 12 days in the roots. No significant differences in concentration were observed between susceptible and resistant species.

Sulistyowati and colleagues (1990) demonstrated that scoparone could only be detected in the heavily invaded, necrotic zones of citrus roots. In the current study, Carrizo and C35 rootstocks showed the highest degree of root rot in the soil-inoculation experiment and also maintained the highest levels of scoparone from day 20 onwards. The inconsistent infection levels between various rootstocks in the current study could have influenced scoparone levels in the samples, resulting in variations in scoparone accumulation patterns.

In the elicitation of scoparone production in a hydroponic system, the zoospore inoculation method was found to be superior pertaining to disease severity than the millet seed inoculation method used in the soil. Uniform infection was achieved with all citrus rootstocks tested in the static aqua cultures where 250 ml plastic containers were used (Figures 7 & 8). In the *Phytophthora* lifecycle, zoospores are the major stage that is responsible for the spread of the pathogen (Stanghellini & Rasmussen, 1994). Zoospores are produced in either a vesicle or a sporangium, and are released in the presence of free water. They can remain motile for a period of time, varying from minutes to hours. The washing of the citrus roots before planting it in a hydroponic system conceivably inflicts some damage on the roots. This low level of artificial wounding, and the presence of zoospores and free water in the hydroponic system is probably ideal conditions for *Phytophthora* infection.

In the second experiment where 10L hydroponic containers were used, the inoculum dosage was probably too high since all rootstocks, regardless of known susceptibility or tolerance, showed extensive root rot within 6 days (Figure 9). Also, none of the different rootstocks or treatments showed any induction of scoparone synthesis. Treatment with fosetyl-Al and acibenzolar-S-methyl had no significant effect on disease severity or scoparone synthesis. This might be as a result of low concentrations of the active ingredients that were used.

In the third experiment, a lower inoculum dose of *P. nicotianae* was used during the infection procedure, in an attempt to prevent the previous "over killing" of the seedling roots. A higher fosetyl-Al dose was also applied at vital times before infection, to facilitate better activation of plant defence responses. The tolerant Swingle rootstock still showed a high level of susceptibility towards *P. nicotianae* under these conditions (Figure 10). This was somewhat unexpected since Swingle is highly tolerant according to the literature and it has been the most tolerant rootstock tested so far in the hydroponic system. Extractions were done at four different times, but no scoparone was induced in any of the rootstocks (Figure 11 & 12). These results therefore indicate that although more uniform infection was achieved in the hydroponic system in comparison to the millet-seed inoculation method in the soil, the severe disease pressure was not suitable for scoparone induction. It is furthermore not likely that this type of severe disease pressure observed in the hydroponic system will be present in normal field situations in the orchards.

In the detached root inoculation study, it was demonstrated that certain fluorescent compounds were excreted by citrus roots upon infection by *P. nicotianae* (Figures 13 – 17). A faint fluorescence was observed in the solutions in the eppendorf tubes with inoculated root pieces from 6 hours onwards, after which it rapidly increased. Similarly, El Modafar *et al*. (1995) showed that phytoalexins were excreted from *Platanus* leaves into tiny droplets of conidial suspension of *Ceratocystis fimbriata* f. sp. *platani*. In their study, a faint fluorescence was observed after 4 hours that rapidly increased to reach a peak at 48 hours. Various unknown fluorescent compounds, but no scoparone were however excreted during our study by any of the rootstocks.

The only time scoparone was detected in the current study was in the roots of citrus rootstocks that were planted in the soil and inoculated with *P. nicotianae* using the millet seed inoculation method. Pathogen or host biotic elicitors like polysaccharides, oligosaccharide fragments, proteins, glycoproteins, and fatty acids can induce defence responses (Dixon & Lamb, 1990). Carbohydrate elicitors from the mycelial walls of *P. megasperma* f.sp *glycinea* are released by soybean β-1,3-endoglucanase. These carbohydrates are potent elicitors that induce phytoalexin (glyceollin) accumulation in infected soybean tissues (Yoshikawa, 1995). Oligosaccharides released from the plant cell walls of *Citrus limon* seedlings by treatment with endo-polygalacturonase, induce the accumulation of phytoalexins in intact lemon seedlings (Roco, 1993). *P. cryptogea* and *P. capsici* produce two amino-acid peptides called cryptogein and capsicein respectively. Application of these peptides can induce defence responses and protect tobacco against *P. nicotianae*, the agent of tobacco black shank, which is unable to produce such an elicitor (Ricci *et al*, 1989). Possibly a specific elicitor is necessary for scoparone excretion or accumulation in citrus roots. This elicitor might therefore only be present during the interaction of citrus roots with mycelium of *P. nicotianae* and not when infection is predominantly caused by zoospores. Furthermore, a slow chronic infection scenario might be necessary for scoparone induction as apposed to an acute, rapid infection situation.

Ochocka *et al*. (1995) found that coumarins are distributed differently, both quantitatively and qualitatively, in the roots and aerial parts of *Chrysanthemum segetum* L. This is in accordance with our findings that scoparone accumulation patterns in the roots of citrus, are different from those previously reported by Afek and Sztejnberg (1988).

A distinctive yellow fluorescent compound (U82) has been discovered that correlates well with resistance of citrus rootstocks to *P. nicotianae* (Table 1). If unknown compound U82 is a viable marker for resistance it will certainly be a breakthrough in rootstock resistance research, especially since it is constitutively produced and does not need induction. Such a unique compound that is only associated with tolerant rootstocks could potentially be used in developing a high throughput screening technique for citrus rootstock resistance.

In the current study, the R_F value of scoparone has experimentally been determined as 0.34 and the hR_F are thus 34. (R_F = distance travelled by solute "x" / distance travelled by the solvent front; $hR_F = R_F \times 100$). Ibrahim and Barron (1989) reported an hR_F value of 31 for scoparone. Due to experimental variation for instance usage of different solvent systems, there is usually a small difference in the R_F - values reported in the literature. A few other examples of coumarins are herniarin (7-methoxycoumarin) that has an hR_F -value of 54, umbelliferone (7-hydroxycoumarin) hR_F = 20, scopoletin (7-hydroxy-6-methoxycoumarin) hR_F = 12, coumarin hR_F = 40, daphnetin (7,8-dihydroxycoumarin) hR_F = 42, and aesculetin (6,7-dihydroxycoumarin) with an hR_F–value of 20 (Wagner *et al*., 1983; Ibrahim & Barron, 1989). Some of these R_F – values corresponds with the unknown compounds in the TLC analysis. It is highly likely that the unknown compounds are structurally related to scoparone and that they probably play a role in host defence, but further investigation of these compounds is necessary.

In conclusion, direct fungicidal activity of scoparone was confirmed against *P. nicotianae*, by means of *in vitro* testing. In the time course study, scoparone accumulated in all of the roots of the different rootstocks that were planted in soil inoculated with *P. nicotianae*, regardless of their degree of tolerance towards *P. nicotianae*. No consistent differences in scoparone

accumulation patterns could be observed between the different rootstocks. Inoculation of citrus roots by means of zoospore suspensions of *P. nicotianae* in a hydroponic system proved to be too severe. No scoparone accumulation was detected in this type of system. Chemical treatment with fosetyl-Al did reduce the amount of root rot caused by *P. nicotianae*, but no increase was noticed on scoparone production. A novel technique was established for the detection of fluorescent compounds that have been excreted from citrus roots upon infection by *P. nicotianae*. This method is rapid and easy in comparison to conventional techniques for the extraction of coumarins. It can therefore be used as a high throughput analytical tool in the analysis of excreted compounds from citrus roots.

The essence of the effectiveness of phytoalexins as disease defence compounds in plants is the timing and intensity of their synthesis and accumulation (Kuc, 1995). Host resistance is therefore related to the time it takes to reach 100% fungicidal concentrations at an infection site. No such accumulation patterns of scoparone were observed in the roots of citrus rootstocks in the present study. Our results therefore show that scoparone, which dictates resistance against *P. citrophthora* collar rot and gummosis, is not the primary agent involved in the mechanism of resistance in the roots against *P. nicotianae*. Full knowledge of host-parasite interactions is necessary for understanding of all possible host-pathogen interactions and results. Further studies are therefore necessary to determine the structure, identity and possible antifungal activity of the other unknown compounds from citrus root extracts, especially compound U82.

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Figure 3. Effect of scoparone on mycelial growth of *Phytophthora nicotianae* on potato-dextrose-agar - assessed by means of the standard agar amendment method.

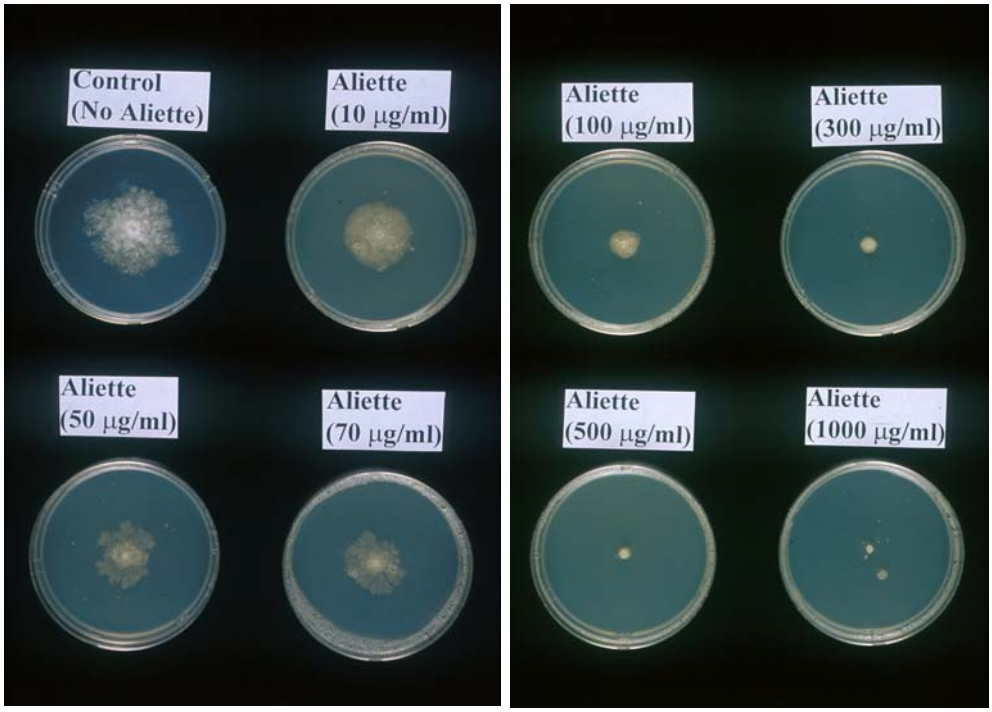

Figure 4. Effect of fosetyl-Al (Aliette) on mycelial growth of *Phytophthora nicotianae* on potato-dextrose-agar - assessed by means of the standard agar amendment method.

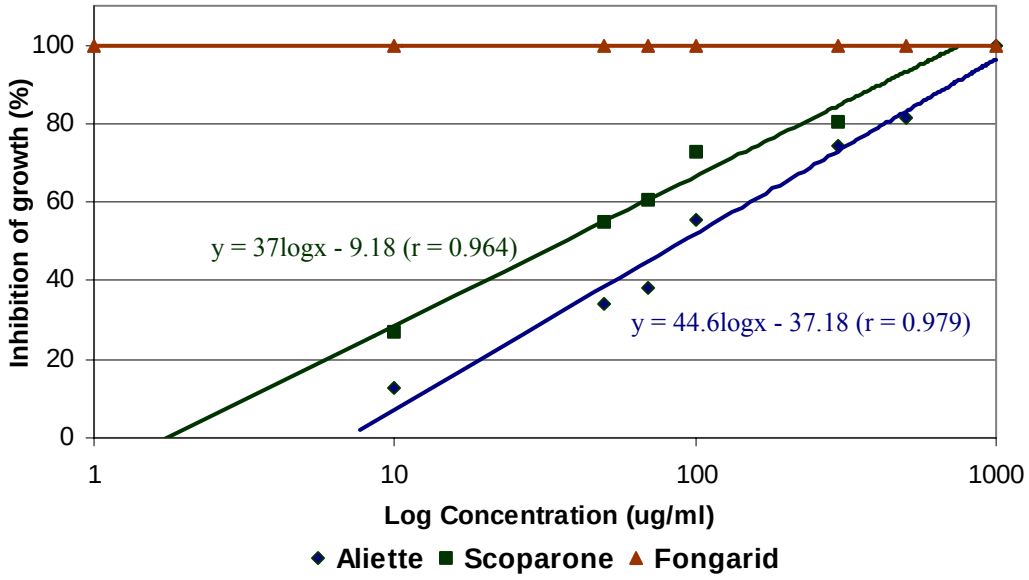

Figure 5. Effect of scoparone, fosetyl-Al (Aliette) and furalaxyl (Fongarid) on i*n vitro* growth of *Phytophthora nicotianae* - dose response expressed as a linear regression.

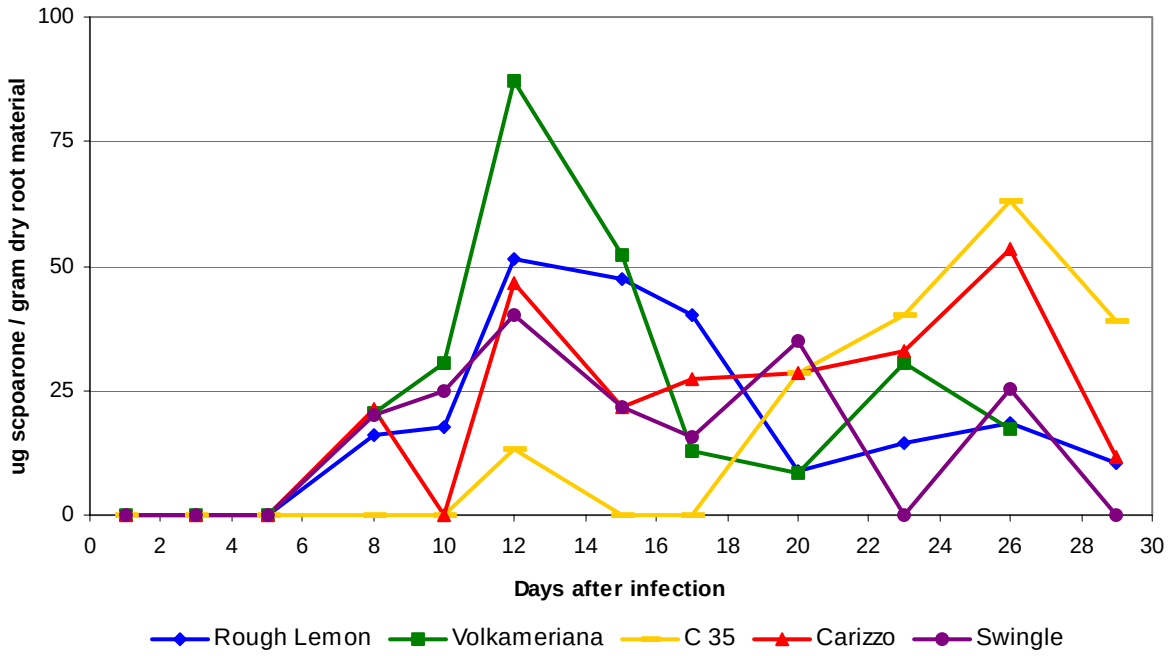

Figure 6. Scoparone levels in the roots of citrus rootstocks following inoculation with *Phytophthora nicotianae*.

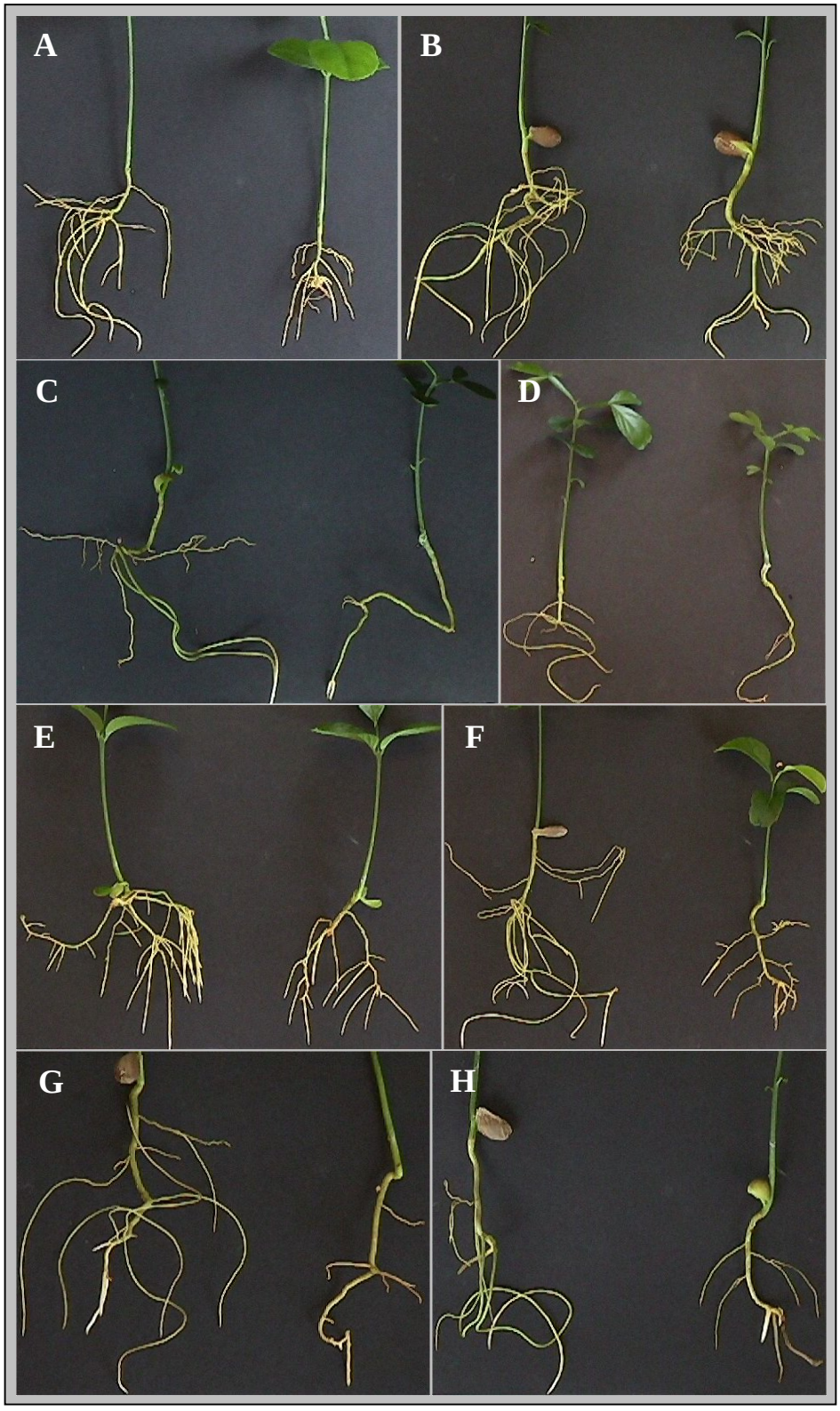

Figure 7. Effect of *Phytophthora nicotianae* infection on the roots of Rough lemon (A), Swingle (B), Troyer (C), Macrophylla (D), Sour orange (E), Volckameriana (F), C35 (G) and Carrizo (H) rootstocks in a aqua culture system, 14 days following inoculation with a zoospore suspension. Left = Uninfected control, Right = Infected.

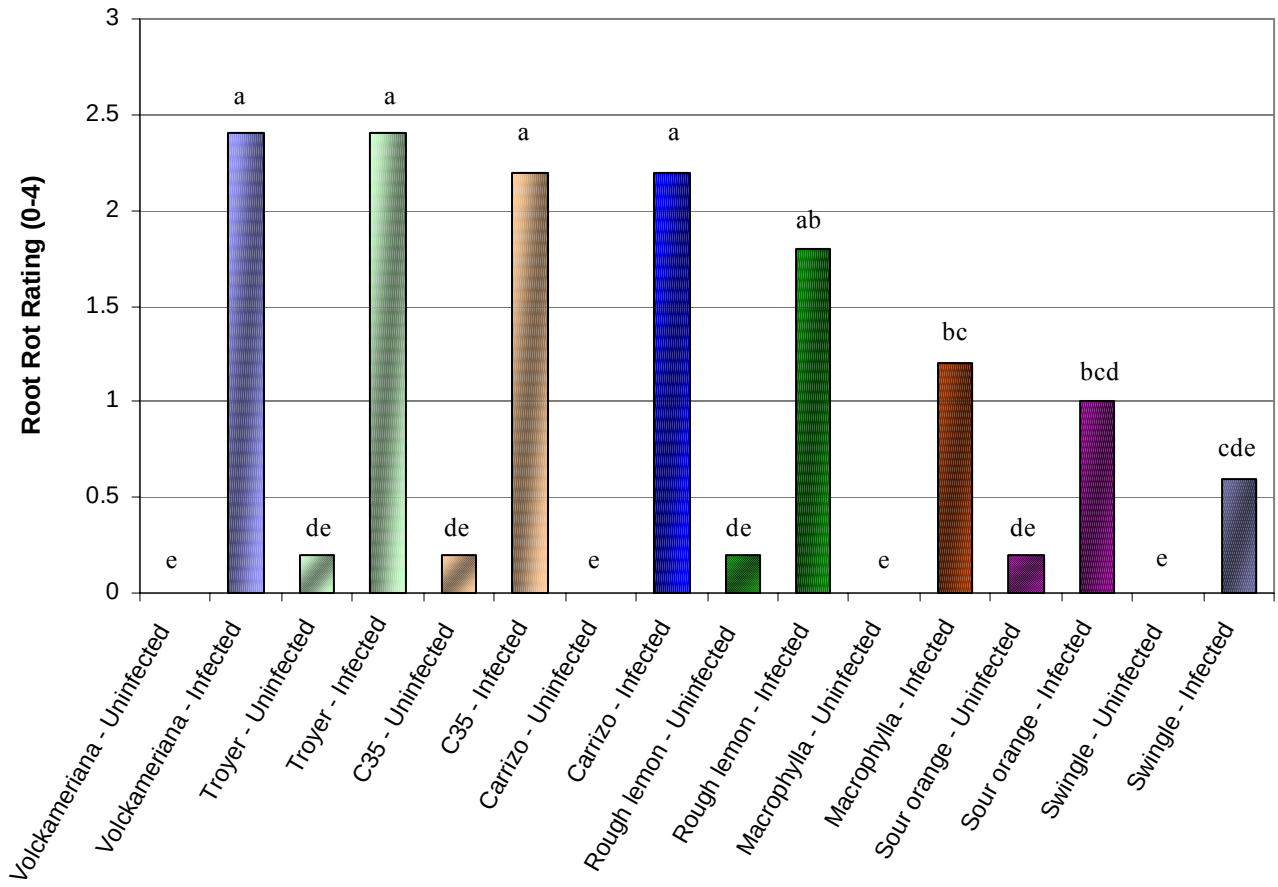

Figure 8. Root rot severity in citrus rootstocks 14 days following inoculation with *Phytophthora nicotianae* in a water culture system (aqua cultures in small 250 ml plastic containers).

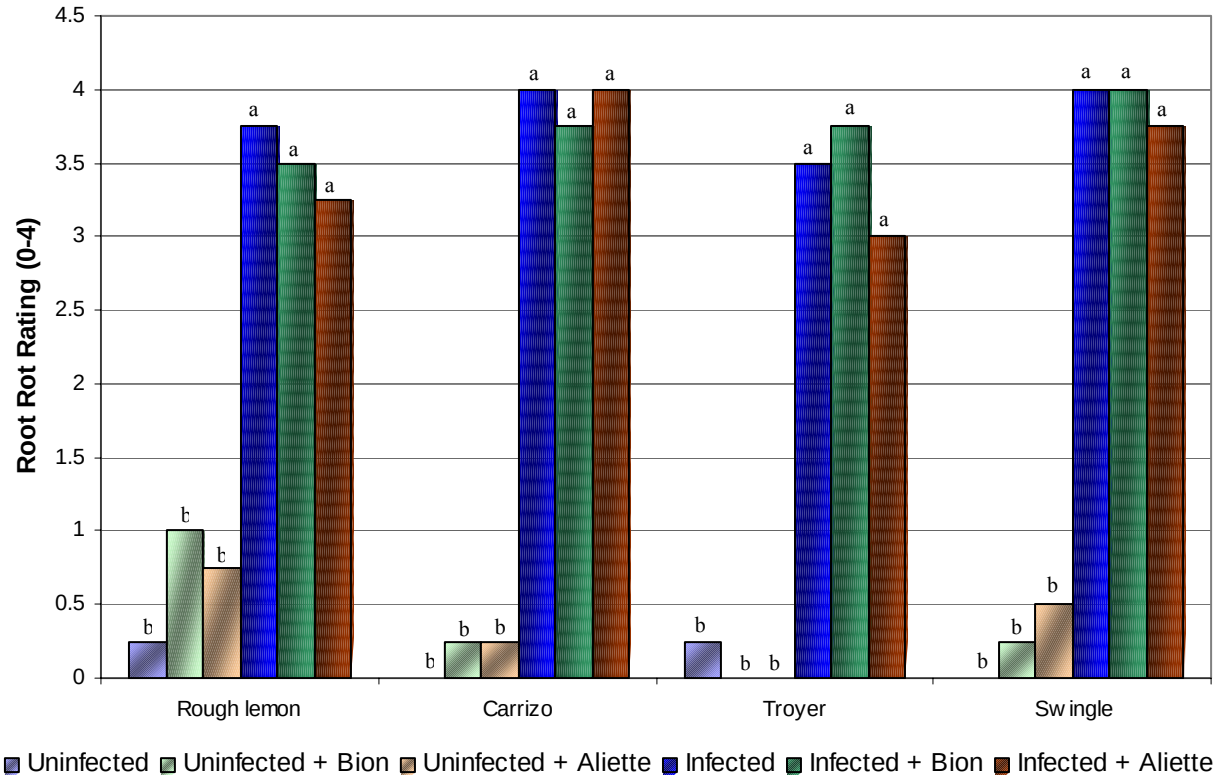

Figure 9. Effect of fungicide treatments on root rot severity in citrus rootstocks, 6 days following inoculation with a zoospore suspension of *Phytophthora nicotianae* in a hydroponic system (first hydroponic experiment in 10 L containers).

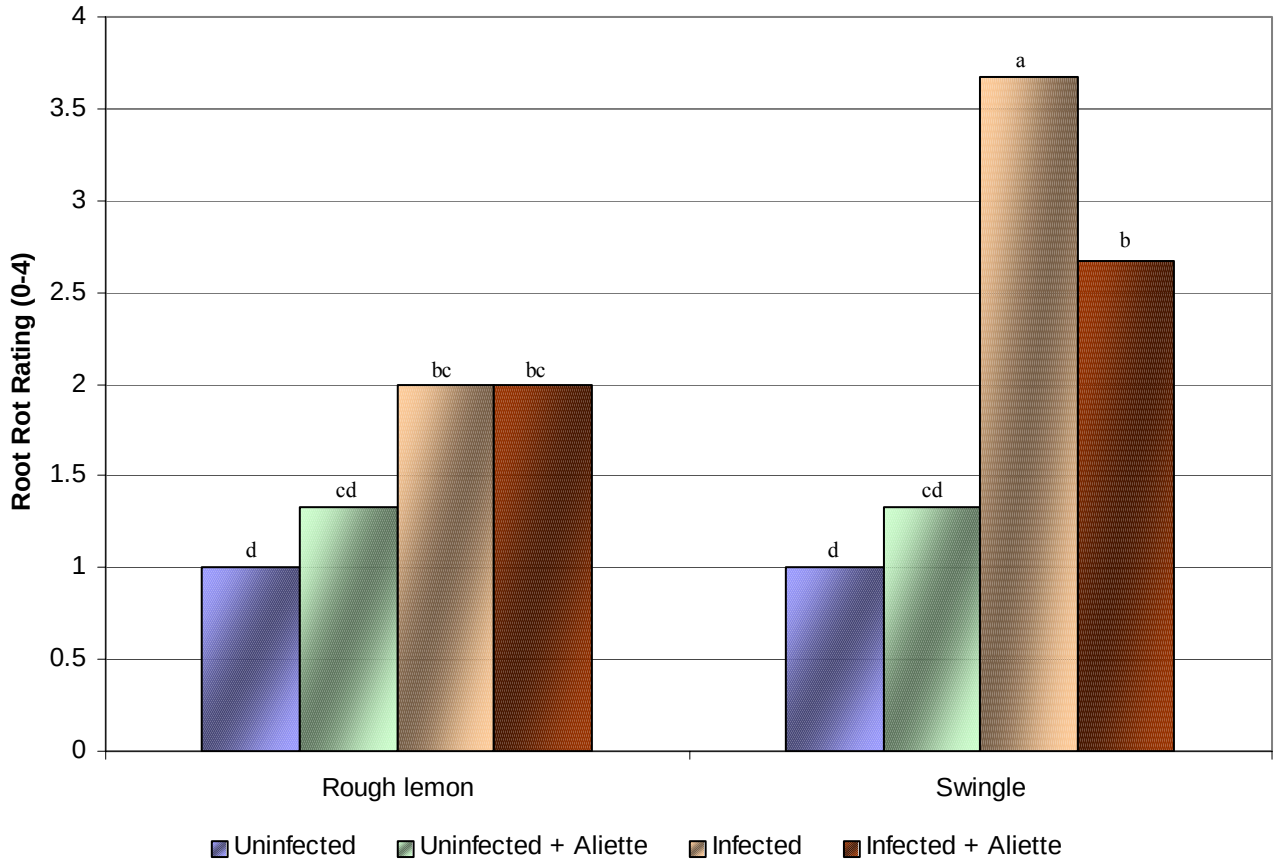

Figure 10. Effect of fungicide treatments on root rot severity of Rough lemon and Swingle rootstocks, 12 days following inoculation with a zoospore suspension of *Phytophthora nicotianae* in a hydroponic system (second hydroponic experiment in 10 L containers).

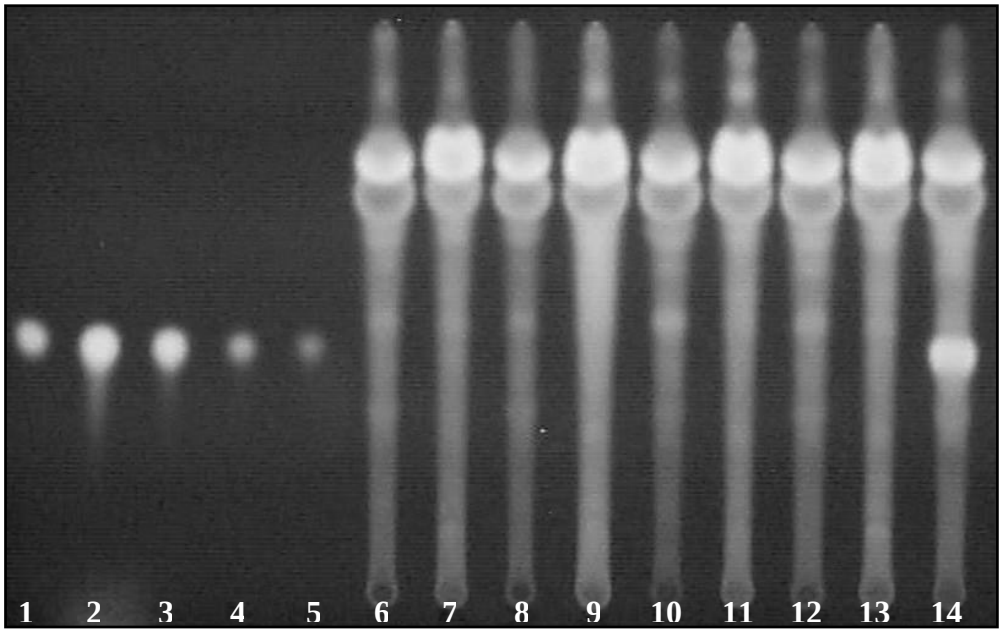

Figure 11. TLC analysis of citrus root extracts 1 day after inoculation of 6-month old seedlings with *P. nicotianae* in a hydroponic system (second hydroponic experiment in 10 L containers). Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: Rough lemon Uninfected; lane 7: Swingle Uninfected; lane 8: Rough lemon Uninfected + Aliette; lane 9: Swingle Uninfected + Aliette; lane 10: Rough lemon Infected; lane 11: Swingle Infected; lane 12: Rough lemon Infected + Aliette; lane 13: Swingle Infected + Aliette; lane 14: Rough lemon spiked with scoparone 200 µg/ml.

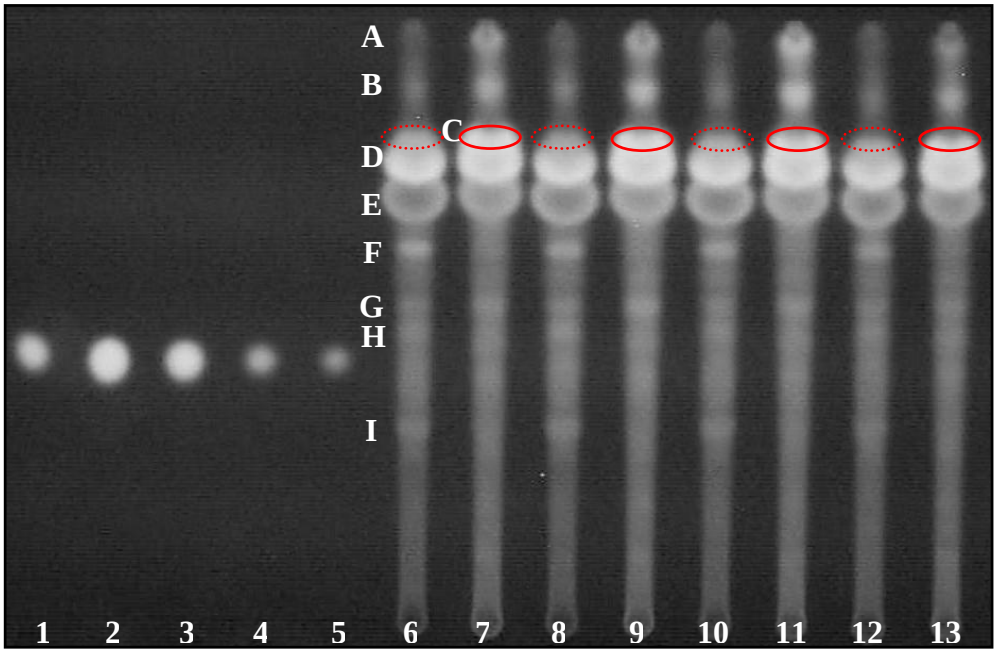

Figure 12. TLC analysis of citrus root extracts 8 days after inoculation of 6-month old seedlings with *P. nicotianae* in a hydroponic system (second hydroponic experiment in 10 L containers). Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: Rough lemon Uninfected; lane 7: Swingle Uninfected; lane 8: Rough lemon Uninfected + Aliette; lane 9: Swingle Uninfected + Aliette; lane 10: Rough lemon Infected; lane 11: Swingle Infected; lane 12: Rough lemon Infected + Aliette; lane 13: Swingle Infected + Aliette; A – I: unknown fluorescent compounds; C: unknown yellow fluorescing compound U82.

Table 1. TLC separation and subsequent quantification of unknown phenolic compound U82, occurring in uninduced citrus rootstocks.

Rootstock	U82 spot length* (mm)	Duncan grouping
Sour orange	4.3	a
Swingle	4.1	a
Troyer	1.42	b
Macrophylla	1.28	bc
Carrizo	1	cd
C35	0.72	d
Volckameriana	0	e
Rough lemon	0	e

* Length of U82 spot on TLC chromatograms was measured. Values are the means of combined averages of four replications. Values followed by the same letter do not differ significantly according to Duncan's multiple range test (P=0.05).

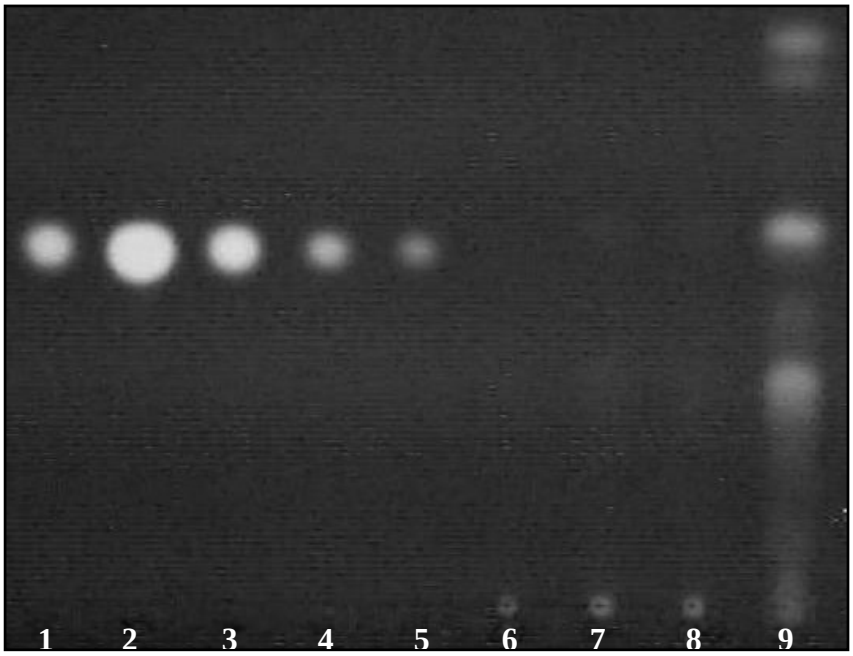

Figure 13. TLC analysis of excreted fluorescent compounds from the excised root pieces of Swingle seedlings, 4 days after exposure to a zoospore suspension of *Phytophthora nicotianae*. Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: uninfected roots (aqueous phase); lane 7: uninfected (organic phase); lane 8: infected roots (aqueous phase); and lane 9: infected roots (organic phase).

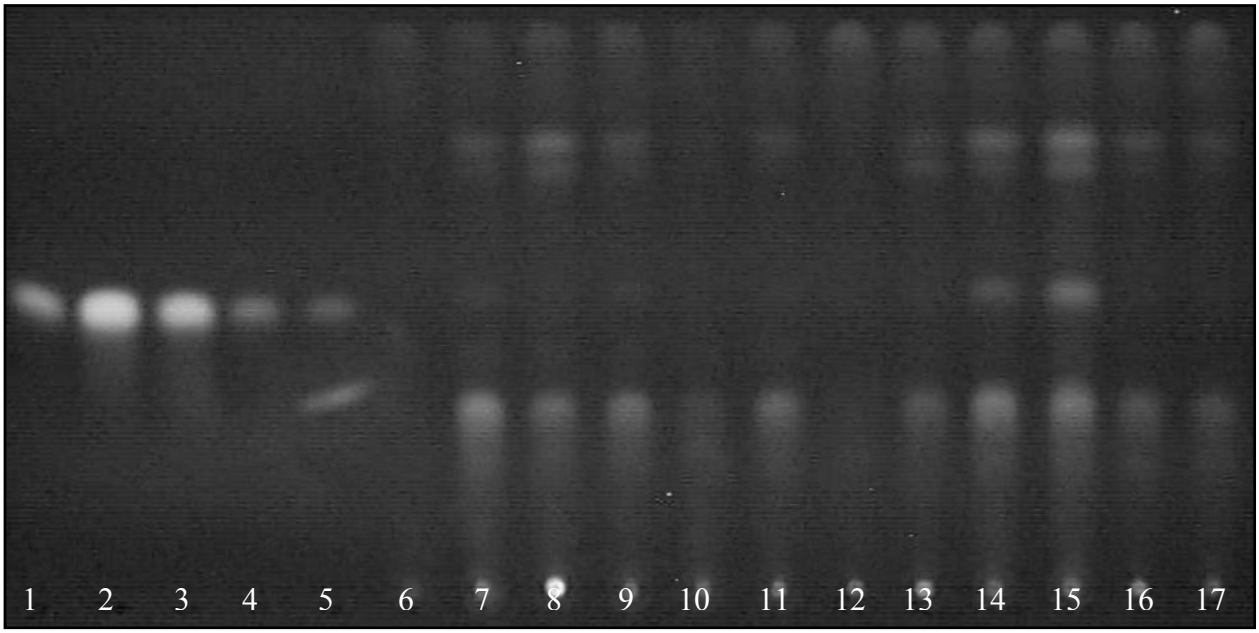

Figure 14. TLC analysis of excreted fluorescent compounds from the excised root pieces of citrus seedlings, 4 days after exposure to a zoospore suspension of *Phytophthora nicotianae*. Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: uninfected roots of *Rough lemon* rootstock (organic phase); lane 7-11: infected roots of *Rough lemon* (organic phase); lane 12: uninfected roots of *Swingle* rootstock; lane 13-17: infected roots of *Swingle* rootstock.

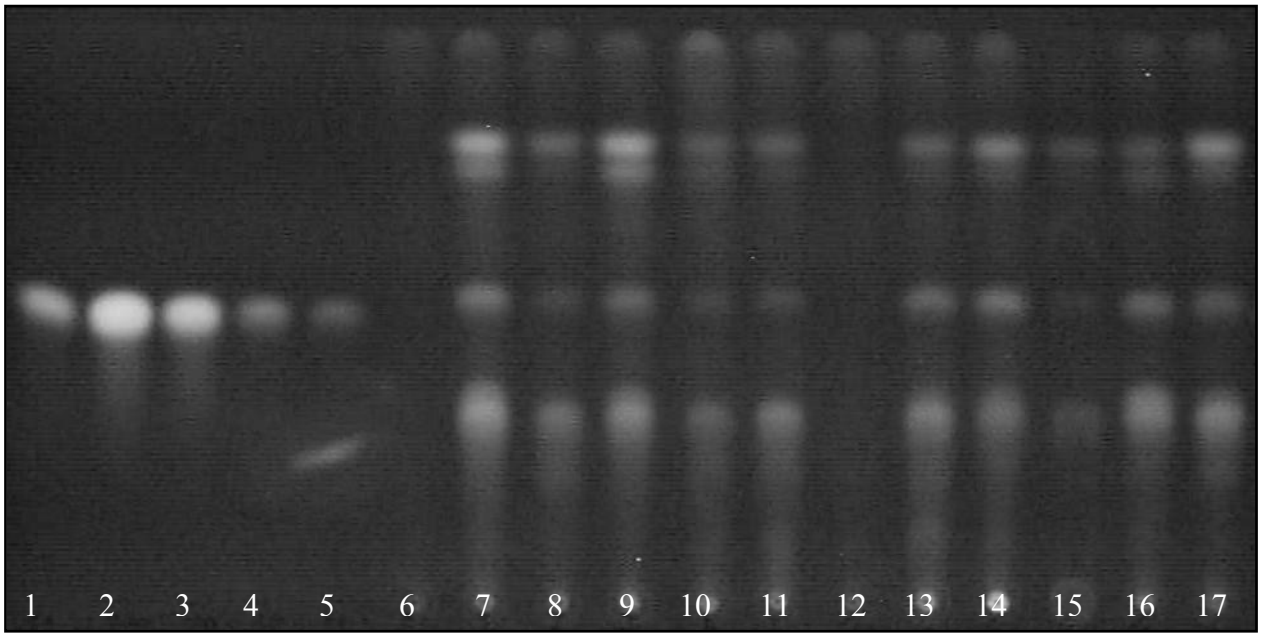

Figure 15. TLC analysis of excreted fluorescent compounds from the excised root pieces of citrus seedlings, 4 days after exposure to a zoospore suspension of *Phytophthora nicotianae*. Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: uninfected roots of *Troyer* rootstock; lane 7-11: infected roots of *Troyer*; lane 12: uninfected roots of *Carrizo* rootstock; lane 13-17: infected roots of *Carrizo* rootstock.

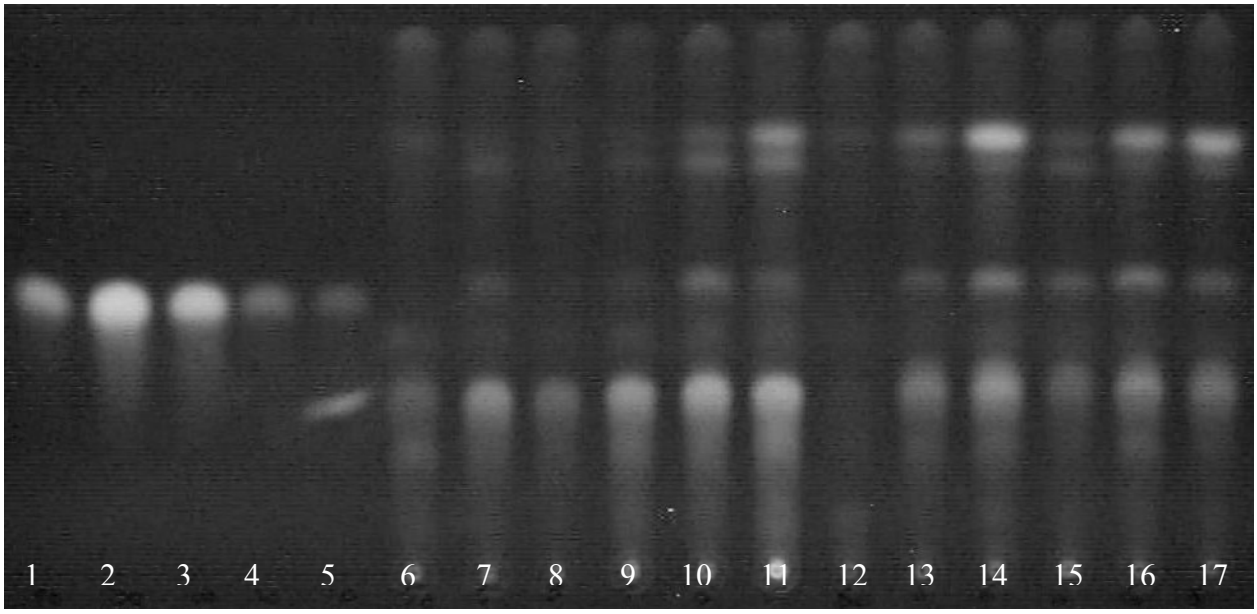

Figure 16. TLC analysis of excreted fluorescent compounds from the excised root pieces of citrus seedlings, 4 days after exposure to a zoospore suspension of *Phytophthora nicotianae*. Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: uninfected roots of *Sour orange* rootstock; lane 7-11: infected roots of *Sour orange*; lane 12: uninfected roots of *Volckameriana* rootstock; lane 13-17: infected roots of *Volckameriana* rootstock

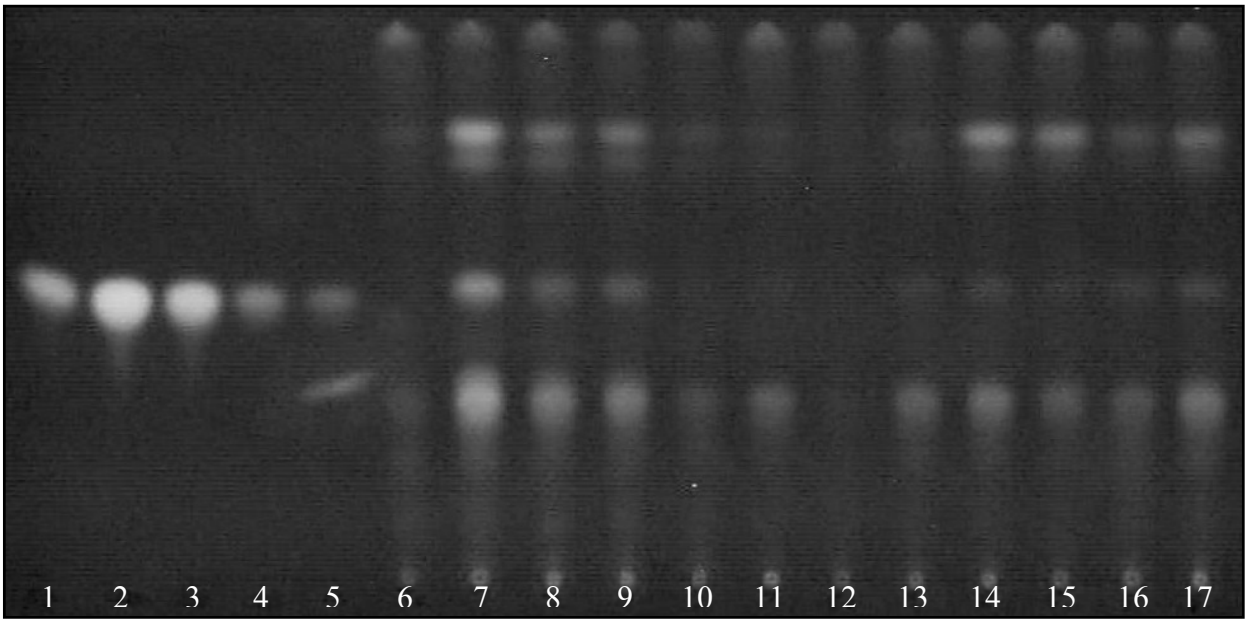

Figure 17. TLC analysis of excreted fluorescent compounds from the excised root pieces of citrus seedlings, 4 days after exposure to a zoospore suspension of *Phytophthora nicotianae*. Lane 1: scoparone standard 50 µg/ml; lane 2: scoparone 200 µg/ml; lane 3: scoparone 100 µg/ml; lane 4: scoparone 20 µg/ml; lane 5: scoparone 10 µg/ml; lane 6: uninfected roots of C35 rootstock; lane 7-11: infected roots of *C35* lane 12: uninfected roots of *Macrophylla* rootstock; lane 13-17: infected roots of *Macrophylla* rootstock.

Chapter 5

GENERAL DISCUSSION

The viability of the citrus industry is to a great extent dependant on the rootstocks in use (Castle, 1987). The systemic fungicides metalaxyl and fosetyl-Al have been effectively used in citrus to control *Phytophthora* diseases, but the excessive dependence on the use of metalaxyl has resulted in the development of resistant *Phytophthora* isolates (Timmer *et al*., 1998). Rootstocks therefore must have a high degree of resistance or tolerance to root and collar rots. Although the use of resistant rootstocks is a good approach for the control of *Phytophthora* diseases, some resistant rootstocks might still be susceptible to other diseases or they might not be suited for specific horticultural conditions.

In the search for alternative rootstocks, it is therefore necessary to develop screening techniques that are rapid and reliable. To identify possible markers for resistance, the objective of the present study was to determine the biochemical mechanisms involved that governs resistance of citrus rootstocks against *P. nicotianae* root rot.

Noteworthy findings that emerged from the current investigation are the following:

1. The Folin-Ciocalteu procedure, which is generally used for the quantification of total phenolic compounds from plant extracts (Bray & Thorpe, 1954; Julkunen-Tiitto, 1985), was used in our study but the reaction mixture was scaled down to enable the use of 96-well ELISA-plates. This modification made the procedure much quicker than the conventional test tube technique, more cost-effective due to smaller reaction volumes and yet sensitive and reproducible for the determination of total phenolic concentration. It is

therefore particularly valuable when screening a large number of samples or during time-course studies of phenolic production

2. The levels of total soluble phenolics were determined in citrus rootstocks tolerant and susceptible to *P. nicotianae* following inoculation with the pathogen. Total phenolic concentrations correlated well with tolerance or susceptibility, which provides evidence that the increase in phenolic concentrations in citrus roots plays a key role in tolerance of citrus rootstocks against *P. nicotianae* root rot.
3. Treatment with the systemic fungicide fosetyl-Al further elevated total phenolic levels in the roots of citrus rootstocks. This indicates that elevation of total phenolics play a role in the mode of action of this fungicide, therefore suggesting an indirect mode of action.
4. Techniques for the detection of the phytoalexin scoparone in citrus roots were evaluated. A new Micellar Electrokinetic chromatography (MEKC) method was developed to determine the amount of scoparone in citrus roots (Aucamp *et al*., 2000). MEKC was found to be very sensitive and selective. Very low levels of compounds can be detected and it can thus be used to analyse complex root extract mixtures.
5. Scoparone accumulation was examined in the roots of citrus rootstocks, with the aim of using scoparone as an indicator of potential resistance, during the rapid screening of citrus rootstocks for resistance against *P. nicotianae*. Scoparone was found in low concentrations in the roots of citrus rootstocks that were planted in soil inoculated with *P. nicotianae*. No correlation between the accumulation of scoparone and citrus root rot resistance could be demonstrated. Our results therefore show that scoparone, which dictates resistance against *P. citrophthora* collar rot and gummosis, is not the primary agent involved in the mechanism of resistance of citrus roots against *P. nicotianae*.
6. TLC-analysis of citrus root extracts revealed a number of other unidentified fluorescent compounds. A distinct yellow fluorescent compound, provisionally labelled U82, was

detected in tolerant Swingle, Sour orange, Troyer and Macrophylla rootstocks. This compound was absent in susceptible Rough lemon and Volckameriana rootstocks. Such a unique compound that is only associated with tolerant rootstocks could potentially be used in developing a high throughput screening technique for citrus rootstock resistance.

Graham (1995) demonstrated that tolerance to *P. nicotianae* root rot might be due to the greater ability of tolerant citrus rootstocks to regenerate roots under certain environmental conditions. There were however rootstocks such as Trifoliate orange that was tolerant, but it did not have a strong root regeneration capability, therefore suggesting a biochemical mechanism of resistance. Pathogen or host biotic elicitors such as polysaccharides, oligosaccharide fragments, proteins, glycoproteins and fatty acids can induce plant defence responses (Dixon & Lamb, 1990). Roco *et al*. (1993) showed that oligosaccharides released from the plant cell walls of *Citrus limon* seedlings by treatment with endo-polygalacturonase, induce the accumulation of phytoalexins in intact lemon seedlings. Successful recognition of these elicitors by the host plants leads to the induction of a variety of defence responses, such as the accumulation of phenolic compounds (Dixon & Lamb, 1990). The production and role of phenolic compounds in the inducible defence mechanisms of plants have been the focus in many investigations (Ebel, 1986; Cahill & McComb, 1992; Kuc, 1995; Purkayastha, 1995; De Ascensao & Dubery, 2003).

In citrus, the production of induced antifungal phenolic compounds has been demonstrated in fruits (Rodov *et al*., 1994), peels (Dubery *et al*., 1999), leaves (Manthey *et al*., 2000) and in citrus roots (Sulistyowati, 1990). Up to now most investigations, concerning the biochemical mechanisms of citrus rootstock resistance against *Phytophthora*, were done by Afek *et al*. (1986; 1988; 1989; 1995). They implicated the phytoalexin scoparone in the resistance

mechanism active within the stem bark of citrus rootstocks against the collar rot fungus *P. citrophthora*. In the present study, scoparone accumulation in the roots as apposed to the stems of citrus rootstocks could not be correlated with tolerance towards *P. nicotianae*. The observed increase in total soluble phenolic concentrations in the roots of citrus rootstocks upon *P. nicotianae* infection, as well as the distinctive accumulation of specific phenolic compounds in tolerant rootstocks, however suggests a biochemical mechanism of resistance.

The mechanisms of resistance of different citrus tissues to different *Phytophthora* species are not fully elucidated. Because of the great variety in the type of tissue affected and the varied response of specific citrus species to infection, there is probably more than one resistance or tolerance mechanism involved. Further studies are therefore necessary to determine the structure, identity and possible antifungal activity of the other unknown compounds from citrus root extracts, especially compound U82.

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APPENDIX

Table 1. A summary of all the citrus rootstocks used during the present study.

CULTIVAR	GENUS AND SPECIES
Rough lemon	*Citrus jambhiri* Lush.
Volkamer lemon	*C. volkameriana* Teng. & Pasq.
Carrizo	*C. sinensis* X *Poncirus trifoliata*
C 35	*C. sinensis* X *P. trifoliata*
Troyer	*C. sinensis* X *P. trifoliata*
Macrophylla	*C. macrophylla* Webster
Sour orange	*C. aurantium* L.
Swingle	*C. paradisi* X *P. trifoliata*

BIOCHEMICAL MECHANISMS FOR TOLERANCE OF CITRUS ROOTSTOCKS AGAINST *PHYTOPHTHORA NICOTIANAE*

by

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RESUMÉ

It was established that although the phytoalexin scoparone is associated with resistance/tolerance of citrus rootstocks to stem cancer caused by *Phytophthora citrophthora*, it does not dictate tolerance to root rot caused by *P. nicotianae*. It can therefore not be used as an indicator for tolerance to root rot. Results of the current study show that increase in total soluble phenolic concentrations in citrus roots after inoculation plays a key role in tolerance of citrus rootstocks against *P. nicotianae* root rot. Elevation of the levels of total phenolic compounds is therefore a part of the mechanism involved in rootstock resistance. As far as could be established, this finding has not been reported before in citrus. Determination of total soluble phenolics in citrus roots can therefore be used as a parameter in the screening of rootstocks for tolerance to *P. nicotianae*.

Application of the systemic fungicide fosetyl-Al increased the total soluble phenolic concentrations in the roots more than infection with the pathogen alone. This provides evidence that the elevation of phenolic levels is involved in the mechanism of action of fosetyl-Al in control of *Phytophthora* root rot, therefore supporting an indirect antifungal mode of action.

A unique chemical compound (U82) has been discovered that is associated only with tolerant rootstocks. If this yellow compound is a viable marker for resistance, it will certainly be a breakthrough in rootstock resistance research. Such a unique compound that is only associated with tolerant rootstocks could potentially be used in developing a more reliable high throughput screening technique for citrus rootstock resistance.

BIOCHEMIESE MEGANISMES VIR WEERSTANDBIEDENDHEID VAN SITRUSONDERSTAMME TEENOOR *PHYTOPHTHORA NICOTIANAE*

deur

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SAMEVATTING

Dit is vasgestel dat die fitoaleksien scoparone wat geassosieer word met weerstandbiedendheid van sitrus onderstamme teen *Phytophthora citrophthora* stam kanker, nie verantwoordelik is vir weerstand teen *P. nicotianae* wortelverotting nie. Dit kan dus nie as ‘n merker vir weerstandbiedendheid teen wortelverotting gebruik word nie. Die resultate van die huidige studie toon aan dat die verhoging in die konsentrasie totale oplosbare fenoliese verbindings in sitrus wortels ‘n rol speel in weerstand en ten minste deel uitmaak van die meganisme van weerstand van sitrus onderstamme teen *P. nicotianae* wortelverotting. Sover vasgestel kon word, is hierdie bevinding nog nie voorheen in sitrus gerapporteer nie. Die konsentrasie totale fenoliese verbindings in sitrus wortels kan dus gebruik word as ‘n merker in die evaluering van onderstamme vir *P. nicotianae* weerstand.

Behandeling met die sistemiese swamdoder fosetyl-Al het die konsentrasies totale fenoliese verbindings in die wortels verder verhoog. Dit verskaf verdere bewys dat die verhoging in totale fenoliese verbindings betrokke is in die meganisme van aksie van fosetyl-Al in die beheer van *Phytophthora* wortelverotting en dui dus op ‘n indirekte meganisme van aksie.

‘n Unieke verbinding (U82) is ontdek wat alleenlik teenwoordig is in weerstandbiedende onderstamme. As die verbinding ‘n werkbare merker vir weerstand is, sal dit beslis ‘n deurbraak wees in navorsing oor sitrus weerstand. So ‘n unieke verbinding, wat met weerstandbiedende onderstamme alleenlik geassosieer word, kan moontlik gebruik word vir ontwikkeling van ‘n vinnige siftingstegniek vir evaluering van sitrus onderstamme ten opsigte van weerstand.