Inhibition of Phytopathogenic Fungi on Selected Vegetable Crops by Catechins, Caffeine, Theanine and Extracts of *Camellia sinensis* (L.) O. Kuntze

by

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DECLARATION

I, the undersigned, declare that the work contained in this dissertation is my own original work and that it has not previously, in its entirety or part, submitted for a degree to any other university.

Michelle Wilmot

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CHAPTER 1

General Introduction

Camellia sinensis as an Alternative Anti-microbial Compound in Plant Disease Management

INTRODUCTION

Plant pathogens are estimated to cause yield reductions of almost 20% in the principal food and cash crops worldwide (Oerke *et al*, 1994). Although these losses may be minimized by the use of disease-tolerant cultivars, crop rotation, or sanitation practices, fungicides are often essential to maximize crop yields. Fungicides also present substantial benefits on food quality. First, they contribute to food safety by controlling many of the fungi that produce mycotoxins. Nearly one quarter of food crops worldwide are affected by mycotoxins such as aflatoxins, ergot toxins, *Fusarium* toxins, patulin, and tenuazonic acid (Pohland, 1993; Schneider and Dickert, 1994). Second, plant pathogenic fungi must be controlled if consumer demand in developed countries for premium quality, diverse foods is to be met; while high-quality fruit and vegetables are an indicator of economic growth in developing countries (Mitchell, 1993).

Disease control using chemicals began during the 1850s when Bordeaux mixture was introduced to control downy mildew ((*Plasmopara viticola* (Berk. and M. A. Curtis) Berl. and De Toni) in French vineyards (Brent, 1985). Paris green (copper acetoarsenite), for instance, originally used in house paint as a fungicide, and was accidentally found to control leaf-eating insects. In the first half of this century, protection of many crops became possible with the introduction of organic fungicides. In the 1960s, the development of systemic products able to penetrate plant tissue and deliver curative properties permitted a more flexible application regime (Brent, 1985; Lyr, 1995). Despite the choice of effective fungicides available, new anti-fungal chemicals are still needed to deliver improved yield and quality benefits.

An important motivation for maintaining research programs is the growing demand by the public for crop protection agents with low use rates, a benign environmental profile, and low toxicity to humans and wildlife. For these reasons, various plant extracts have been screened *in vitro*. According to Abab (1996) the osmotic proteins in tobacco plants are able to inhibit spore germination of some phytopathogenic fungi. The anti-microbial activity of tea (Arora and Bharwaj, 1997; Arora and Ohlan, 1997) and tea polyphenols has also been demonstrated. The inhibitory activity of essential oils on fungi has been investigated (Manohar *et al*, 2001). Recently it has been shown that certain compounds in olives (Del Rio *et al*, 2003) namely: tyrosol, catechins and oleuropein have anti-microbial activity. Jasso de Rodríguez (2005) tested *Aloe vera* (L.) Miller pulp on the mycelium development of *Rhizoctonia solani* Kühn, *Fusarium oxysporum* Schlechtend, and *Colletotrichum coccodes* Berk and Broome.

We decided to expand on the earlier *in vitro* work of Arora (1997), testing *Camellia sinensis* (L.) O. Kuntze water extracts as well as some individual compounds in tea for possible anti-fungal activity *in vitro* and *in vivo*. The research was also encouraged by positive results obtained by farmer's claiming that Polyphenon G (a dried concentrated green tea extract) prevents bacterial infections on vegetable crops (Hara, Personal communications). We also aimed to identify the mode of action by which our treatment compounds control or alleviate the effect of plant pathogens with a specific focus on induced resistance.

CAMELLIA SINENSIS

DESCRIPTION AND ORIGIN

Tea is the common name for certain *Camellia* species belonging to a family consisting of mostly woody flowering plants. This family of plants is distributed throughout tropical and subtropical areas, but most species occur in eastern Asia and South America. *C. sinensis* is considered to be the most important plant in the Camellia genus, particularly from a commercial point of view, as it has become the second most popular beverage, after water, throughout the world (Wang *et al*, 2000). Tea was first discovered in China where it has been consumed for medicinal properties since 3000 BC (Balentine *et al*, 1997; Ferrara *et al*, 2001). The first exclusive book on tea, Ch'a Ching meaning 'Tea Classic' by the Chinese tea expert Lu Yu was published in AD 780 in which he has described various kinds of tea, their cultivation and manufacturing in China.

Tea may vary in flavour and characteristics according to the genotype, type of soil, altitude and climatic conditions of the area in which it is grown (Gramza and Korczak, 2005). Processing methods also affect the flavour and characteristics, as does the blending of different teas from different cultures and geographic regions. Three main categories of tea are produced from *C. sinensis*, namely green-, oolong- and black tea. These vary only in the processing, which results in differences in the chemical make up of the individual types of tea. The processing of green tea is based on the inactivation of enzymes in fresh leaves, either by firing or steaming, to prevent the enzymatic oxidation of catechins. In black tea the polyphenol oxidase catalysed oxidation of fresh leaf catechins is encouraged (Wang *et al*,

2000). Oolong tea is semi-fermented and the characteristics of this tea are between that of green and black tea.

CHEMISTRY

The beverage made from the leaves of the *C. sinensis* plant that is an aromatic stimulant, containing various polyphenols, essential oils such as caffeine (Figure 1.1), theobromine, and theophylline, the principal alkaloids, phenolic acids such as gallic acid and the characteristic amino acid, theanine (Figure 1.2) (Hara, 2001).

Polyphenols in green tea include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of the dry weight in young tea leaves. Most of the green tea polyphenols are flavonols, commonly known as catechins. As previously mentioned the processing of tea changes the tea chemistry, thus changing the polyphenolic component of the tea. The polyphenols that occur in tea are divided into two main groups namely the crude catechins and the crude theaflavins and thearubigins (Fukai *et al*, 1991; Harbowy *et al*, 1997), with the catechins predominantly occurring in green tea and the theaflavins and thearubigins predominantly occurring in black tea (Harbowy *et al*, 1997; Wang *et el*, 2000; Hara, 2001). The main catechins (Figure 1.3) in green tea are epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC) and epicatechin (EC) (Wang *et al*, 2000).

Herbal teas, which contain various known, or unknown components, are quite different from tea (*C. sinensis*). Rooibos (*Aspalatus linearis*, (L) Kuntze) tea is an herbal tea that was also included in our study because it is indigenous to South Africa and it is

considered to have medicinal properties. Rooibos tea contains no caffeine and very small quantities of flavonoids. Aspalathin is the main polyphenol in Rooibos (Gadow et al, 1997).

It is important to have an understanding of the chemical composition of different teas and the variance in composition of chemicals in the different teas to eventually understand the mechanisms by which microbial growth is inhibited.

MEDICINAL PROPERTIES

C. sinensis extracts as well as specific compounds in these extracts have well documented medicinal properties.

Green tea is known to be very rich in flouride. A study using natural toothpaste (containing green tea bioflavonoid/zinc ascorbate) was conducted to determine the effect on bacterial plaque accumulation. The results showed a significant decrease in total viable plaque biomass when compared with non-active control toothpaste (Wolinsky *et al*, 2000). Another study showed the epicatechins to have properties that prevent bacterial adherence to teeth, inhibit human and bacterial amylases and inhibit glucosyl transferase, thereby limiting the biosynthesis of sticky glucan (Wang, 2000).

Using different animal models, many laboratories have shown that green tea extract, taken orally or applied to the skin, inhibits skin tumour formation induced by chemical carcinogens or ultra-violet radiation (UVB). The extracts also possess anti-inflammatory activity (Kakegawa *et al*, 1985; Wagner, 1989) that, similarly to the anti-cancer activity (Wang *et al*, 1994), is owed to the polyphenolic constituents present therein. The polyphenol

mainly responsible for the prevention of cancer formation is EGCg. When applied to mouse skin, EGCg prevents UVB-induced oxidative stress. Mouse skin models have illustrated extensive beneficial effects of green tea extracts and although only a few human skin studies have been conducted, many cosmetic and pharmaceutical companies are supplementing their skin care products with green tea extracts (Katiyar and Elmets, 2001)

Tea has also shown to have anti-carcinogenic activity (Sakamoto, 2000; Lambert and Yang, 2003). A study (Nakachi *et al*, 2000) using 8552 residents, representative of Japan's population, tested whether or not green tea was an effective anti-carcinogenic. Results showed a decreased relative risk of cancer incidence for those consuming over ten cups, compared with those consuming below three cups of green tea per day. In addition, increased consumption was associated with a significant delay in the onset of cancer. The tumour incidence and average tumour yield in rats with chemically induced colon cancer were significantly reduced when the rats received (-)-epigallocatechin gallate, a major polyphenolic constituent of green tea (Kim *et al*, 1994). In a study conducted at the New Jersey Medical School, extracts of both black and green tea significantly inhibited leukaemia and liver tumour cells from synthesizing DNA (Lee *et al*, 1993).

C. sinensis extracts have also shown to have antioxidant activity which is known to boost the immune system (Serafini *et al*, 1996; Campanella *et al*, 2003). Some antioxidants are known to reverse endothelial dysfunction which could lead to the reduced risk of cardiovascular disease (Duffy *et al*, 2000)

Although tea has proven medical benefits there are also possible health risks. Caffeine can induce insomnia and nervousness in some individuals. The U.S. Food and Drug

Administration does not include caffeine on its "generally recognized as safe" (GRAS) list, but acknowledges no clear evidence of hazard at normal levels of use. Phenolic-rich tea extracts has been shown to reduce the ability of humans to utilise dietary iron. Thus excessive intake of tea should be avoided by individuals who are prone to anaemia and also by vegetarians (Samman *et al*, 2001).

ANTI-MICROBIAL PROPERTIES

Human and animal pathogens

Green and black teas have been reported to possess anti-fungal, antibacterial, and antiviral activity (Dreosti, 1996; Anna *et al*., 2003). Green tea extract have been shown to inhibit bacterial species such as *Escherichia coli* (IFO 3301), *Streptococcus mutans* (IFO 13955), *Helicobacter pylori* (ATCC 6919), *Propionibacter acnes* (ATCC 6919), *Bacillus staerothermophilus* (ATCC 12980) and *Staphylococcus aureus* (ATCC 25923) *in vitro* to name but a few (Yamamoto *et al*, 1997; Katsuhiro *et al*, 1999). Glucan synthesis by the bacterial glucosyltransferase is inhibited by ECg and EGCg (Kubo *et al*, 1992). These gallated catechins were also shown to interfere with the sucrose-dependent adherence of bacterial cells at much smaller concentrations than those which were needed to inhibit the growth of the bacteria (Muroi *et al*, 1993). *C. sinensis* extract have been shown to have some anti-fungal activity on human pathogens such as *Candida albicans* and other filamentous fungi (Yam *et al*, 1998). Catechins, caffeine and tannic acid have proven activity against viruses such as influenza virus (Nakayama *et al*, 1990), rotavirus (Yamamoto *et al*, 1997) and human immunodeficiency virus (Nakane and Ono, 1990).

Plant pathogens

Fukai, Ishigami and Hara (1991) tested the anti-bacterial activity of catechins (EC, EGC, ECg and EGCg) against phytopathogenic bacteria *in vitro*. The strains that were tested included *Erwinia* spp., *Pseudomonas* spp., *Clavibacter* spp., *Xanthomonas* spp. and *Agrobacterium* spp. *Erwinia* spp.was the most inhibited while *Clavibacter* spp.was the least inhibited of the bacteria. EGCg had the most anti-bacterial activity of the catechins tested. Black and green tea extract have also been shown to have activity against *Bacillus* spp., *Pseudomonas* spp. and *Xanthomonas* spp. *in vitro* (Arora and Bharwaj, 1997). Caffeine has also been documented as being insecticidal, larvicidal and inhibitory to moulds, yeast and bacteria (Nathanson, 1984; Pearson and Marth, 1990 and Aneja and Gianfagna, 2001). Anti-fungal activity of catechins against *Bipolaris carbonum* Nelson has also been reported (Chakraborty and Saha, 1994). El-Gammal *et al*. (1986) showed that the flavonols quercitin, kaempferol and myricetin had activity against gram-positive bacteria and phytopathogenic fungi in a screening test. Theaflavins, the oxidized products formed from tea leaf catechins during black tea fermentation, showed an anti-viral activity on tobacco mosaic virus (TMV) (Okada *et al*, 1977). Little work has been done on the effectiveness of *C. sinesis* extracts and some of the individual components thereof on phytopathogens. This has therefore been main focus of our study.

MECHANISMS WHICH CONTRIBUTES TO *CAMELLIA SINENSIS* ANTI-MICROBIAL ACTIVITY

The major anti-microbial activity of *C. sinensis* extracts has been contributed to caffeine and the catechins in these extracts. Caffeine is a known inhibitor of normal cell division in plant and animal cells that often results in binucleate cells (Aneja and Gianfagna, 2001), thus inhibiting spore germination and growth of microbes. Gallated catechins (EGCg and ECg) have been reported to deactivate proteins and disrupts the bacterial lipid bilayer by changing the membranes fluidity and morphology (Ikigai *et al*, 1993).

During the current study we aimed at identifying whether caffeine and a catechin rich *C. sinensis* extract (PolyphenonG) induces phytoalexins in tomato and lettuce plants. Induced resistance is the phenomenon that a plant, when given an appropriate stimulation by a harmless, inducer, will become able to combat more efficiently the infection by virulent pathogens through enhancement of its naturally inherent defence mechanisms. Efforts were concentrated on the identification of ferulic acid, caffeic acid in the lettuce and tomato root and shoot extracts, as these acids are known to be in these plants or reported to be induced as part of the plants natural defence mechanisms (Caspersen *et al*, 2000 and Romani *et al*, 2002).

CONCLUSIONS

Previous studies demonstrating some anti-microbial activity of *C. sinensis* extracts and some of the major compounds in these extracts provided the motivation for the current study, with the aim of investigating wider agricultural application of these natural products. Furthermore, understanding the underlying mode of action of these compounds will contribute towards improved application of these products. The aims of the current study was:

1. To test whether Polyphenon G (PPG), a concentrated extract from green tea (*C. sinesis*) and the individual compounds in PPG had activity against phytopathogenic fungi *in vitro* and *in vivo*.
2. To determine the mode of action of these compounds using specific biochemical methods aimed at identifying possible induced resistance.

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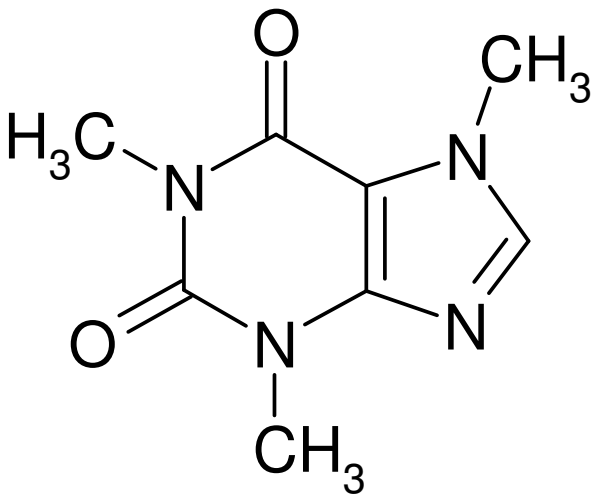

Figure 1.1

Chemical structure of caffeine

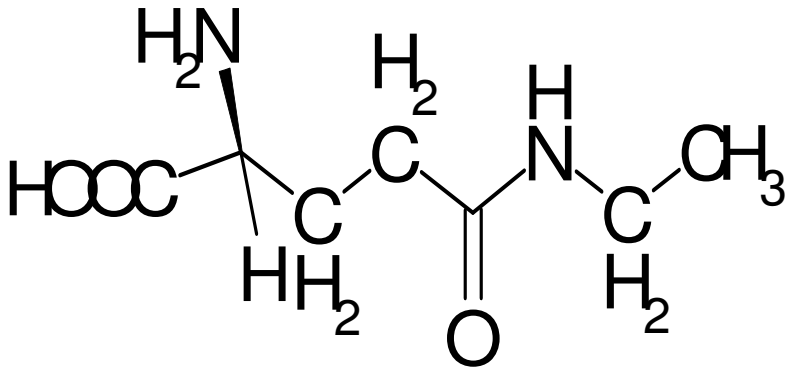

Figure 1.2

Chemical structure of theanine (γ-ethyl amine glutamic acid)

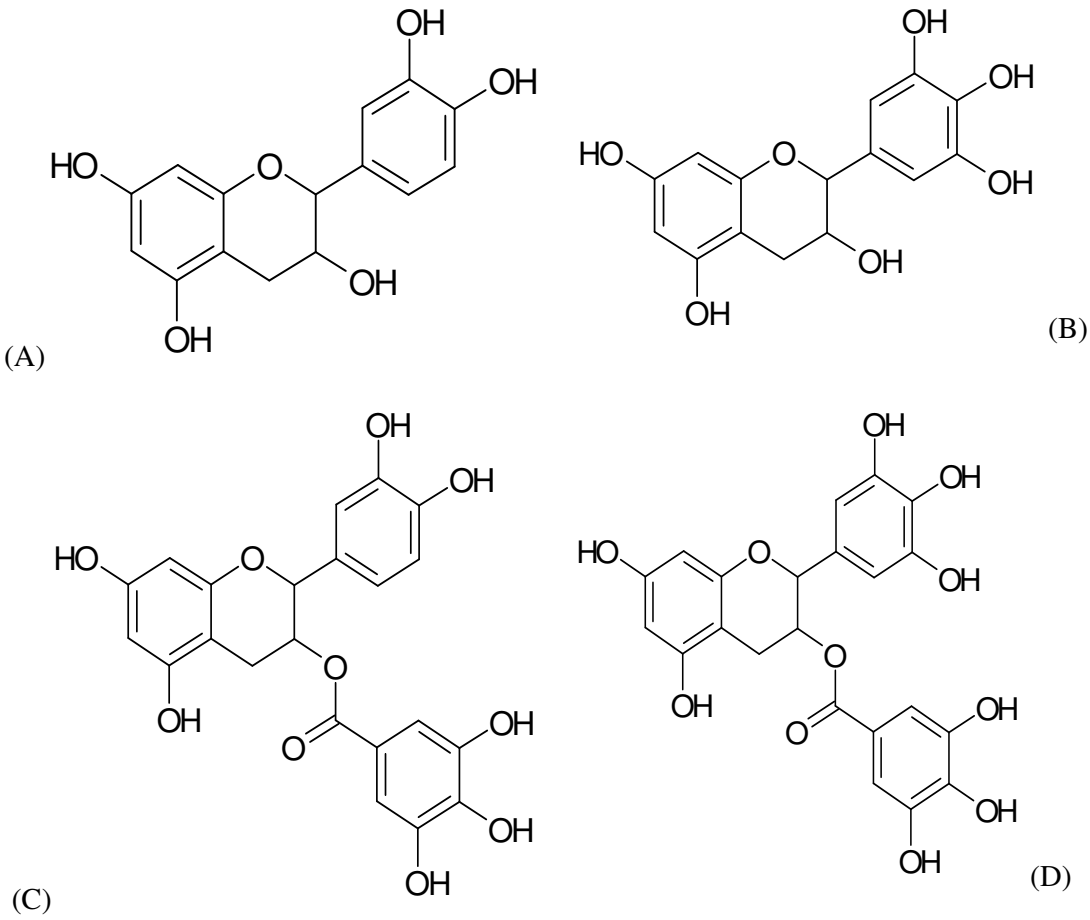

Figure 1.3

Chemical structures of (A) – Epicatechin (EC) (B) – Epigallocatechin (EGC)

(C) – Epicatechin gallate (ECg) and (D) – Epigallocatechin gallate (EGCg)

CHAPTER 2*

In vitro Inhibition of Phytopathogenic Fungi by Caffeine, Tea Catechins, Theanine, Polyphenon G, Black-, Green - and Rooibos tea extracts

* The use of Polyphenon G and caffeine as a fungicide has been patented in 2006, ref number 05P328 MN

ABSTRACT

Commercial agriculture relies heavily upon high inputs of chemical pesticides to protect crops against pathogens and pests. These practices are currently being re-evaluated due to concern about the possible health and environmental consequences. One option for an environmentally safe, antimicrobial compound is the use of tea extracts for control of phytopathogenic fungi. The present study reports on the sensitivity of twenty different phytopathogenic fungal species to extracts from black-, green- and rooibos tea extracts, concentrated green tea extract (Polyphenon G), caffeine, theanine, epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC), and epicatechin (EC), and Polyphenon G combined with caffeine. The fungi included in the study were the following: *Alternaria solani*, *Aspergillus flavus*, *Botrytis cinerea*, *Chalara elegans*, *Colletotrichum coccoides*, *Curvularia lunata*, *Dreschlera* sp., *Fusarium oxysporum*, *F. solani*, *Mucor hiemalis*, *M. pusillus*, *Penicillium expansum*, *Phytophthora capsici*, *P. nicotianae*, *Pythium* F-group, *Rhizopus stolonifer*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Stemphylium herbarum* and *Verticillium fungicola*. *In vitro* anti-fungal activity was determined by means of the agar-amendment method. For each fungus, the dose response was recorded and the IC_{50} value to the compounds was calculated. The inhibition of fungal growth by the compounds was as follows (in decreasing order): caffeine > EGCg ≥ ECg > EGC ≥ EC > Polyphenon G > green tea extracts ≥ black tea extracts > rooibos tea extracts ≥ theanine. In some cases the Polyphenon G and caffeine combination reduced the IC_{50} values for both the compounds, indicating a synergistic effect. *P. nicotianae* and *P. capsici* were most sensitive to all the compounds, while *R. stolonifer* and *P. expansum* were least sensitive. These results suggest

the possibility that these compounds will be effective against fungal diseases of crops when applied *in vivo*.

INTRODUCTION

Crop growers continually battle with fungal diseases affecting their crops. Chemical insecticides and fungicides are helpful in improving the quality and dependability of food supplies when used correctly and with care. The resistance of pests and plant pathogens against pesticides and fungicides is rapidly becoming a serious problem, and some chemicals have detrimental effects on the environment. Growing consumer awareness towards the use of organically grown produce is beginning to direct farmers into the investigation of alternative chemical control measures such as natural plant extracts (Quiroga *et al*, 2001).

In an effort to search for novel environmentally friendly antimicrobial agents, various plant extracts are being screened *in vitro*. According to Abad (1996), the osmotic proteins in tobacco plants are able to inhibit spore germination of some phytopathogenic fungi. The antimicrobial activity of tea (Arora and Bhardwaj, 1997) and tea polyphenols has also been demonstrated (Fukai *et al*., 1991; Kubo *et al*., 1992). Recently it has been shown that certain compounds in olives (Del Rio *et al*., 2003), such as tyrosol, catechins and oleuropein, have antimicrobial activity. The antimicrobial properties of caffeine and polyphenols are known to be part of the chemical defence mechanism in certain plants (Harborne, 1993).

Three compounds, namely catechins, caffeine and theanine (respectively 15%, 5% and 4% of dry weight) are found in relatively high concentrations in tea (*Camellia sinensis* (L.) Kuntze) (Hara, 2001). Tea cultivars that synthesize high amounts of these compounds have been selected over centuries for commercial tea production, due to their good taste and

disease resistance. Despite the considerable metabolic energy cost of producing such a large amount of secondary metabolites (Harborne, 1993), the scarcity of pathogens on tea plants is probably due to the abundance of these compounds. The low toxicity of tea on humans is well established, since adverse effects have rarely been reported. Investigation into the anti-fungal properties of tea extracts on fungal pathogens of selected vegetable crops, all with low phenolic- and no caffeine content, was therefore undertaken. In Japan, farmers have observed positive effects against bacterial diseases on vegetable crops when using Polyphenon G (Hara, personal communication). In the current study, the anti-fungal activity of tea extracts and individual compounds was assessed against a broad spectrum of fungi. The anti-fungal activity of Rooibos tea extract (*Aspalathus linearis*) was also investigated. Rooibos leaves may be brewed into an herbal tea that contains no caffeine and small quantities of catechins. Aspalathin is the main flavonoid in the rooibos plant (Gadow *et al*, 1997).

The *in vitro* anti-fungal activity of extracts from black-, green- and rooibos tea extracts, concentrated green tea extract (Polyphenon G) and the principle components thereof, namely caffeine, theanine, epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC) and epicatechin (EC) were assessed individually, using the agar-amendment method (Arora and Ohlan, 1997). The combined effect of Polyphenon G and caffeine was then investigated after it was determined that these two compounds were the most effective and suitable for *in vivo* application. All of the above-mentioned compounds were tested against selected fungal phytopathogens of vegetable crops and post-harvest pathogens, including *Penicillium expansum* Link and *Aspergillus flavus* Link: Fr, both mycotoxin producers. *P. expansum* is known to produce the toxin patulin in apples (Errampalli, 2004), whilst *A. flavus* produces aflatoxins in cereal crops such as maize and

wheat (Patkar *et al*, 1994) under warm and humid storage conditions. Inhibition of these fungi by tea compounds could have both economic and health importance.

MATERIALS AND METHODS

Fungal phytopathogens tested

The following fungal isolates were obtained from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria and maintained on potato dextrose agar (PDA) (Biolab, Johannesburg, RSA): *Alternaria solani* (Sorauer), *A. flavus* (Link), *Botrytis cinerea* (de Bary), *Chalara elegans* (Naj Raj and W.B. Kendr.), *Colletotrichum coccoides* (Berk and Broome), *Curvularia lunata* (Wakker), *Dreschlera* sp., *Fusarium oxysporum* (Schltdl. Em. W.C. Snyder and H.N. Hansen), *F. solani* (Mart.), *Mucor hiemalis* (Link.), *M. pusillus* (Link.), *P. expansum* (Link), *Phytophthora capsici* (Leonian), *P. nicotianae* (Brenda de Haan), *Pythium* F-group, *Rhizopus stolonifer* (Ehrenb.: Fr), *Sclerotinia sclerotiorum* ((Lib.) de Bary), *Sclerotium rolfsii* ((Curzi) Tu and Kimbr), *Stemphylium herbarum* (E. Simmons) and *Verticillium fungicola* (Kleb.). These fungi were selected on the basis of their host specificity and taxonomic diversity. The diseases caused by these pathogens are shown in Table 2.1.

Chemical compounds tested

The chemicals and concentrations thereof used in the fungal inhibition studies are given in Table 2.2. The composition of Polyphenon G, as provided by Mitsui Norin Co. Ltd. (MN), Japan, is given in Table 2.3. Polyphenon G in combination with caffeine was tested at

concentrations that varied between pathogens. The average IC_{50} values, obtained in previous single compound inhibition studies, were used in the Polyphenon G and caffeine combination tests. The fungi were divided into three categories namely: sensitive, intermediate and tolerant. The average IC_{50} value for each group of fungi was then quartered, halved, doubled and quadrupled to give a total of five doses per compound per fungus. These phytopathogens and the investigated concentrations are listed in Table 2.4. Polyphenon G, theanine, EGCg, ECg, EGC and EC were donated by MN. Caffeine was purchased from Sigma. Lipton black tea, Lipton rooibos tea and Eve's green tea were purchased from a local supermarket.

Preparation of the tea extracts

The tea extracts for black-, green- and rooibos tea were prepared by brewing in hot water. The required amount of each tea sample was weighed off, placed in a muslin cloth bag and suspended in boiling water for 15 minutes. Excess tea extract was then manually squeezed out of the cloth bag. The extract was allowed to stand for 10 minutes after which it was decanted into an Erlenmeyer flask. The volume was adjusted to 1L. PDA was amended with the tea extracts at the required concentrations and autoclaved for 12 minutes at 121°C.

Assay of the anti-fungal activity

The agar-amendment method was used to assess anti-fungal activity (Arora and Ohlan, 1997). Concentrations of the compounds used are given in Table 2.2 and Table 2.4. Each agar plate (60 mm diameter) was inoculated in the centre with a fungal disc (5 mm diameter) cut from the edge of an actively growing fungal colony (seven days old) on PDA. The plates were incubated at 25°C in the dark. The colony diameter of *M. hiemalis*, *M.*

pusillus, *Pythium* F-group, *R. stolonifer* and *S. rolfsii* was measured after three days, and the colony diameters of the remaining fungi were measured after seven days due to their slower growth rate. Fungi were grown on unamended PDA as controls. PDA amended with selected commercial fungicides was used for comparative purposes. Fungicides were selected on the basis of known efficacy against the representative groups of fungi included in the current tests. All fungicides were tested at a concentration of 0.1g a.i.L^{-1}. The trade name, manufacturer and recommended dosage of the fungicides are given in parentheses: benomyl WP, 500g a.i.kg^{-1} (Benlate, du Pont, Halfway House, RSA - 0.2 g.L^{-1}) for *A. flavus*, *B. cinerea*, *C. elegans*, *C. coccoides*, *F. oxysporum*, *F. solani*, *P. expansum*, *S. sclerotiorum*, and *V. fungicola*; difenoconazole EC, 250g a.i.L^{-1} (Score, Novartis, Isando, RSA - 0.4 g.L^{-1}) for A. solani, *C. lunata*, *Dreschlera* sp. and *S. herbarum*; propamocarb SL, 722g a.i.L^{-1} (Previcur, Sanachem, Durban, RSA - 0.14 g.L^{-1}) for *Pythium* F-group; tolclofos-methyl WP,500g a.i.kg^{-1} (Rizolex, Sanachem, Durban, RSA - 0.2 g.L^{-1}) for *M. hiemalis*, *M. pusillus*, *R. stolonifer* and *S. rolfsii*; and metalaxyl GR, 50g a.i.kg^{-1} (Ridomil, Novartis, Isando, RSA - 2 g.L^{-1}) for *P. capsici* and *P. nicotianae*. Each experiment was repeated twice and three replicate plates were used for each.

Calculations

The percentage inhibition was calculated according to the following formula:

$$\text{Percentage inhibition} = (C - T) \times \frac{100}{C} \qquad \text{Equation 1}$$

C = the diameter of growth of the unamended control.

T = the diameter of growth on the test plate.

The IC_{50} values for green and black tea were determined visually from dose response graphs. The IC_{50} values for caffeine, EGCg, Polyphenon G and combinations of Polyphenon G and caffeine were calculated using the Calcusyn 1.1 (Biosoft) software package (Chou and Hayball, 1996). The general equation for dose-effect as derived by Chou (1996) correlates dose and the effect in the simplest possible form:

$$f_a/f_u = (D/D_m)^m \qquad \text{Equation 2}$$

D = the dose of the compound

D_m = the median-effect dose signifying the potency

f_a = the fraction effected by the dose

f_u = the fraction unaffected, fu = 1- f_a

m = an exponent signifying the sigmoidicity of the dose curve.

The combination results were processed according to the Chou-Talalay combination index (CI) equation using the Calcusyn program version 2 (Chou and Hayball, 1996). The equation determines only the additive effect rather than synergistic or antagonistic effect. However, synergism is defined as a more than additive effect, whilst antagonism is defined as a less than additive effect. Thus, CI < 1 indicates synergism, CI = 1 indicates additive effect and CI > 1 indicates antagonism. Using this CI equation has the added benefit of the automated calculation of the dose reduction index (DRI). DRI is an important parameter as a favourable DRI (i.e. > 1) reduces toxicity while retaining the therapeutic effect (Han *et al*, 2004).

RESULTS

Theanine had no inhibitory effect on any of the fungi tested. The rooibos tea extract inhibited only *P. nicotianae* at an IC_{50} value of less than 100 $g.L^{-1}$ whilst the IC_{50} values for all the other pathogens was higher than $200g.L^{-1}$. The IC_{50} values for caffeine, EGCg, Polyphenon G, combinations of Polyphenon G and caffeine, black and green tea extracts are indicated in Table 2.5. The *in vitro* fungal inhibition obtained with four catechins at $2.5g.L^{-1}$ is shown in Table 2.6. The calculated CI and DRI values for various concentrations of the Polyphenon G and caffeine combinations are given in Table 2.7.

DISCUSSION

The present study investigated the *in vitro* sensitivity of twenty different fungi to tea extracts, and to various individual compounds from tea. The amount of inhibition of fungal growth by the compounds was as follows (in decreasing order): caffeine > EGCg ≥ ECg > EGC ≥ EC > Polyphenon G > green tea extracts ≥ black tea extracts > rooibos tea extracts ≥ theanine. The combined effect of Polyphenon G and caffeine was also investigated and in some instances the combination of Polyphenon G and caffeine reduced the IC_{50} values for both the compounds individually thereby indicating a synergistic effect.

Caffeine is the most abundant alkaloid in tea (Hara, 2001). The anti-fungal activity of caffeine against wood-rotting pathogens was previously reported by Arora and Ohlan (1997). It has also been reported that caffeine accumulates around wound areas in coffee plants as a natural, antimicrobial defence mechanism (Aneja and Gianfagna, 2001). With the exception

of *P. expansum* and *F. solani*, all the fungi were completely inhibited at concentrations of less than 5 $g.L^{-1}$ of caffeine. The effect of caffeine on *S. rolfsii* is shown in Figure 2.2.

During the studies by Arora and Bhardwaj (1997) on the anti-fungal activity of black and green teas on phytopathogens, only one concentration of tea extract was used, namely 150 $g.L^{-1}$. Their results showed a 49% inhibition of *F. solani* with green tea extract and 45% inhibition by black tea extract, while the current study found a 51% inhibition of the same fungus with green tea extract and 40% inhibition with black tea extract, when tested at the same concentration, indicating similar findings. Of the three teas tested green tea extracts showed the highest anti-fungal activity followed by black tea extracts, while rooibos tea extracts exhibited very little or no anti-fungal activity (effect on *S. sclerotiorum* illustrated in Figure 2.3). Caffeine, EGCg and Polyphenon G showed much higher anti-fungal activity than the black-, green- and rooibos tea extracts.

Theanine had no anti-fungal effect at the highest dose tested (20 $g.L^{-1}$). Rooibos tea extracts had negligible anti-fungal effect. The black tea extracts exhibited less anti-fungal activity than green tea extracts. Black tea is enzymatically oxidised and contains fewer monomeric polyphenols (Chun *et al.*, 1999) thus, even though both black- and green tea extracts contain caffeine, green tea extracts still exhibited greater anti-fungal activity, possibly due to the presence of the monomeric catechins. PPG was five times more effective than green tea extracts. EGCg was ten times more effective than green tea extracts. The effect of EGCg on *F. oxysporum* is shown in Figure 2.4. Caffeine was 100 times more effective than green tea extracts. Thus caffeine acts synergistically with PPG to make this crude extract of green tea as effective as pure EGCg. The monomeric catechins tested included EC, EGC, ECg and EGCg. All of these compounds were tested at a concentration of 2.5 $g.L^{-1}$ to determine which catechin in Polyphenon G exhibits the greatest anti-fungal properties. Overall, the

gallated catechins exhibited a greater anti-fungal activity than the non-gallated catechins. The anti-fungal efficacy seems to increase with molecular mass and number of hydroxyl groups on the catechin i.e. EGCg ≥ ECg > EGC ≥ EC. This relationship can be seen for *C. coccodes* (Figure 2.5), *Dreschlera* sp., *P. capsici* and *P. nicotianae*. The increase in percent growth inhibition by catechins with increase in molecular weight is shown in Figure 2.1. This agrees with the antibacterial properties reported by Fukai *et al*. (1991) and Yamamoto *et al*, (1997) and the antimutagenic properties reported by Apostolides *et al.* (1997). This near linear relationship may be useful in predicting the antimicrobial properties of other bioflavonoids.

Polyphenon G had an IC_{50} of < 16 $g.L^{-1}$ for 11 of the 20 pathogens. EGCg had an IC_{50} of < 5$g.L^{-1}$ for 11 of the 20 pathogens. Caffeine had an IC_{50} of < 2 $g.L^{-1}$ for 18 of the 20 pathogens again indicating a broad-spectrum of activity. Overall, the *in vitro* efficacies of commercial fungicides (at lower concentration than the experimental compounds) were better than the experimental compounds. In some cases the experimental compounds used at higher concentrations than the fungicides preformed as well as the fungicides.

The combination index (CI) equation is based on the multiple drug-effects derived from enzyme kinetic models. The DRI defines the degree of dose reduction that is possible in a combination for a given degree of effect as compared with the concentration of each drug alone. Thus a CI value less than one and DRI value for both chemicals greater than one indicates synergism. The CI and DRI values for all concentrations of the PPG caffeine combinations are noted in Table 2.7. The combinations that showed a synergistic effect are indicated in bold (orange), whilst those that showed an antagonistic effect are indicated in italics (blue). Those that are neither synergistic nor antagonistic are indicated in black.

The combinations of Polyphenon G and caffeine reduced the amount of one or both compounds required to obtain the IC_{50} doses for 17 of the 20 pathogens. The PPG + caffeine mixture halved the concentrations needed for each chemical individually to obtain IC_{50} values in the case of *A. solani, B. cinerea, M. pusillus* and *V. fungicola*. Thus the PPG + caffeine mixture showed significant synergism for four of the 20 fungi tested and showed mild synergism for 13 of the 20 fungi tested.

It can be concluded from these studies that tea extracts, and some of the major compounds in tea, have the potential to be effective anti-fungal agents with specific use against fungal diseases on vegetable crops. These compounds may be of economic importance and also have potential use as broad-spectrum anti-microbial agents. Greenhouse trials will have to be conducted to determine whether these compounds will be effective against the diseases caused by these pathogens on their respective hosts.

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Table 2.1

Fungi that were tested *in vitro*, the hosts they infect and the diseases they cause

Host	Disease	Pathogen
Cucumber diseases	Wilt	*Fusarium oxysporum*
(*Cucumis sativus* L.)	Wilt	*Fusarium solani*
Lettuce diseases	Rot	*Sclerotinia sclerotiorum*
(*Lactuca sativa* L.)	Root rot	*Pythium* F-group
	Leaf spot	*Curvularia lunata*
	Leaf spot	*Stemphylium herbarum*
Tomato diseases	Early blight	*Alternaria solani*
(*Lycopersicon*	Grey mould	*Botrytis cinerea*
esculentum Mill.)	Black root rot	*Chalara elegans*
	Anthracnose	*Colletotrichum coccoides*
	Wilt	*Sclerotium rolfsii*
	Wilt	*Verticillium fungicola*
	Root rot	*Phytophthora capsici*
	Buckeye rot	*Phytophthora nicotianae*
	Leaf spot	*Dreschlera* sp.
Various hosts	Post-harvest rot	*Mucor hiemalis*
		Mucor pusillus
		Rhizopus stolonifer
	Mycotoxin	*Penicillium expansum*
	producers	*Aspergillus flavus*

Table 2.2

Compounds and concentration ranges that were tested for *in vitro* anti-fungal activity

Compound	Concentration ($g.L^{-1}$)
Polyphenon G	2.5, 5, 10, 15 and 20
Caffeine	0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 5 and 10
Theanine	2.5, 5, 10, 15 and 20
Black tea extract (Lipton)	100, 150 and 200
Green tea extract (Eve's)	100, 150 and 200
Rooibos tea extract (Lipton)	100, 150 and 200
Epigallocatechin gallate (EGCg)	0.5, 1, 2, 2.5 and 5
Epicatechin gallate (ECg)	2.5
Epigallocatechin (EGC)	2.5
Epicatechin (EC)	2.5

Table 2.3

Composition of Polyphenon G*

Compound	% Dry weight
Epigallocatechin (EGC)	8.4
Epicatechin (EC)	2.1
Epigallocatechin gallate (EGCg)	13.8
Epicatechin gallate (ECg)	2.6
Gallocatechin gallate (ECg)	0.3
Catechin gallate (CG)	0.2
Caffeine	6.1
Total	33.2

* Provided by Mitsui Norin Co. Ltd. (MN), Japan

Table 2.4

Polyphenon G, caffeine and combinations of the two compounds and concentration ranges that were tested against the selected pathogens

	Fungal pathogens	Concentration ($g.L^{-1}$) Polyphenon G (PPG)	Caffeine (CAF)	PPG+CAF*
Tolerant fungi	*Alternaria solani*, *Botrytis cinerea*, *Colletotrichum coccoides*, *Chalara elegans*, *Fusarium solani*, *Penicillium expansum*, *Rhizopus stolonifer*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*	5 10 20 40 80	0.5 1 2 4 8	5:0.5 10:1 20:2 40:4 80:8
Intermediatly sensitive	*Aspergillus flavus*, *Dreschlera* sp., *Fusarium oxysporum*, *Mucor pusillus* and *Verticillium fungicola*	3.75 7.5 15 30 60	0.375 0.75 1.5 3 6	3.75:0.375 7.5:0.75 15:1.5 30:3 60:6
Sensitive fungi	*Curvularia lunata*, *Mucor hiemalis*, *Phytophthora capsici*, *Pythium* F-group, *Phytophthora nicotianae* and *Stemphylium herbarum*	1.25 2.5 5 10 20	0.25 0.5 1 2 4	1.25:0.25 2.5:0.5 5:1 10:2 20:4

* PPG:CAF signifies the concentration of Polyphenon G and caffeine used in each case, e.g. 5:0.5 is a combination of 5g of Polyphenon G and 0.5 g of caffeine in 1L of potato dextrose agar (PDA).

Table 2.5

The IC_{50} values for *in vitro* inhibition of fungal growth by caffeine, epigallocatechin gallate (EGCg), Polyphenon G, combinations of caffeine and Polyphenon G, green-, black- and rooibos tea extracts

	$IC_{50}(g.L^{-1})$						
Fungal Pathogens	PPG	Caffeine	PPG:CAF***	EGCg	Green tea extracts	Black tea extracts	Rooibos tea extracts
Alternaria solani	20	0.99	5:0.5	1.33	>200	>200	>200
Aspergillus flavus	18	1.54	15:1.5	8.18	200	>200	>200
Botrytis cinerea	28	1.89	10:1	40.76	>200	>200	>200
Chalara elegans	20	1.66	20:2	12.04	100	150	>200
Colletotrichum coccoides	21	1.23	10:1	1.37	< 100	150	>200
Curvularia lunata	3.0	1.25	2.5:0.25	1.28	100	200	>200
Dreschlera sp.	17	0.92	7.5:0.75	3.46	< 100	200	>200
Fusarium oxysporum	18	0.92	7.5:0.75	3.17	100	100	>200
Fusarium solani	22	1.86	15:1.5	3.62	150	>200	>200
Mucor hiemalis	5.0	1.26	5:0.5	2.03	< 100	100	>200
Mucor pusillus	15	1.20	3.75:0.37	5.84	< 100	150	>200
Penicillium expansum	75	4.53	40:4	8.03	>200	>200	>200
Phytophthora capsici	5.0	0.10	< 1.25:0.25*	4.02	< 100	< 100	>200
Phytophthora nicotianae	1.5	0.01	< 1.25:0.25*	0.10	< 100	< 100	< 100
Pythium F-group	3.0	0.31	1.25:0.25	6.89	< 100	< 100	>200

Rhizopus stolonifer	35	2.28	20:2	>5.0*	>200	>200	>200
Sclerotinia sclerotiorum	25	1.47	15:1	85.69	175	>200	>200
Sclerotium rolfsii	40	0.89	15:1	>5.0*	100	< 100	>200
Stemphylium herbarum	2.5	0.80	2.5:0.25	2.31	< 100	< 100	>200
Verticillium fungicola	16	0.83	3.75: 0.37	3.16	< 100	< 100	>200
Average IC_{50} (g.L-1)	19.5	1.3	10.8	10.7	**	**	**

* Indicated values that were excluded from average IC_{50} calculations as they could not be calculated from the data.

** The average IC_{50} values could not be calculated for these compounds as most of the values are < or > values.

*** PPG:CAF signifies the concentration of Polyphenon G and caffeine used in each case. Eg. 5:0.5 is a combination of 5g of Polyphenon G and 0.5 g of caffeine in 1L of potato dextrose agar (PDA).

Table 2.6

In vitro inhibition of fungal growth by epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg) at a concentration of 2.5g.L^{-1}

	Percentage inhibition by catechin type			
Fungal Pathogens	EC	EGC	ECg	EGCg
Alternaria solani	51	50	51	76
Aspergillus flavus	10	10	12	12
Botrytis cinerea	0	0	0	0
Chalara elegans	0	0	0	0
Colletotrichum coccoides	43	50	51	70
Curvularia lunata	53	36	60	60
Dreschlera sp.	24	27	39	43
Fusarium oxysporum	50	50	62	55
Fusarium solani	31	49	39	51
Mucor hiemalis	0	14	100	100
Mucor pusillus	0	0	41	43
Penicillium expansum	34	34	45	39
Phytophthora capsici	29	36	40	44
Phytophthora nicotianae	53	70	74	100
Pythium F-group	0	0	13	17
Rhizopus stolonifer	0	0	0	0
Sclerotinia sclerotiorum	0	0	0	0
Sclerotium rolfsii	48	47	60	49
Stemphylium herbarum	0	0	0	0
Verticillium fungicola	24	38	24	40
Average percentage inhibition	22.5	25.55	36.05	39.95

Table 2.7

Combination index (CI) and dose reduction index (DRI) values for concentrations indicating synergism during anti-fungal assays with Polyphenon G (PPG) and caffeine (CAF) in various combinations. These values were calculated using the Calcusyn 1.1 (Biosoft) package

	Pathogens	Concentrations ($g.L^{-1}$)														
		PPG 5 CAF 0.5			PPG 10 CAF 1			PPG 20 CAF 2			PPG 40 CAF 4			PPG 80 CAF 8		
		CI	DRI PPG	DRI CAF	CI	DRI PPG	DRI CAF	CI	DRI PPG	DRI CAF	CI	DRI PPG	DRI CAF	CI	DRI PPG	DRI CAF
High dose Tolerant fungi	*Alternaria solani*	1.1	2.3	1.4	1.8	1.5	0.9	1.0	2.2	1.8	1.7	1.3	1.1	***2.4***	***0.9***	***0.8***
	Botrytis cinerea	**0.7**	**4.3**	**2.0**	1.3	2.4	1.1	2.0	1.5	0.7	**0.9**	**2.8**	**1.8**	1.8	1.4	0.9
	Colletotrichum coccodes	1.7	1.6	1.0	2.4	1.1	0.7	**0.7**	**3.7**	**2.5**	1.3	1.9	1.2	***2.7***	***0.9***	***0.6***
	Chalara elegans	**0.8**	**2.7**	**3.1**	1.2	1.6	1.7	1.9	1.0	1.0	1.0	2.3	1.6	2.1	1.1	0.8
	Fusarium solani	**0.8**	**2.0**	**3.3**	1.4	1.2	1.9	2.2	0.8	1.1	**0.7**	**3.3**	**2.5**	1.4	1.3	1.2
	Penicillium expansum	1.6	1.3	1.2	***3.2***	***0.6***	***0.6***	1.2	1.6	1.5	1.4	1.5	1.4	***2.4***	***0.8***	***0.8***
	Rhizopus stolonifer	1.4	1.6	1.2	***2.9***	***0.8***	***0.6***	1.7	1.3	1.0	***2.9***	***0.8***	***0.6***	2.3	1.0	0.8
	Sclerotium rolfsii	***3.8***	***0.4***	***0.8***	1.1	2.9	1.2	**0.4**	**60.0**	**2.9**	**0.7**	**30.0**	**1.4**	1.4	15.0	0.7
	Sclerotinia sclerotiorum	2.7	1.3	0.4	**0.3**	**10.1**	**4.4**	**0.6**	**5.0**	**2.2**	1.2	2.5	1.1	2.6	1.3	0.5

		PPG 3.75 CAF 0.375			PPG 7.5 CAF 0.75			PPG 15 CAF 1.5			PPG 30 CAF 3			PPG 60 CAF 6		
Medium dose Intermediately sensitive fungi	*Aspergillus flavus*	1.3	1.6	1.5	***2.3***	***0.8***	***0.8***	***3.7***	***0.5***	***0.4***	**0.7**	**4.8**	**1.8**	1.4	2.4	0.9
	Dreschlera spp.	1.4	1.4	1.3	1.8	1.0	1.0	**0.4**	**3.7**	**7.2**	**0.8**	**1.8**	**3.6**	1.6	0.9	1.8
	Fusarium oxysporium	1.4	2.1	1.0	2.4	1.2	0.6	**0.5**	**10.2**	**2.4**	1.0	5.1	1.2	2.0	2.5	0.6
	Mucor pusillus	**0.9**	**3.1**	**1.5**	1.6	1.7	0.9	**0.7**	**2.9**	**2.3**	1.5	1.4	1.1	***3.1***	***0.7***	***0.5***
	Verticillium fungicola	1.6	1.9	0.8	**0.3**	**7.4**	**4.8**	**0.6**	**3.7**	**2.4**	1.3	1.8	1.2	***2.7***	***0.9***	***0.6***
		PPG 1.25 CAF 0.25			PPG 2.5 CAF 0.5			PPG 5 CAF 1			PPG 10 CAF 2			PPG 20 CAF 4		
Low dose Sensitive fungi	*Curvularia lunata*	1.4	0.9	3.4	1.1	1.3	2.2	1.2	1.7	1.4	**0.5**	**39.4**	**2.0**	***3.5***	***0.7***	***0.4***
	Mucor hiemalis	1.9	0.8	1.3	1.1	1.5	2.1	1.5	1.0	1.4	**0.8**	**2.2**	**2.6**	1.6	1.1	1.3
	Phytophthora capsici	**0.3**	**7.9**	**5.2**	**0.5**	**4.1**	**2.9**	***11.4***	***0.6***	***0.1***	1.7	1.2	1.0	1.2	1.2	2.3
	Pythium F-group	1.0	3.7	1.2	1.4	2.3	0.9	**0.7**	**2.9**	**2.4**	1.5	1.4	1.2	***3.0***	***0.7***	***0.6***
	Photosphere nicotianae	**0.3**	**5.5**	**5.1**	**0.6**	**3.0**	**2.8**	18.5	0.1	0.1	1.9	1.0	1.0	**0.9**	**1.9**	**2.5**
	Stemphylium herbarum	1.1	2.3	1.3	1.4	1.9	1.1	2.1	1.2	0.7	**0.9**	**2.6**	**1.7**	1.9	1.3	0.8

* CI values < 1 and DRI values > than 1 indicates synergism

** Values in bold (orange) indicate synergism, while values in italics (blue) indicate antagonism

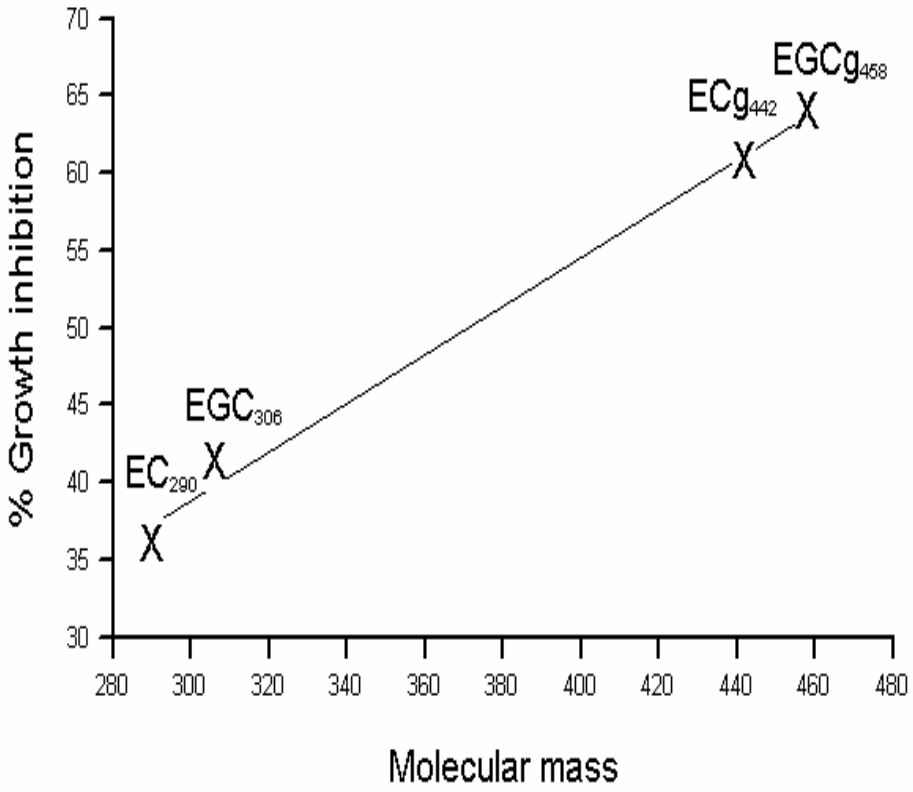

Figure 2.1

The anti-fungal efficacy of epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg) at a concentration of 2.5g.L^{-1} with regards to molecular mass

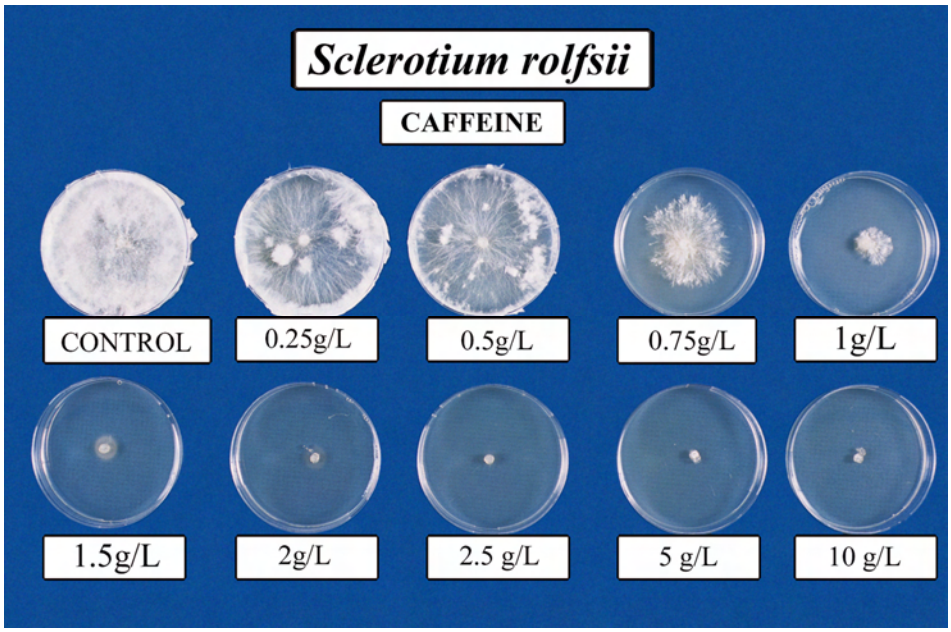

Figure 2.2

The *in vitro* effect of caffeine on the colony diameter of *Sclerotium rolfsii*. The treatments ($g.L^{-1}$) are indicated on the labels

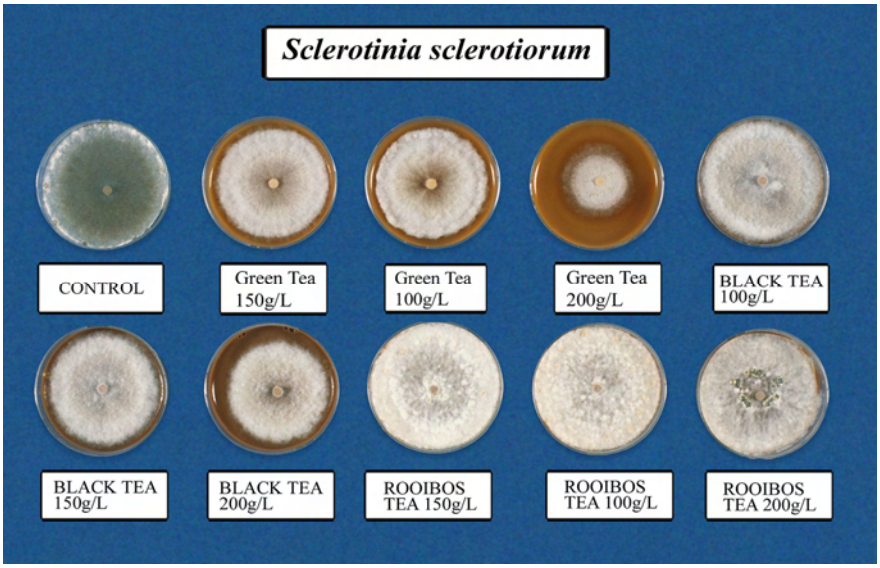

Figure 2.3

The *in vitro* effect of black-, green- and rooibos- tea extracts on the colony diameter of *Sclerotinia sclerotiorum*. The treatments ($g.L^{-1}$) are indicated on the labels

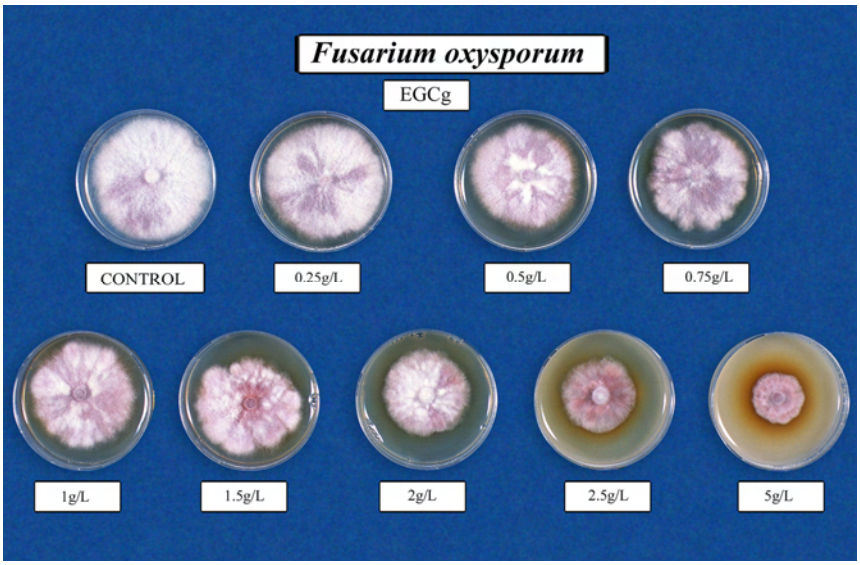

Figure 2.4

The *in vitro* effect of epigallocatechin gallate (EGCg) on the colony diameter of *Fusarium oxysporum*. The treatments ($g.L^{-1}$) are indicated on the labels

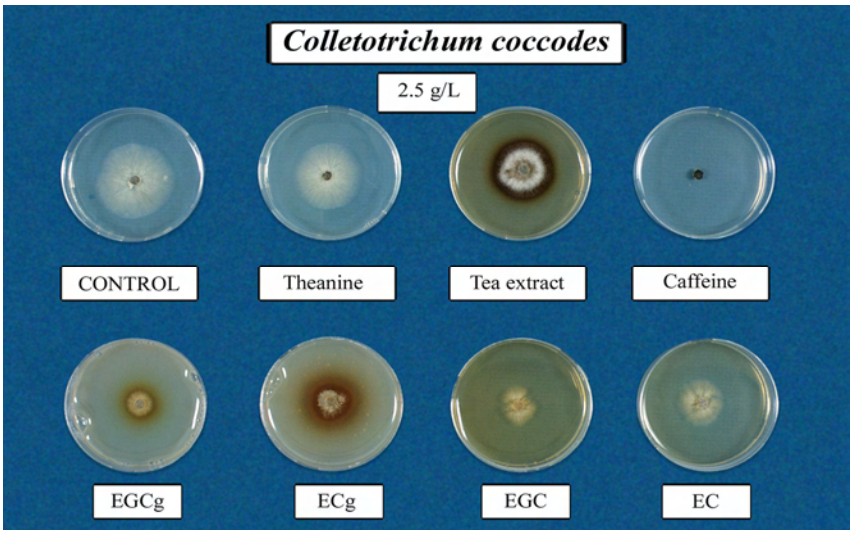

Figure 2.5

The *in vitro* effect of theanine, Polyphenon G, caffeine, epigallocatechin gallate (EGCg), epigallocatechin (EGC), epicatechin gallate (ECg) and epicatechin (EC) at a concentration of $2.5g.L^{-1}$, on the colony diameter of *Colletotrichum coccodes*

CHAPTER 3*

Control of Phytopathogenic Fungi on Selected Vegetable Crops with Caffeine and Green Tea (*Camellia sinensis*) Extracts Under Greenhouse Conditions

* The use of Polyphenon G and caffeine as a fungicide has been patented in 2006, ref number 05P328 MN

ABSTRACT

In the current study tea (*Camellia sinensis*) extracts have been evaluated for their *in vivo* activity against phytopathogenic fungi. These compounds have potential as alternative, environmentally friendly plant disease control agents. The fungicidal activity of Polyphenon G, caffeine, and combinations of Polyphenon G and caffeine were tested for *in vivo* activity against seven phytopathogenic fungi including *Fusarium oxysporum* and *Fusarium solani* on cucumber, *Phytophthora capsici* and *Sclerotium rolfsii* on tomato, *Sclerotinia sclerotiorum* and *Pythium* F-group on lettuce and *Sphaerotheca fuliginea* on zucchini squash plants. For each pathogen, treatment with a commercial fungicide effective against that particular pathogen was included as standards. The combinations of Polyphenon G and caffeine gave the best overall results and effectively controlled *Fusarium solani* on cucumber, *Phytophthora capsici* and *Sclerotium rolfsii* on tomato, *Sclerotinia sclerotiorum* and *Pythium* F-group on lettuce. This is the first report of *in vivo* fungicidal activity of Polyphenon G, caffeine and combinations of these compounds. In some instances the test compounds performed better than the commercial fungicides at the recommended doses. The results indicate that Polyphenon G, caffeine or combinations of these compounds have potential as alternative, environmentally friendly plant disease control agents on selected vegetable crops.

INTRODUCTION

Alternative strategies employing the products of plant extracts for plant disease control have gained popularity in recent years (Abad *et al* 1996; Singh *et al*, 1998; Lee *et al*, 2001; Quiroga *et al*, 2001; Banos-Bautista *et al*, 2003; Del Rio *et al*, 2003; Jasso de Rodríguez *et al*, 2004). Plant extracts are rich in bioactive chemicals and may be potential alternative to synthetic fungicides (Hedin, 1982). These natural compounds are biodegradable, resulting in the production of non-toxic intermediates (Lee *et al*, 2001). Certain plant extracts and phytochemicals act on different types of plant disease complexes through various mechanisms and may be applied to a crop in the same way as other synthetic chemicals. Various other plant derived compounds, such as phenolics, terpenoids, alkaloids and lignans, may also have anti-microbial activity (Harborne, 1993).

Anti-microbial activity of commercial teas and some of the individual compounds in tea have previously been reported (Fukai *et al*, 1991; Arora and Bhardwaj, 1997). During previous *in vitro* studies (Chapter 2), we assessed the anti-fungal activity of extracts from black (*Camellia sinensis* (L) Kuntze), green, and rooibos (*Aspalatus linearis* (L) Kuntze) teas, concentrated green tea extract (Polyphenon G), and the principle components thereof, including: caffeine, theanine, epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC) and epicatechin (EC). In the previous tests, caffeine (CAF) showed the greatest anti-fungal activity followed by EGCg, Polyphenon G (PPG), green-, black- and rooibos tea extracts in decreasing order. Theanine had no anti-fungal effect. Polyphenon G and caffeine being the most effective compounds *in vitro*, were tested in these trials

for their efficacy *in vivo*, on a range of fungal pathogens on selected vegetable crops under greenhouse conditions.

MATERIALS AND METHODS

Fungal phytopathogens

Pure cultures of the following six fungal species were obtained from the University of Pretoria culture collection and maintained on potato dextrose agar (PDA) (Biolab, Merck) plates: *Fusarium oxysporum* (Schltdl. Em. W.C. Snyder and H.N. Hansen), *Fusarium solani* (Mart.), *Phytophthora capsici* (Leonian), *Pythium* F-group, *Sclerotinia sclerotiorum* ((Lib.) de Bary) and *Sclerotium rolfsii* ((Curzi) Tu and Kimbr). Cultures were stored on PDA slants at 25°C. *Sphaerotheca fuliginea* ((Schlecht.) Pollacci) was maintained on live host plants (Zucchini squash (*Cucurbita pepo* L.)) under greenhouse conditions, at temperatures varying between 25°C and 31°C, for the duration of the study. The pathogens and diseases caused on the respective hosts are shown in Table 3.1.

Inoculum preparation and Inoculation

The soilborne phytopathogens (*F. solani*, *F. oxysporum*, *P. capsici*, *Pythium* F-group, *S. sclerotiorum* and *S. rolfsii*) were grown on 90 mm diameter PDA plates at 25ºC until the plates were completely covered. Millet (*Penniselum glauceam* L.) seeds (200g) were steeped in water (200ml) for 24h, transferred to autoclavable polyethylene bags, and autoclaved twice for 45min at 120°C on two consecutive days.

Ten 5mm diameter agar discs were cut with a cork borer from the actively growing edge of each fungal colony and mycelial discs were aseptically transferred to each of the sterilized millet seed bags. The bags were incubated for 28 days at 25°C. The millet seed inoculum of each individual pathogen was added to the soil (Weideman and Wehner, 1993) at the following concentrations: 100 $g.L^{-1}$ for *F. oxysporum*, *F. solani* and *P.* F-group; 70, 50 and 20 $g.L^{-1}$ for *P. capsici*, *S. sclerotiorum* and *S. rolfsii,* respectively.

Zucchini squash plants were naturally infected with *S. fuliginea* by transferring healthy plants to a greenhouse with infected plants and placing the healthy plants amongst the infected plants (Bettiol, 1999).

Soil preparation

Sandy loam soil (Conradie Organics, Pretoria, Gauteng, South Africa) and potting soil mix consisting of composted kraal manure and bark (Just Nature Organics, Pretoria, Gauteng, South Africa) was steam pasteurized separately and mixed in a 1:1 ratio (v:v) based on results obtained during preliminary trials.

Host plants

Seeds of a susceptible commercial varieties of cucumber (*Cucumis sativus* L., cv Volcano), lettuce (*Lactuca sativa* L., cv Nadine), tomato (*Lycopersicon esculentum* Mill., cv Money Maker) and zucchini squash (*Cucurbita pepo*, cv. Caserta) were obtained from Hygrotec (Pretoria-North, Gauteng, South Africa). The cucumber and

zucchini squash seeds were pre-germinated in sterile vermiculite in a Conviron (Fisons, Fi-totron 600H) growth cabinet at 25 ºC and subsequently transplanted into pasteurized potting soil. The seedlings were grown until four true leaves had developed before inoculation. The tomato and lettuce seeds were germinated and grown at a commercial nursery (Multiplant, Brits, North West South Africa) until five weeks old.

Green house trials

Treatments:

The treatments included in this study were untreated uninfected and infected controls respectively, a fungicide standard and five concentrations of Polyphenon G, caffeine and combinations of the two compounds (total of 18 treatments per experiment). The treatment concentrations are give in Table 3.2.

All the synthetic fungicide standards were tested at a concentration of 0.1g a.i.L^{-1}. The trade name, manufacturer and recommended dosage of the fungicides are given in parentheses: benomyl WP, 500g a.i.kg^{-1} (Benlate, du Pont, Halfway House, RSA - 0.2 g.L^{-1}) for *F. oxysporum*, *F. solani* and *S. sclerotiorum*, propamocarb SL, 722g a.i.L^{-1} (Previcur, Sanachem, Durban, RSA - 0.14 g.L^{-1}) for *P.* F-group; tolclofos-methyl WP, 500g a.i.kg^{-1} (Rizolex, Sanachem, Durban, RSA - 0.2 g.L^{-1}) for *S. rolfsii*; metalaxyl GR, 50g a.i.kg^{-1} (Ridomyl, Novartis, Isando, RSA - 2 g.L^{-1}) for *P. capsici*; triforine SL, 190 g a.i.L^{-1} (Funginex, Efekto, Pretoria, RSA – 0.53 g.L^{-1}) for *S. fuliginea*.

Soilborne pathogens:

Soil containing millet seed inoculum of the various soilborne pathogens was transferred to 700 ml plastic pots. Seedlings were transplanted into the inoculated soil and treated with 100 ml of the various compounds as a soil drench. Soil inoculation, transplanting of the plants into the inoculated soil and first treatment of plants were all done on the same day. Treatments were applied a second time after 14 days. The experimental design consisted of a randomised block design with 18 treatments and 8 replicates per treatment. Each replicate comprised of a single plant per pot. Pots were rotated once a week to ensure even exposure to greenhouse conditions. Each experiment was repeated twice. Greenhouse temperatures were maintained at 25 to 35ºC during the experiments.

Twenty-seven days after inoculation the plants were removed from the soil and any soil adhering to the roots was rinsed off under running tap water. Roots and shoots of plants were excised and weighed separately. Root rot was visually assessed according to a 0-4 scale (0 = healthy, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% root rot). Randomly selected root segments (approximately 5 mm long) were excised from each plant, surface sterilized in 70% ethanol for 1 min and plated on the appropriate medium as described below. Three plates were used per plant with ten root segments plated on each. From this, the percentage infection was calculated and pathogens were re-isolated (Stanghellini and Kronland, 1986). Root segments from seedlings inoculated with *F. solani*, *F. oxysporum* and *S. sclerotiorum* were plated onto semi-selective RBGU medium (Van Wyk *et al* 1986), containing: glycerol (UniLab, Merck) 10 ml, urea (Biolab, Merck) 1g, L-Alanine (Sigma) 0.5g, quintozene (750g

(active ingredient (a.i.))kg^{-1}, Plaaschem) 1g, Rose Bengal (Sigma) 0.5g and Chloramphenicol (RoLab, Norvatis) 0.25g. These chemical compounds were dissolved in 20ml 70% Ethanol and added to 12g Agar bacteriological (Biolab, Merck) after it has been autoclaved. Root segments from seedlings inoculated with *P.* F-group (Figure 3.1) were plated onto BNPRA medium (Masago *et al*, 1977): tolclofos-methyl (500g a.i.kg^{-1}, Sanachem) 0.2g, quintozene (750g a.i.kg^{-1}, Plaaschem) 0.05g, benomyl (500g a.i.kg^{-1}, Du Pont) 0.01g, nystatin (Sigma) 0.025g, ampicillin (BeTabs pharmaceuticals) 0.25g, rifampicin (Rolab, Norvatis) 0.025g These chemical compounds were dissolved in 20ml 70% Ethanol and added to 12g Agar bacteriological after it has been autoclaved. Roots inoculated with *P. capsici* were plated on BNPRAH medium which contains the same ingredients as the BNPRA media with an addition of 1ml hymexazol (360 g (a.i)L^{-1}, Agrishel) (Masago *et al*, 1977). Roots inoculated with *S. rolfsii* were plated on PDA amended with 0.05g.L^{-1} rifampicin.

Foliar pathogen:

The zucchini plants infected with *S. fuliginea* were treated only after the onset of disease (seven days after being moved to the greenhouse compartment with high inoculum density). Zucchini squash plants were sprayed with the various compounds until runoff, on a weekly basis. No uninfected control was included during this trial (McGrath and Shiskoff, 1996). Experiments were laid out in a randomized design with five replicates per treatment. Each replication consisted of one pot containing one plant. The pots were rotated on the greenhouse bench on a weekly basis to ensure even exposure to conditions in greenhouse. The duration of the experiment extended

over a period of three weeks. Each independent experiment was repeated twice. The temperatures in the greenhouse during the experiments were maintained at 26 to 32°C. The severity of the *S. fuliginea* infestation was visually evaluated on each individual leaf, by rating according to a 0-4 scale (0 = healthy, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% disease development). The disease rating scale for *S. fuliginea* on zucchini squash used during the experiments is shown in Figure 3.2 (Tzeng *et al*., 1996; Reuveni *et al*, 1996).

STATISTICAL ANALYSIS

Significant differences were determined using Fisher's protected least significant difference test (F-test), and are indicated on the graphs using letter notation. The comparison wise error rate was $P<0.05$. Standard error of the means are indicated as error bars on the graphs.

RESULTS

Data on the average fresh shoot and root mass is given in Figures 3.3, 3.5, 3.7, 3.9, 3.11 and 3.13. The data on the average percentage infection and root rot ratings are shown in Figure 3.4, 3.6, 3.8, 3.10, 3.12 and 3.14. The average percentage disease assessment ratings for *S. fuliginea* on zucchini squash plants are shown in Figure 3.15.

Lettuce infected with Pythium F-group

Treatment of *Pythium* infected lettuce plants with the combinations of PPG and caffeine at concentrations of 2.5 $g.L^{-1}$ + 0.25 $g.L^{-1}$ and 5 $g.L^{-1}$ + 0.5 $g.L^{-1}$ respectively resulted in shoot mass comparable to that of the untreated uninfected control as well as zero percent root rot (Figure 3.3; 3.16). Treatment with PPG at a concentration of 12.5 $g.L^{-1}$ resulted in significantly higher shoot mass in comparison with the propamocarb treatment. Treatments with PPG at concentrations of 7.5 $g.L^{-1}$ and 10 $g.L^{-1}$, caffeine at 0.25 $g.L^{-1}$, 0.5 $g.L^{-1}$, 1 $g.L^{-1}$ and 1.25 $g.L^{-1}$ as well as combinations of PPG and caffeine at concentrations of 7.5 $g.L^{-1}$ + 0.75 $g.L^{-1}$, 10 $g.L^{-1}$ + 1 $g.L^{-1}$ and 12.5 $g.L^{-1}$ + 1.25 $g.L^{-1}$ respectively, gave shoot mass comparable to that of the propamocarb treatment. The latter combination treatments also resulted in percentage infection comparable to that of the propamocarb treatment, while all other treatments gave significantly higher infection values (Figure 3.4). Significant differences were not as prevalent with regards to root mass and were, for most treatments, comparable to that of the uninfected untreated control. Exceptions to this were the untreated infected control and treatments with PPG at 12.5 $g.L^{-1}$, caffeine at 1.25 $g.L^{-1}$ and combinations of PPG and caffeine at concentrations of 7.5 $g.L^{-1}$ + 0.75 $g.L^{-1}$, 10 $g.L^{-1}$ + 1 g.L-1 and 12.5 $g.L^{-1}$ + 1.25 $g.L^{-1}$ respectively. Excluding the treatments with PPG at 12.5 $g.L^{-1}$ and caffeine at 1.25 $g.L^{-1}$ all other treatments resulted in percentage root rot comparable to or significantly lower than that of the propamocarb treatment (Figure 3.4). Lettuce plants infected with *Pythium* resulted in a 50% reduction in shoot and root mass respectively when comparing the untreated uninfected and untreated infected controls.

Lettuce plants infected with Sclerotinia sclerotiorum

Treatment of *S. sclerotiorum* infected lettuce plants with PPG at a concentration of 10 $g.L^{-1}$ and the combination of PPG and caffeine at the concentration of 25 $g.L^{-1}$ + 02.5 $g.L^{-1}$ respectively resulted in shoot mass comparable to that of the untreated uninfected control (Figure 3.5). Treatments with PPG at concentrations of 15 $g.L^{-1}$, 20 $g.L^{-1}$ and 25 $g.L^{-1}$ and the combinations of treatments with PPG and caffeine at concentrations of 5 $g.L^{-1}$ + 1 $g.L^{-1}$, 10 $g.L^{-1}$ + 1.5 $g.L^{-1}$, 15 $g.L^{-1}$ + 2 $g.L^{-1}$ and 25 $g.L^{-1}$ + 2.5 $g.L^{-1}$ respectively, gave shoot mass comparable to that of the benomyl treatment. The root mass of the uninfected control as well as treatments with benomyl, PPG at 10 $g.L^{-1}$ and all the combination treatments did not significantly differ. The percentage infection (Figure 3.6) decreased as the concentrations of the treatments increased for all treatments included. The lowest percentage root rot (Figure 3.6) was observed with treatments of benomyl, PPG at 10 $g.L^{-1}$ and the combination of PPG and caffeine at a concentration of 5 $g.L^{-1}$ + 1 $g.L^{-1}$ respectively. Lettuce plants infected with *S. sclerotiorum* resulted in a 50% reduction in shoot and root mass respectively when comparing the untreated uninfected and untreated infected controls.

Tomatoes infected with Phytophthora capsici

Treatment of *P. capsici* infected tomato plants with the combinations of PPG and caffeine at the concentrations of 5 $g.L^{-1}$ + 0.5 $g.L^{-1}$, 7.5 $g.L^{-1}$ + 0.75 $g.L^{-1}$, 10 $g.L^{-1}$ + 1 $g.L^{-1}$ and 12.5 $g.L^{-1}$ + 1.25 $g.L^{-1}$ respectively resulted in shoot mass comparable to that of the untreated uninfected control (Figure 3.7, 3.17). The treatment with the

combination of PPG and caffeine at a concentration of 7.5 $g.L^{-1}$ + 0.75 $g.L^{-1}$ resulted in root mass comparable to that of the uninfected untreated control. The shoot and root mass of the 2.5 $g.L^{-1}$ + 0.75 $g.L^{-1}$ PPG and caffeine combination were not significantly different from that of the commercial fungicide control (metalaxyl). The percentage root rot (Figure 3.8) for all combination treatments was zero although the percentage infection still remained between 57 and 85%. The treatments with PPG and caffeine individually did not effectively control the disease. Tomato plants infected with *Phytophthora* resulted in 90% reduction in shoot and root mass respectively when comparing the untreated uninfected and untreated infected controls.

Tomatoes infected with Sclerotium rolfsii

Treatment of *S. rolfsii* infected tomato plants with all but the treatments with 5 $g.L^{-1}$ and 10 $g.L^{-1}$ PPG, respectively, and the combination of PPG and caffeine at the concentration of 25 $g.L^{-1}$ + 3 $g.L^{-1}$ respectively resulted in shoot mass comparable to that of the untreated uninfected control (Figure 3.9). Treatments with 1 $g.L^{-1}$, 1.5 $g.L^{-1}$ 2 $g.L^{-1}$ and 3 $g.L^{-1}$ caffeine and the combination with PPG and caffeine at a concentration of 25 $g.L^{-1}$ + 3 $g.L^{-1}$ respectively resulted in root mass comparable to the infected untreated control while all other treatments resulted in root mass comparable to the uninfected untreated control. The percentage infection (Figure 3.10) decreased as the concentrations of the treatments increased for all treatments included. The percentage root rot of the tolchlofos-methyl treatment, the PPG treatments and the combination treatments did not differ significantly from each other. Tomato plants infected with *S. rolfsii* resulted in a 45% reduction in shoot mass and a 50% reduction in root mass when comparing the untreated uninfected and untreated infected controls.

Cucumber plants infected with Fusarium solani

Treatment of *F. solani* infected cucumber plants with the combinations of PPG and caffeine at the concentrations of 20 $g.L^{-1}$ + 2.5 $g.L^{-1}$ and 25 $g.L^{-1}$ + 3 g.L-1, respectively, resulted in shoot and root mass not significantly higher than that of the untreated uninfected control (Figure 3.11; 3.18). Treatments with caffeine at a concentration of 2 and 2 $g.L^{-1}$ as well as the PPG and caffeine combinations of 5 $g.L^{-1}$ + 1 $g.L^{-1}$, 10 $g.L^{-1}$ + 1.5 $g.L^{-1}$ and 15 $g.L^{-1}$ + 2 $g.L^{-1}$ resulted in shoot mass that did not differ significantly from the commercial fungicide, benomyl. PPG and caffeine combination treatments at the concentrations of 20 $g.L^{-1}$ + 2.5 $g.L^{-1}$ and 25 $g.L^{-1}$ + 3 $g.L^{-1}$, both resulted in a zero percent root rot (Figure 3.12), while the latter treatment also gave the lowest percentage infection of all infected plants. Cucumber plants infected with *F. solani* resulted in a 77% reduction in shoot mass and a 60% reduction in root mass when comparing the untreated uninfected and untreated infected controls.

Cucumber plants infected with Fusarium oxysporum

Treatment of *F. oxysporum* infected cucumber plants with combination treatments of PPG and caffeine as well as caffeine at concentrations of 1 and 1.5 $g.L^{-1}$ did showed disease control, giving shoot mass significantly higher than the infected control, which still only resulted in 50% of the potential mass when compared to the uninfected untreated control (Figure 3.13). Percentage infection decreased as the concentrations of the treatment compounds increased (Figure 3.14). Percentage root rot decrease for PPG and the PPG and caffeine combination treated plants as the concentrations of the treatments increased. Percentage root rot initially decreased after

treatments with caffeine between 1g.L^{-1} and 2g.L^{-1} and then increased for between treatments of 2g.L^{-1} and 3 g.L^{-1} Cucumber plants infected with *F. oxysporum* resulted in a 73% reduction in shoot mass and a 60% reduction in root mass when comparing the untreated uninfected and untreated infected controls.

Zucchini squash plants infected with Sphaerotheca fuliginea

Treatment of *S. fuliginea* infected zucchini plants with PPG significantly reduced the percentage infection (Figure 3.15). The lowest disease incidence was achieved with PPG at 12.5 g.L^{-1}. Treatments with 7.5 and 10 g.L^{-1} PPG and the PPG and caffeine combinations at concentrations of 2.5 g.L^{-1} + 0.25 g.L^{-1} , 5 g.L^{-1} + 0.5 g.L^{-1} and 7.5 g.L^{-1} and 0.75 g.L^{-1} resulted in a percentage infection not significantly different from that of the commercial fungicide treatment, triforine.

DISCUSSION

The antimicrobial activity of tea and tea polyphenols has been demonstrated before (Fukai *et al*., 1991; Arora and Bhardwaj, 1997. Harborne (1993) showed the antimicrobial properties of caffeine and polyphenols as part of the chemical defence mechanism in certain plants. However, no scientific publications courewqld be foiurewnd reporting on in vivo efficacy of tea extract or its' individual components against plant diseases.

The present study on the *in vivo* sensitivity of seven different fungi to PPG, caffeine and combinations thereof indicate that the combination of PPG and caffeine

is the most effective treatment followed by individual treatments with caffeine and then PPG. These results identified the most effective concentrations against the various pathogens assessed during the study.

The combination of Polyphenon G and caffeine effectively controlled *F. solani* on cucumber, *P. capsici* and *S. rolfsii* on tomato, *S. sclerotiorum* and *Pythium* F-group on lettuce. PPG individually significantly inhibited the development of *S. fuliginea* on zucchini squash plants. These results confirmed Arora and Ohlan (1997) *in vitro* work on the anti-fungal activity of *C. sinenis* and caffeine.

Phytotoxicity symptoms were observed in infected lettuce and cucumber plants treated with caffeine (applied as a soil drench) at concentrations of 2.5g.L^{-1} and higher. The results also show that in these instances, the combination of PPG and caffeine does appear to reduce the phytotoxicity of caffeine, while caffeine appears to augment the disease suppressing efficacy of PPG. On zucchini squash plants phytotoxicity was visible at a concentration of 1g.L^{-1} caffeine (applied as a foliar spray) and at higher concentrations, whether it was amended with PPG or not. These results indicate that phytotoxic concentrations are dependant on both crop type and method of application. A possible solution to the problem of phytotoxicity and still maintaining effective control could be to reduce the concentrations of the compounds whilst increasing the number of applications.

Cucumber plants inoculated with *F. solani* showed no disease symptoms when treated with a combination of PPG and caffeine. Cucumber plants infected with *F. oxysporum* showed an average loss in shoot mass of 50% when compared to the

uninfected control. These results indicate that treatments are spescie spesific and that individual host/pathogen trails will have to be preformed to accurately determine efficacy and treatment concentration.

All the pathogens tested during the greenhouse pot trials were inhibited by one or more of the applied treatments, with the exception of *F. oxysporum* which was less affected. In some instances the treatments only delayed the onset of disease. This is still of importance as a delay in the onset of disease could still affect the trial yield.

Comparing treatment compounds to fungicide controls it was observed that the combination of PPG and caffeine performed similar to or better than the commercial fungicide in six of the seven host/pathogen studies. PPG individually performed similar to or better than the commercial fungicide in four of the seven host/pathogen studies. Caffeine individually preformed similar to or better than the commercial fungicide in three of the seven host/pathogen studies.

The increasing incidence of resistance to fungicides by plant pathogens and the loss of existing chemical compounds for disease control are two important factors driving the need to search for new agricultural fungicides. In addition, the desire for safer agrochemicals with less environmental and human toxicity is a major concern. Particularly desirable is the discovery of novel anti-microbial agents representing new chemical classes that lack cross-resistance to chemicals currently used. Based on the results and earlier findings (Arora and Ohlan, 1997) in the *in vitro* activity of these compounds, we conclude that the PPG and caffeine combinations have potential to act as environmentally friendly plant disease control agents.

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Table 3.1

List of the fungal pathogens, their host plants and the diseases caused

Host	Pathogen	Disease
Cucumber (*Cucumis sativus* L.)	*Fusarium oxysporum*	Root rot
	Fusarium solani	Root rot
Lettuce (*Lactuca sativa* L.)	*Sclerotinia sclerotiorum*	Root rot
	Pythium F-group	Root rot
Tomato (*Lycopersicon esculentum* L.)	*Sclerotium rolfsii*	Root and stem rot
	Phytophthora capsici	Root rot
Zucchini (*Cucurbita pepo* L.)	*Sphaerotheca fuliginea*	Powdery mildew on leaves

Table 3.2

Applied treatments and included controls for each phytopathogenic fungi used during *in vivo* studies

Pathogen	Treatments ($g.L^{-1}$)					
	Uninfected control	Infected control	Fungicide control (a.i.)	PPG	Caffeine	Combinations of PPG and Caffeine
Fusarium oxysporum	Water*	Water	Benomyl 0.1	5, 10, 15, 20, 25	1, 1.5, 2, 2.5, 3	5:1, 10:1, 15:1.5, 20:2, 25:2.5
Fusarium solani	Water	Water	Benomyl 0.1	5, 10, 15, 20, 25	1, 1.5, 2, 2.5, 3	5:1, 10:1, 15:1.5, 20:2, 25:2.5
Phytophthora capsici	Water	Water	Metalaxyl 0.1	2.5, 5, 7.5, 10, 12.5	0.25, 0.5, 0.75, 1, 1.25	2.5:0.25, 5:0.5, 7.5:0.75, 10:1, 12.5:1.25
Pythium F-group	Water	Water	Propamocarb 0.1	2.5, 5, 7.5, 10, 12.5	0.25, 0.5, 0.75, 1, 1.25	2.5:0.25, 5:0.5, 7.5:0.75, 10:1, 12.5:1.25
Sclerotinia sclerotiorum	Water	Water	Benomyl 0.1	5, 10, 15, 20, 25	1, 1.5, 2, 2.5, 3	5:1, 10:1, 15:1.5, 20:2, 25:2.5
Sclerotium rolfsii	Water	Water	Tolclofos-methyl 0.1	5, 10, 15, 20, 25	1, 1.5, 2, 2.5, 3	5:1, 10:1, 15:1.5, 20:2, 25:2.5

- PPG = Polyphenon G
- a.i. = active ingredient

* Tap water

Figure 3.1

Colonies of *Pythium* growing from root segments of inoculated lettuce plants after plating on BNPRA medium

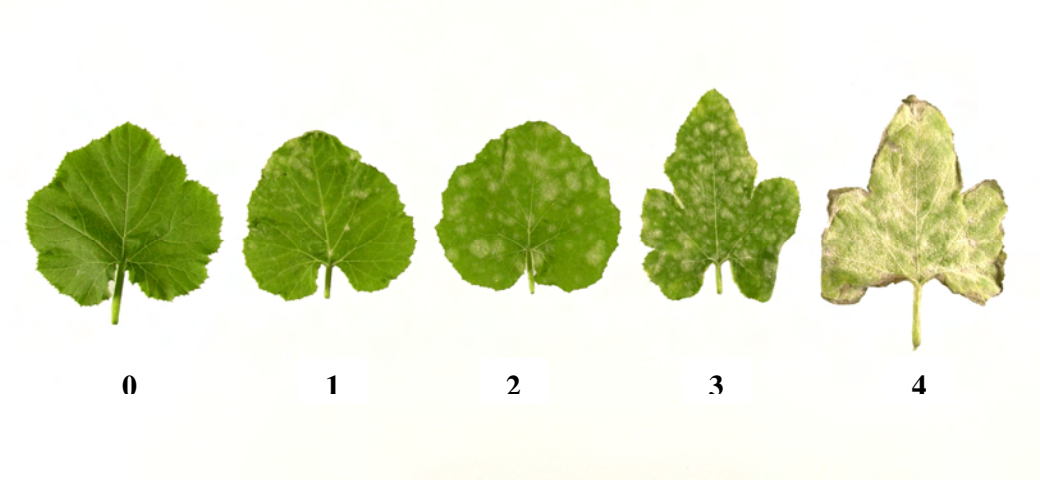

Figure 3.2

Disease rating scale used for zucchini squash plants infected with *S. fuliginea*. 0 = healthy, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% disease severity

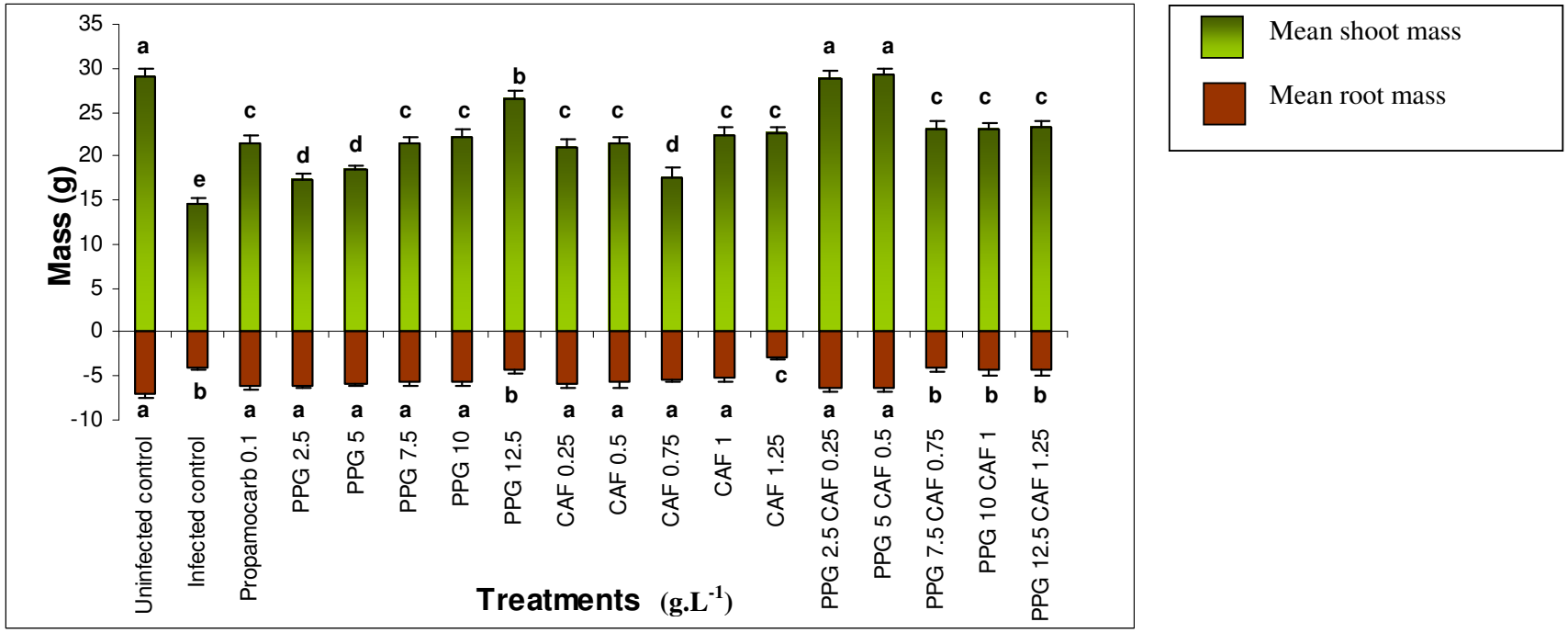

Figure 3.3

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Pythium* F-group inoculated lettuce plants under greenhouse conditions. Uninfected and infected controls were included. Propamocarb treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

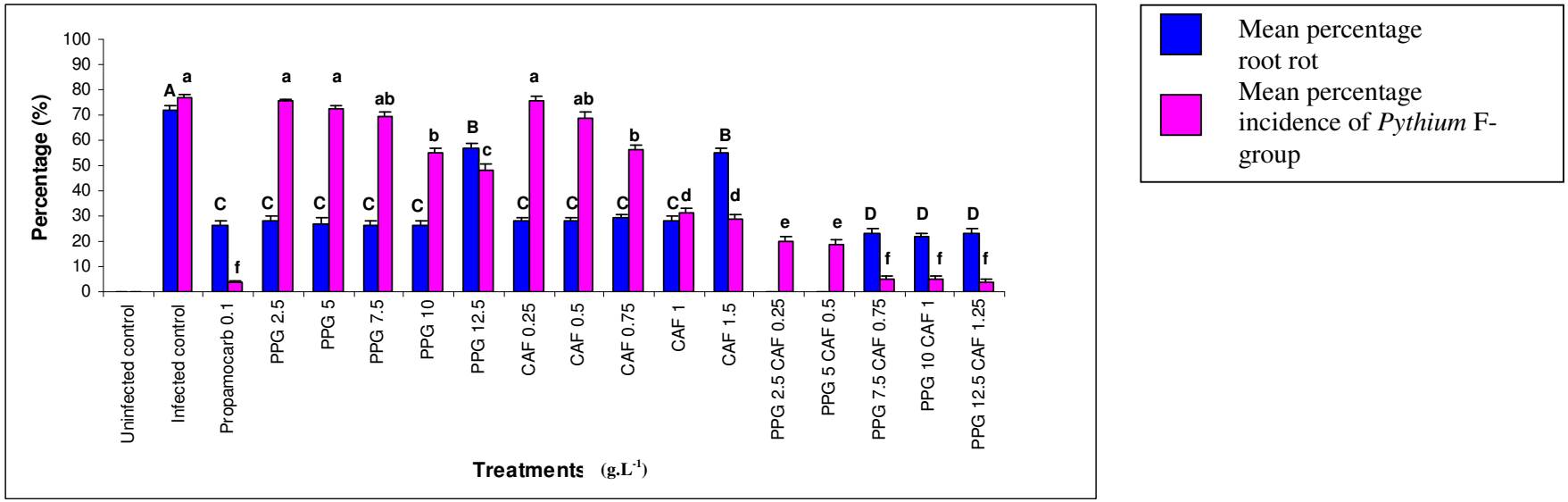

Figure 3.4

Percentage root rot and percentage infection on lettuce plants inoculated with *Pythium* F-group. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Propamocarb treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

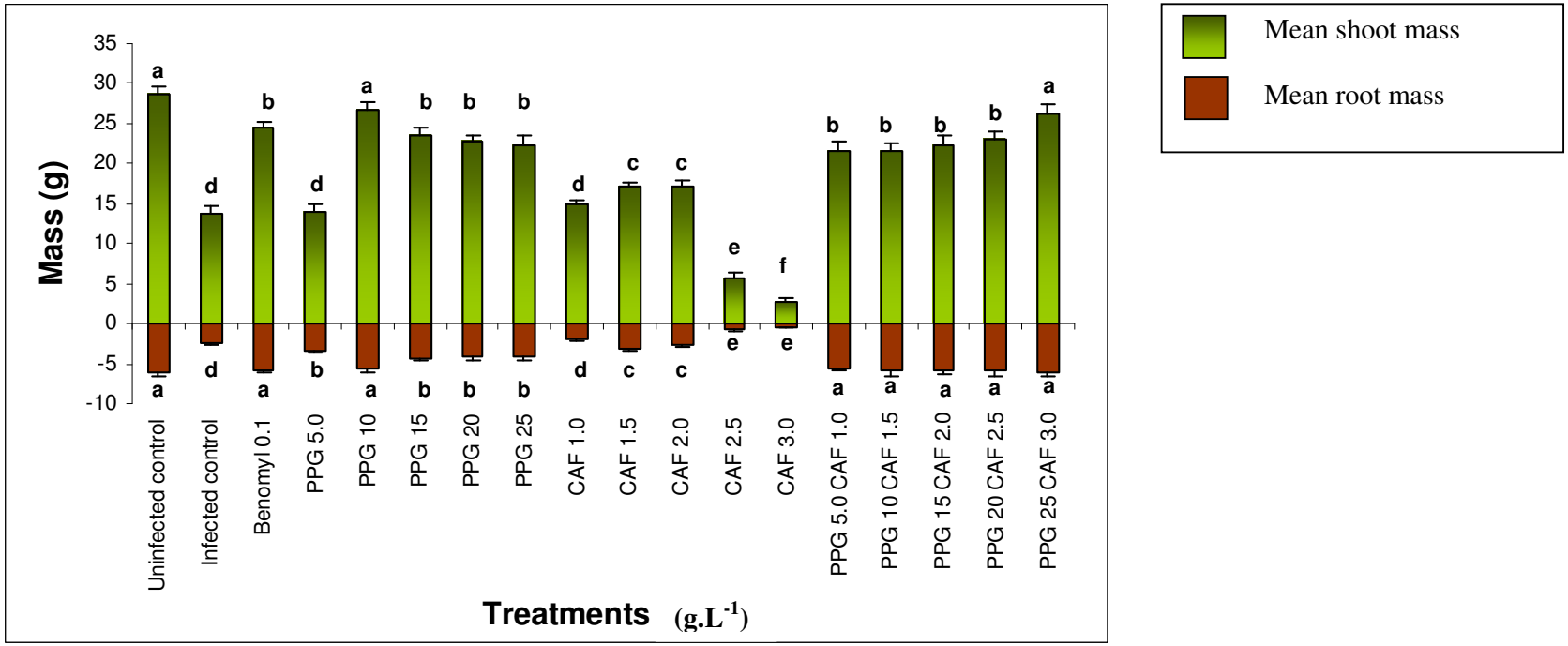

Figure 3.5

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Sclerotinia sclerotiorum* inoculated lettuce plants under greenhouse conditions. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

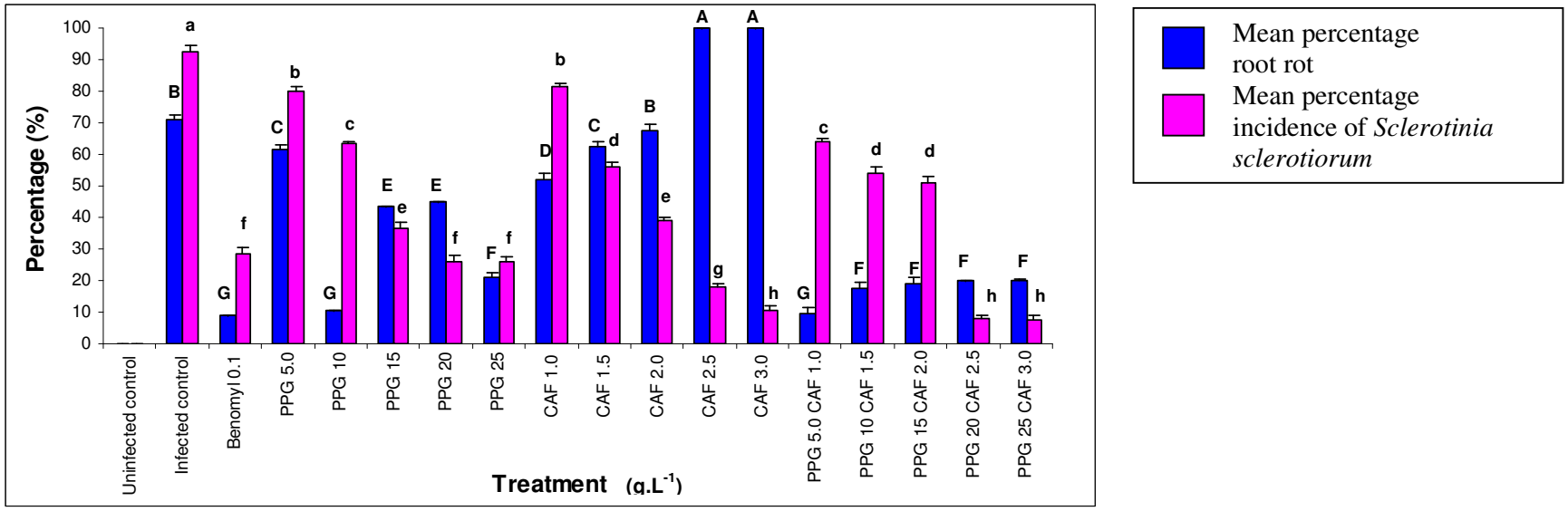

Figure 3.6

Percentage root rot and percentage infection on lettuce plants inoculated with *Sclerotinia sclerotiorum*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

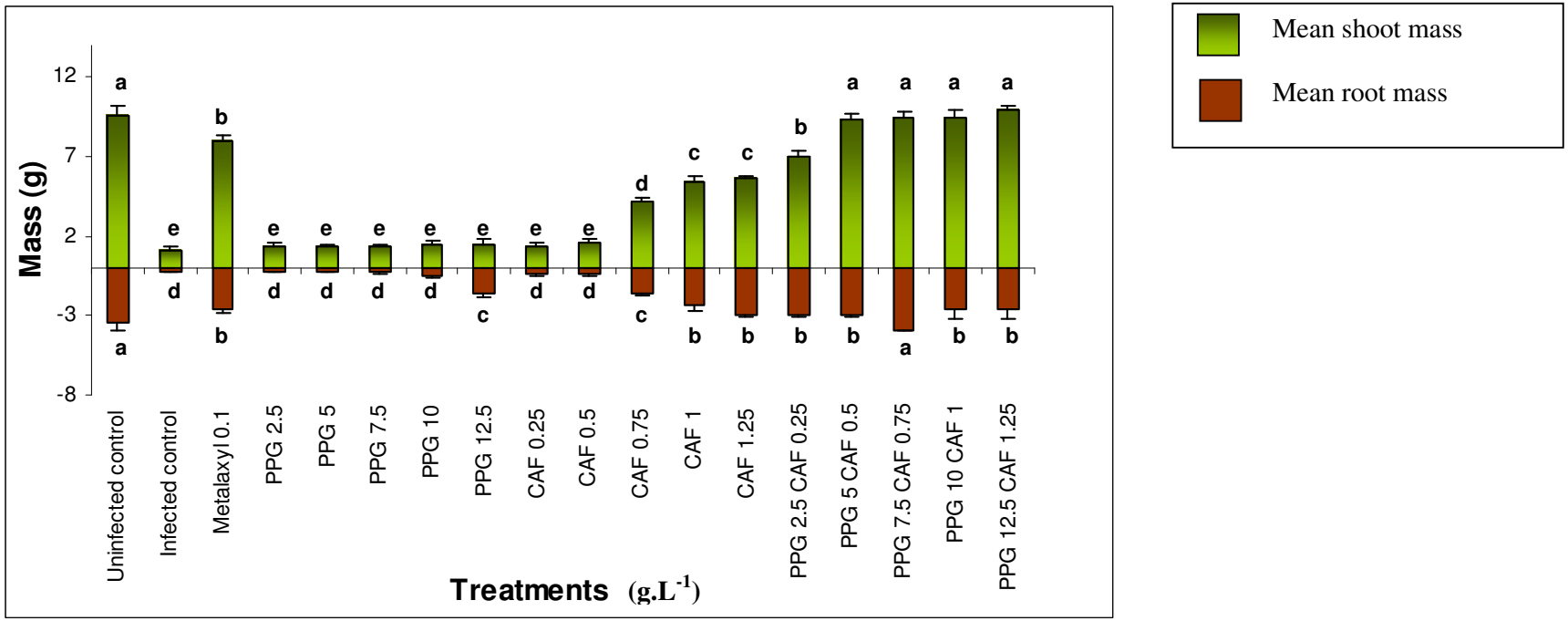

Figure 3.7

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Phytophthora capsici* inoculated tomato plants under greenhouse conditions. Uninfected and infected controls were included. Propamocarb treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

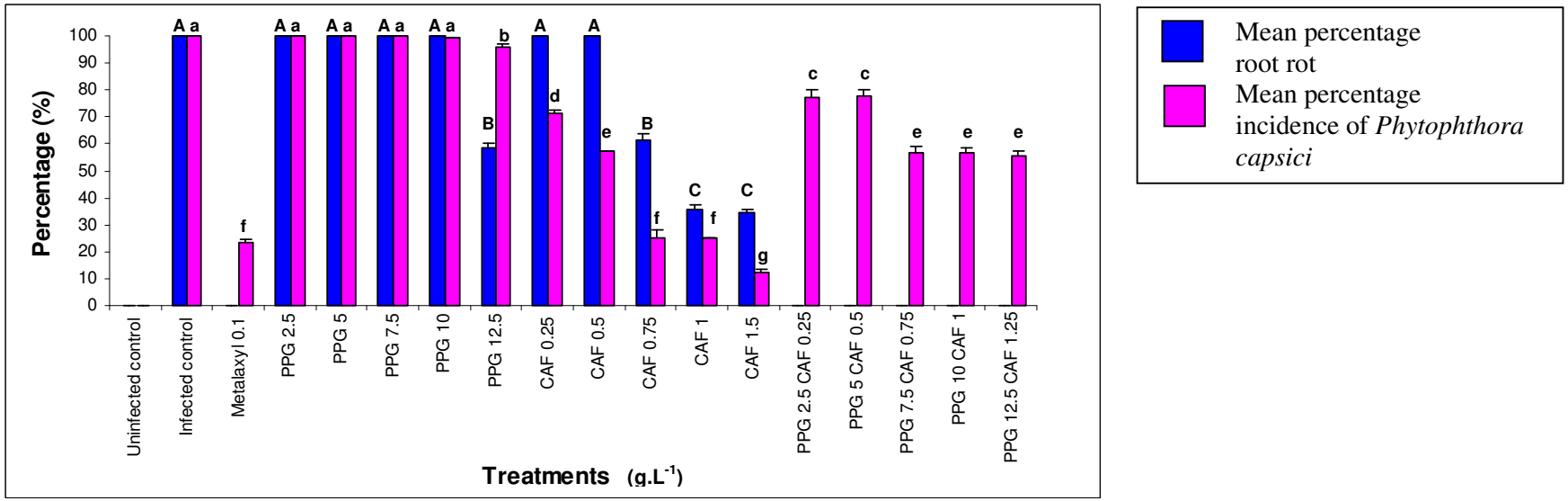

Figure 3.8

Percentage root rot and percentage infection on tomato plants inoculated with *Phytophthora capsici*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Metalaxyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

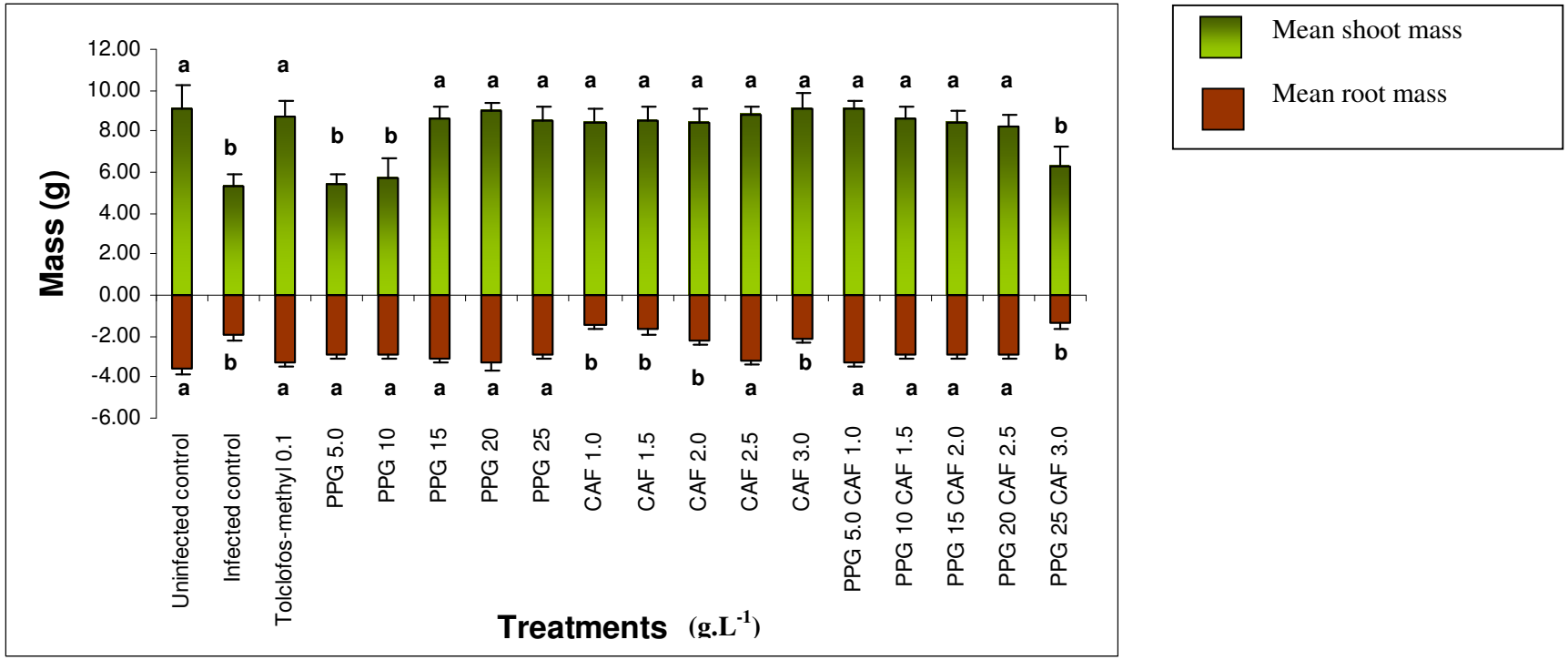

Figure 3.9

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Sclerotium rolfsii* inoculated tomato plants under greenhouse conditions. Uninfected and infected controls were included. Propamocarb treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

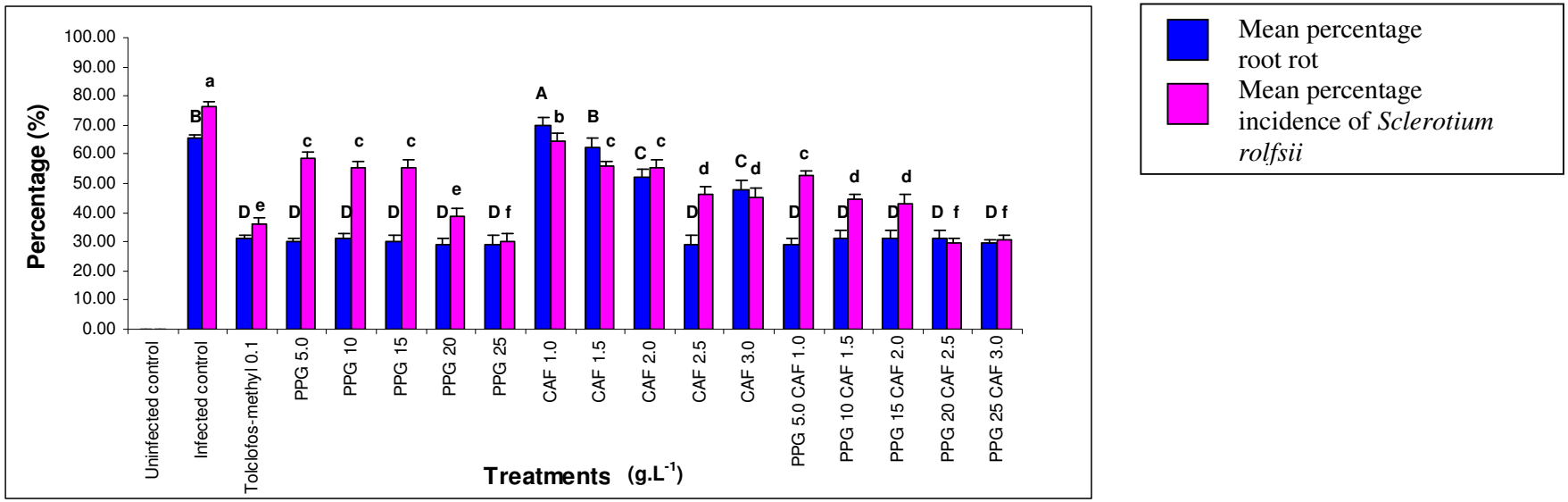

Figure 3.10

Percentage root rot and percentage infection on tomato plants inoculated with *Sclerotium rolfsii*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Tolclofos-methyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

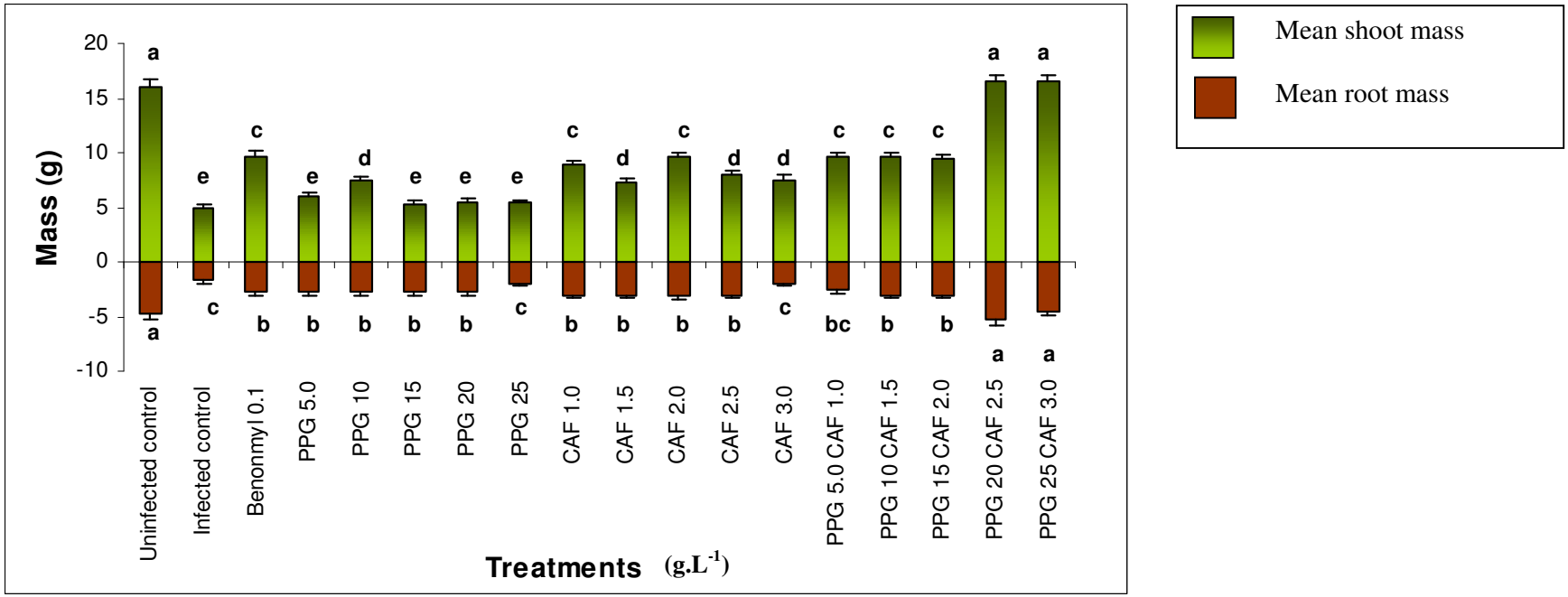

Figure 3.11

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Fusarium solani* inoculated cucumber plants under greenhouse conditions. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

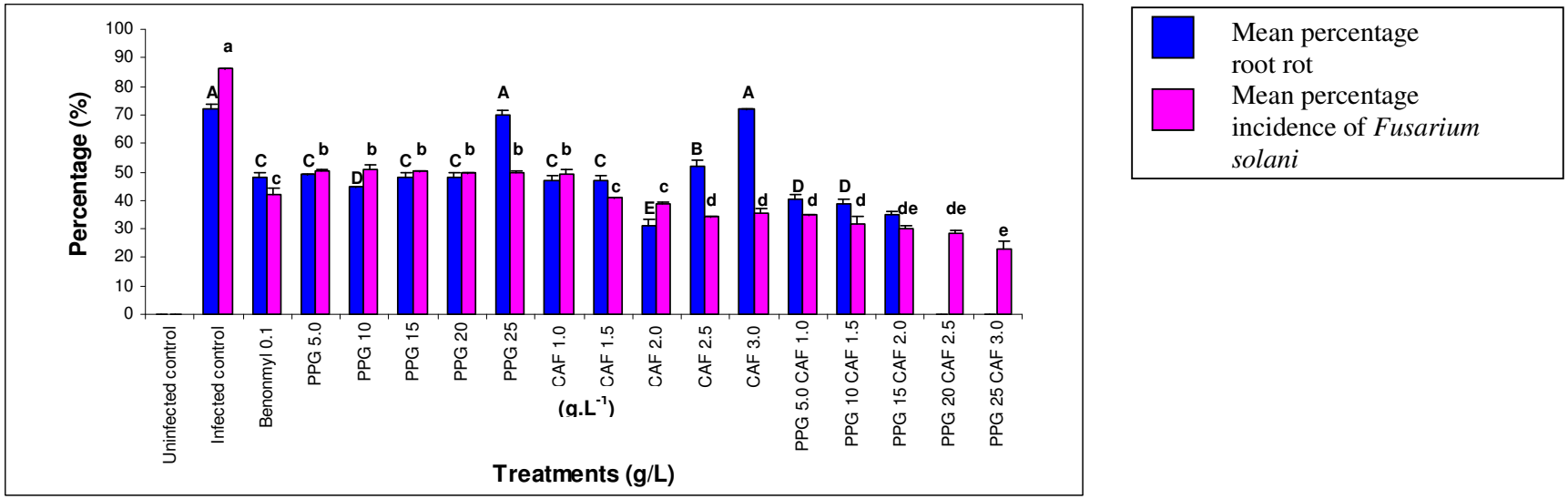

Figure 3.12

Percentage root rot and percentage infection on cucumber plants inoculated with *Fusarium solani*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

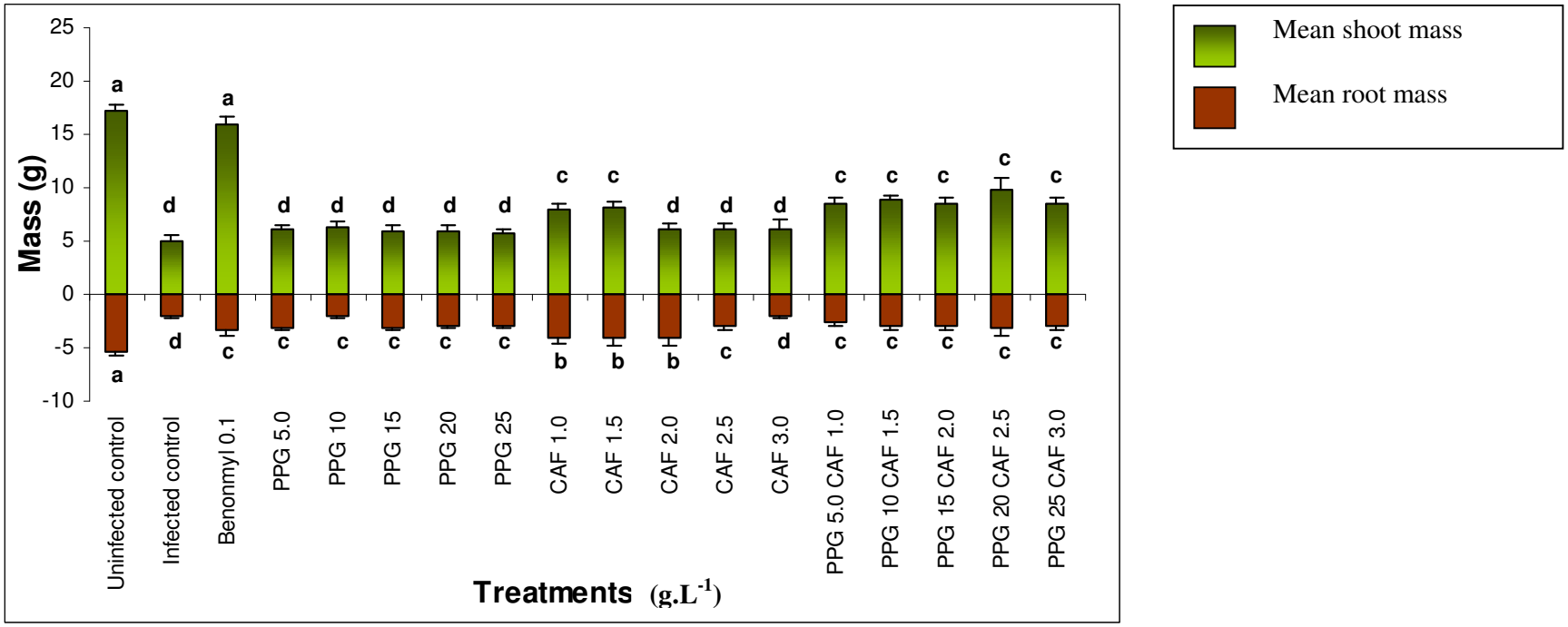

Figure 3.13

Effect of soil drench treatments with Polyphenon G (PPG), caffeine (CAF) and combinations of the two compounds on fresh shoot and root mass of *Fusarium oxysporum* inoculated cucumber plants under greenhouse conditions. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

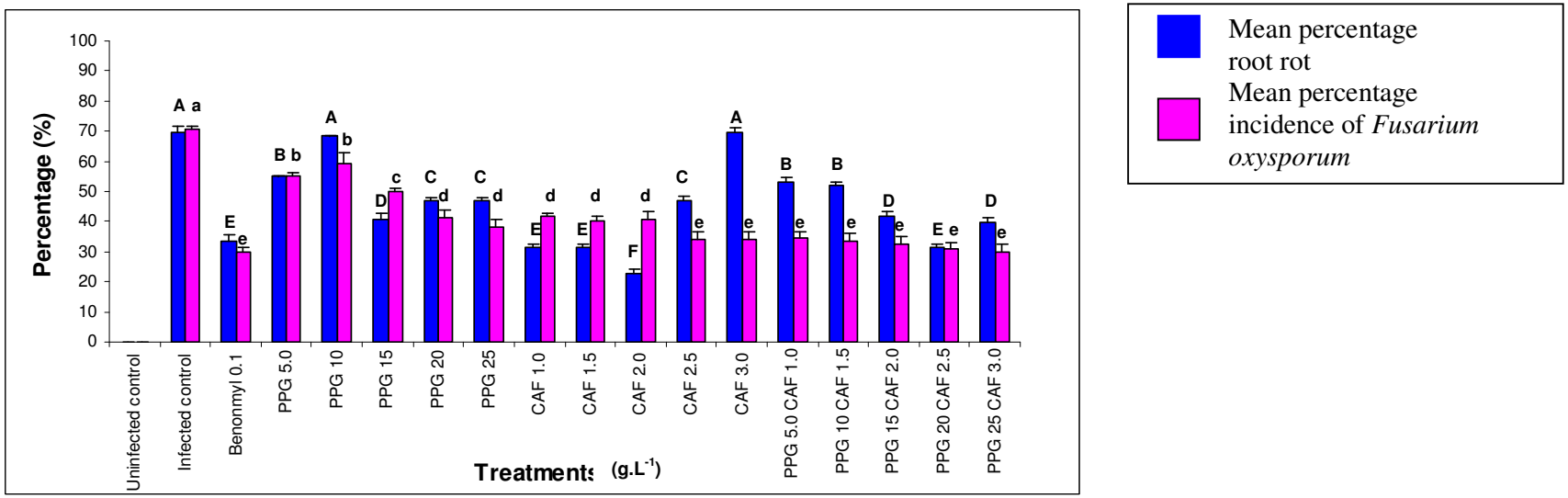

Figure 3.14

Percentage root rot and percentage infection on lettuce plants inoculated with *Fusarium oxysporum*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Uninfected and infected controls were included. Benomyl treatment was included as a commercial fungicide standard. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test). Lower case – percentage incidence. Upper case – percentage root rot

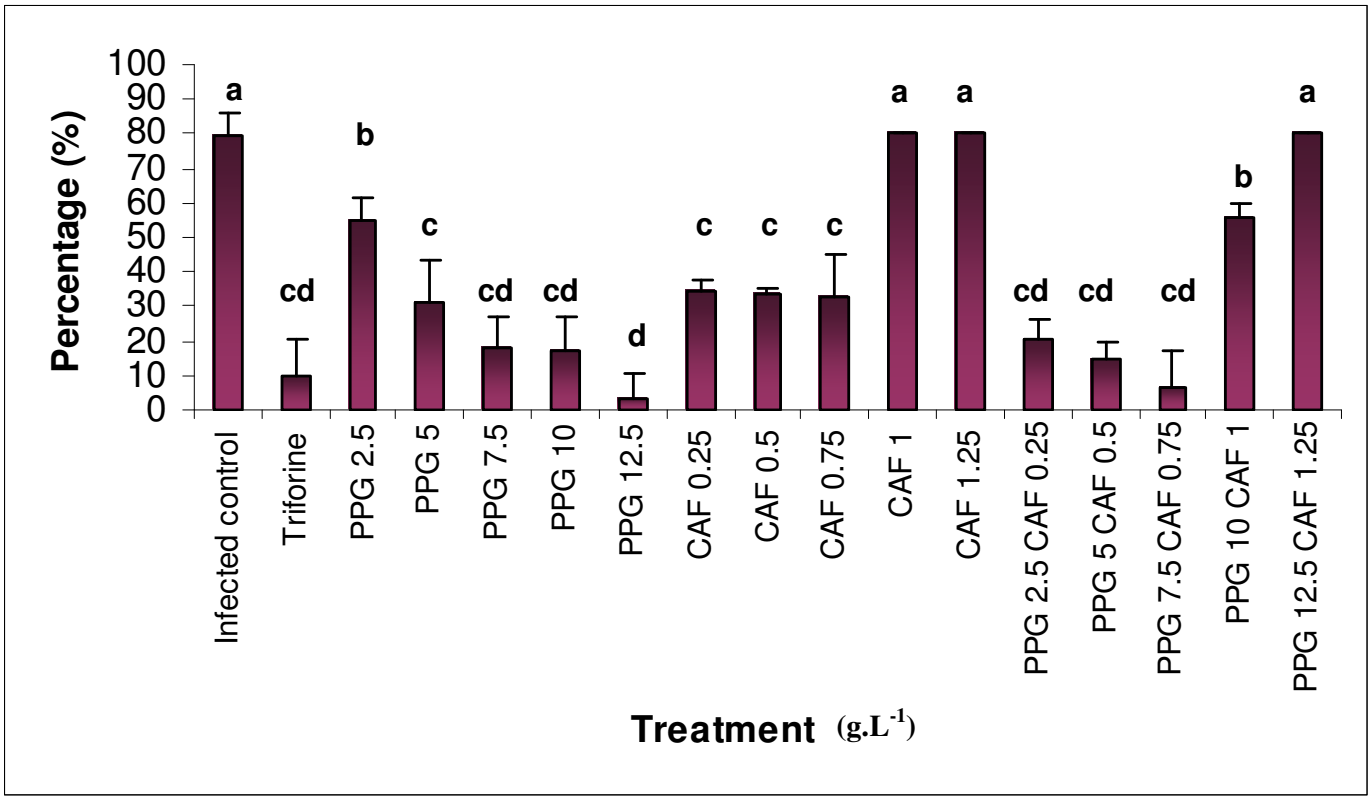

Figure 3.15

Mean percentage disease incidence on zucchini squash plants infected with *Sphaerotheca fuliginea*. Treatments included Polyphenon G (PPG), caffeine (CAF) and mixtures of the two compounds. Infected untreated plants and a commercial fungicide (triforine) treatment were included as controls. Columns represent the means of three independent experiments with eight plants per treatment per experiment. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$ using the F-test)

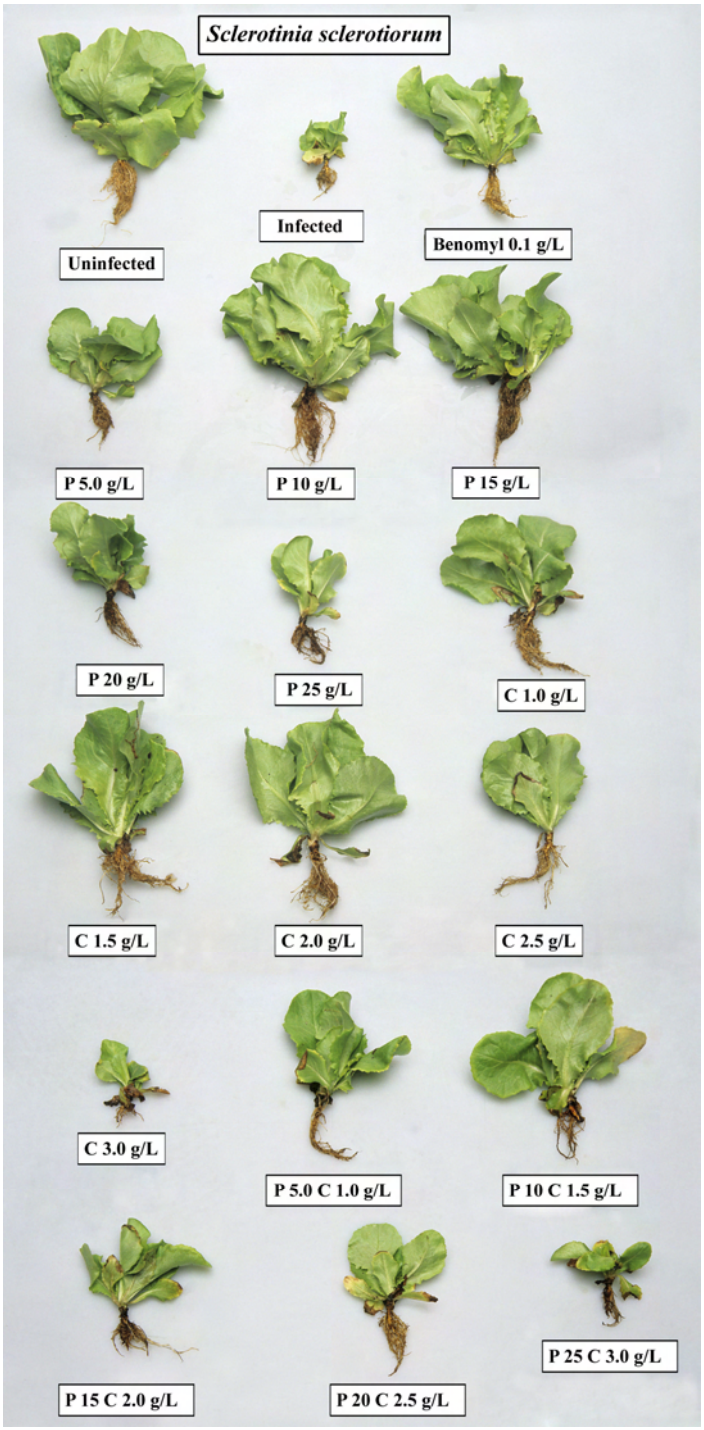

Figure 3.16

Effect of soil drench treatments with Polyphenon G (P), caffeine (C) and combinations of the two compounds on lettuce plants inoculated with *Sclerotinia sclerotiorum*. Concentrations of compounds are indicated on labels

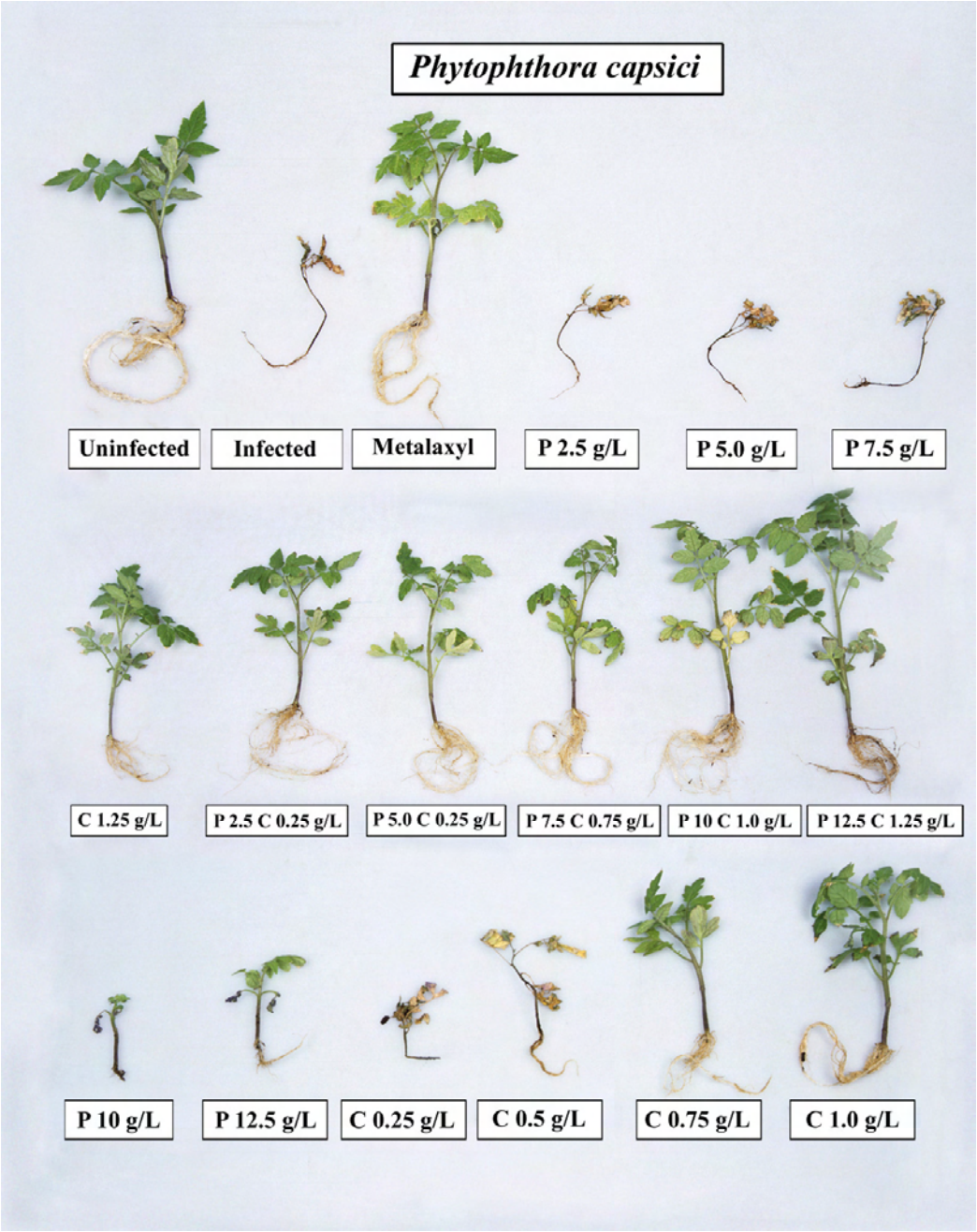

Figure 3.17

Effect of soil drench treatments with Polyphenon G (P), caffeine (C) and combinations of the two compounds on tomato plants inoculated with *Phytophthora capsici*. Concentrations of compounds are indicated on labels

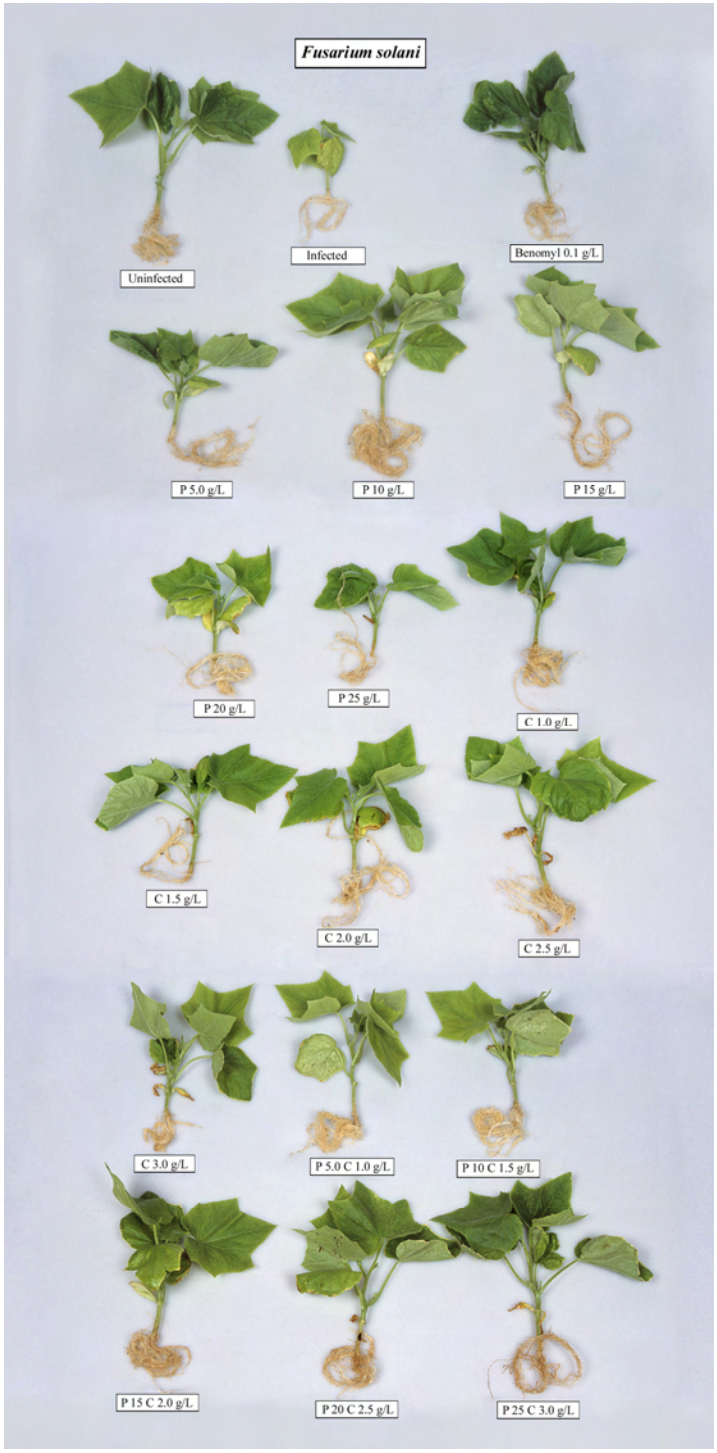

Figure 3.18

Effect of soil drench treatments with Polyphenon G (P), caffeine (C) and combinations of the two compounds on cucumber plants inoculated with *Fusarium solani*. Concentrations of compounds are indicated on labels

CHAPTER 4

Induction of Defence Reactions in Tomato and Lettuce Plants by Application of Tea (*Camellia sinensis*) Extracts and Caffeine

ABSTRACT

One of the major problems concerning the production of food crops is the difficulty of controlling plant diseases. One approach to the control of plant diseases is through the induction and enhancement of the plant's own defence mechanisms which would not involve the application of toxic compounds to plants. Recently, the activity of Polyphenon G (PPG) and caffeine against phytopathogenic fungi has been demonstrated (Chapter 2 and 3). During the current study the aim was to investigate induced resistance as a possible mode of action in tomato and lettuce plants. Total phenolic content (TPC) was determined in root and shoot extracts of tomato and lettuce plants after treatment with PPG, caffeine and combinations of PPG and caffeine as well as a commercial plant defence inducing compound (fosetyl-al for tomatoes and potassium phosphonate for lettuce) as a positive control. Samples were taken at 0, 24, 48 and 96 hours after treatments were applied. In the case of lettuce *Pythium* inoculated plants were also included. The TPC assay showed an increase in phenolic levels in root extracts from tomato and lettuce plants for all treatments when compared to the untreated plants. TLC analyses coupled with a bioassay revealed the presence of a fungitoxic compound in all infected lettuce samples as well as the uninfected combination treatment and potassium phosphonate treatment. The fungitoxic compound was identified as ferulic acid by comparing Rf-values to those of standards and by retention time on reversed phase HPLC. The presence of salicylic acid and caffeic acid in selected root and shoot samples was also indicated by HPLC. These results confirm that tomato and lettuce plants treated with PPG, caffeine and a combination of PPG and caffeine can induce compounds in plants similar to those produced by commercial defence system inducers.

INTRODUCTION

Application of chemicals to plants in order to prevent or inhibit disease development is a fundamental means of managing diseases caused by fungi. However, these chemical methods of disease management are expensive and can detrimentally affect the ecosystem. The obvious pollution problems due to indiscriminate use of synthetic pesticides and their toxic effect on non-target organisms have prompted investigations on exploiting pesticides of plant origin. Resistance to fungicides in plant pathogen populations is one of the most significant problems in the area of chemical disease management. The use of fungicides will continue to play a major role in disease management for the foreseeable future, and development of strategies for resistance management will be necessary to maintain a useful arsenal of the most effective fungicides. Such strategies are required if we are to prolong the useful life of these disease control agents. Natural plant products are important sources of new agrochemicals for the control of plant diseases (Cardellina, 1988; Gulter, 1988). Furthermore, biocides of plant origin are non-phytotoxic, systemic, easily biodegradable and possibly augment existing fungicides (Mason and Mathew, 1996; Qasem and Abu-Blan, 1966)

Knowledge of the effectiveness of particular compounds is important for achieving effective disease control. Equally important is an understanding of the underlying physiological mode of action of plant disease management materials (Schmourlo *et al*, 2005). Investigations into mechanisms of disease suppression by plant products have suggested that these plant products may either act on the pathogen directly (Amadioha, 2000), or induce systemic resistance in host plants resulting in

reduction of disease development (Olivieri *et al*, 1996; Narwal *et al*, 2000; Paul and Sharma, 2002; Schneider and Ullrich, 1994).

The main anti-microbial activity of *C. sinensis* extracts has been contributed to caffeine and the catechins present in these extracts. Caffeine is a known inhibitor of normal cell division in plant and animal cells that often results in binucleate cells (Aneja and Gianfagna, 2001), thus inhibiting spore germination and growth of microbes. Gallated catechins (EGCg and ECg) have been reported to deactivate proteins and disrupt the bacterial lipid bilayer by changing the membranes' fluidity and morphology (Ikigai *et al*, 1993). In an effort to find alternative or additive modes of action, we investigated induced resistance as possible mechanism of action of Polyphenon G (PPG) and caffeine as well as combinations of the two compouds.

Induced mechanisms have received considerable attention, having been studied by means of molecular, biochemical and phytochemical methods (Ng *et al*., 1987, Wu *et al*., 1995; Zang and Lewis, 1997, Romani *et al*, 2002). Induced resistance is the phenomenon whereby a plant, when given an appropriate stimulation by harmless inducers, will become able to combat more efficiently the infection by virulent pathogens through enhancement of its naturally inherent defence mechanisms. It is our hypothesis that the profile of phytochemicals that are present in tea (*Camellia sinensis* (L.), Kuntze) present a broad spectrum of anti-microbial ability and potentially can induce disease resistance in plants.

The present study was thus undertaken to determine if plants treated with PPG (*C. sinensis* extract), caffeine and mixtures of the two compounds increases the total

soluble phenolic content in plants using the Folin-Ciocalteu method. Thin layer chromatography bioassays were used for the detection of active anti-fungal compounds in plant extract while changes in phenolic profiles were analyzed using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Inoculum preparation

Pythium F-group and *Cladosporium cladosporioides* (Fres.) de Vries were obtained from the University of Pretoria culture collection and maintained on potato dextrose agar (PDA) (Biolab, Merck) plates.

Pythium F-group was grown on 90 mm diameter PDA plates at 25ºC until the plates were completely covered. Millet (*Penniselum glauceam* L.) seeds (200g) were steeped in water (200 ml) for 24h, transferred to autoclavable polyethylene bags, and autoclaved twice for 45min at 120°C on two consecutive days. Ten 5 mm diameter agar discs were cut with a cork borer from the actively growing edge of each fungal colony. Mycelial discs were aseptically transferred to each of the sterilized millet seed bags and the bags were incubated for 28 days at 25°C. The millet seed inoculum ($100g.L^{-1}$ soil) was added to the soil (Weideman and Weher, 1993).

Soil preparation

Sandy loam soil (Conradie Organics, Pretoria, Gauteng, South Africa) and potting soil consisting of composted kraal manure and bark (Just Nature Organics, Pretoria, Gauteng, South Africa) was steam pasteurized separately and mixed in a 1:1 ratio (v:v) based on results obtained during preliminary trials.

Plant preparation

Lettuce (*Lactuca sativa* L., cv Nadine) and tomato (*Lycopersicon esculentum* Mill., cv Money Maker) seeds were obtained from Hygrotec (Pretoria-North, Gauteng, South Africa). The tomato and lettuce seeds were germinated and grown at a commercial nursery (Multiplant, Brits, North-West, South Africa) until five weeks old.

The five week old tomato plants were transplanted into seedling trays in the soil mixture and drenched with 7 ml/cell of (i) 12.5 $g.L^{-1}$ Polyphenon G, (ii) 1.25 $g.L^{-1}$ caffeine (Sigma), a mixture of (iii) 12.5 $g.L^{-1}$ Polyphenon G and 1.25 $g.L^{-1}$ caffeine, and (iv) 100 $ml.L^{-1}$ fosetyl-al WP 800 $g.kg^{-1}$ active ingredient (a.i.) (Aliette, Rhone-Poulenc, RSA) respectively. Untreated (v) (treated with water only) plants were included as controls. Untreated tomato plants were harvested at time 0h. Thereafter plants from all treatments were harvested at 24h, 48h and 96h respectively. Fourty plants with an approximate combined fresh weight of 35g were harvested per treatment per day. A total of 640 plants were used per experiment. Plant roots and

shoots were separated and frozen at -4 ºC before being freeze dried for further analysis. Each experiment was repeated once.

Lettuce plants were transplanted into seedling trays in the above mentioned soil mixture. Greenhouse temperatures were maintained between 23 and 28 ºC, with ambient humidity and natural daylight for the duration of the experiment. Inoculation of specific plants with *P.* F-group was done 24h before treatment of the plants with the various compounds. Time 0h started after the 24h infection period and untreated (treated with water only) lettuce plants were harvested at this time. Uninfected and infected plants were drenched with 7 ml/cell of (i) 12.5 $g.L^{-1}$ Polyphenon G, (ii)1.25 $g.L^{-1}$ caffeine, the mixture of (iii) 12.5 $g.L^{-1}$ Polyphenon G and 1.25 $g.L^{-1}$ caffeine, (iv) and 100 $ml.L^{-1}$ potassium phosphonate 200$g.L^{-1}$ a.i. SL (Phytex, Horticura, RSA) respectively. Uninfected and infected controls (treated with water only) were also included. Thereafter plants from all treatments were harvested at 24h, 48h and 96h respectively. Thirty plants with an approximate combined weight of 50g were harvested per treatment per day. A total of 930 plants were used per experiment. Plant roots and shoots were separated and frozen at -4 ºC before being freeze dried for further analysis. Each experiment was repeated once.

Extractions

For extraction of root tissue, freeze dried roots were ground using a mortar and pestle. The ground root tissue (0.25g) was then added to 5ml of methanol (MeOH) containing 0.1% butylated hydroxyl-tolune (BHT, Sigma) and then sonicated in a sonifer bath for 30 minutes. The suspensions were subsequently centrifuged at 3000

rpm for 10 min. The supernatants were used as the test solution (Simonovska *et al*, 2003).

For the extraction of leaf tissue, freeze dried leaves were ground using a mortar and pestle and 8ml methanol was then added to 1.0g of the ground leaf tissue. The suspension was then sonicated (50% duty cycle; power setting 3 microtip on Branson 30 sonifier) for 5min. The solutions were vacuum filtered once through Whatman No 1 filter paper and the MeOH was removed by roto-evaporation at 70±2 °C (Rémus-Borel *et al*, 2005). After evaporation, extracts were dissolved in 12ml water. Chlorophyll was removed during extractions (3 x 6 ml) with petroleum ether (Analar, Merck), retaining the bottom layer. The phenolic compounds were then extracted (5 x 6ml) with ethyl acetate (Analar, Merck), retaining the top layer (Simonovska *et al*, 2003).

Total phenolic content

Total phenolic content (TPC) of the extracts were determined using the Folin–Ciocalteau reagent (Sigma) (Yu *et al*, 2003). The volumes were modified for the wells in microtiter plates. Each reaction mixture contained 5µl sample solution, 175µl distilled-deionized water, 25µl Folin–Ciocalteau reagent and 50µl of aqueous sodium carbonate (Na_2CO_3) ($20g.100ml^{-1}$). The reagent blank contained 5µl distilled deionised water instead of sample. The solution of each well was mixed with a micropipette until the yellow color faded. Plates were then incubated in a microplate incubator at 40°C for 30min. The absorbency of the various extracts was measured with a Multiscan Ascent VI 24 354-00973 (version 1.3.1) at 690 nm. The phenolic

content was expressed in mg gallic acid equivalents (GEA)/ ml extract from the standard curve using the equation:

$$GAE/ml\ extract = 1.3527x + 0.0109\ (R^2 = 0.9989) \quad \text{Equation 1}$$

Absorbency at 690 nm

TPC was expressed as (GAE) and calculated as a percentage of the dry weight using the following equation:

$$mg\ GAE/g\ dry\ weight = \left[\frac{GAE\ mg/ml}{M} \times V \right] \times 100 \quad \text{Equation 2}$$

V = Volume of original sample in ml

M = Mass of original sample in g

The analysis was repeated for each sample, i.e., four independent experiments, with three replicates per sample.

Thin-layer chromatography (TLC) bio-autography

The TLC bio-autography was preformed on all the samples. Thin-layer chromatography was preformed on Silica gel 60 (Merck) plates. Plates were developed in chloroform-methanol (1:1, v/v) to the top and dried for 30min at 110 ºC

to activates the plates. Each sample (0.1ml) was spotted on a line 10mm from the bottom of the TLC plates. Plates were developed in a developing chamber using n-hexane : ethyl acetate : formic acid (20:19:1) (Simonovska *et al*, 2003). Salicylic acid (Sigma), Caffeic acid (Sigma) and Ferulic acid (Fluka) at concentrations of 20 $g.L^{-1}$, $5g.L^{-1}$ and 2.5 $g.L^{-1}$ respectively, were included in the study after these compounds were identified through HPLC to be present in the extracts.

After the TLC plates were developed, they were assessed for the presence of anti-fungal compounds according to the bio-assay procedure decribed by Homans and Fuchs, (1970). A suspension comprising of 7g KH_2PO_4 (Merck), 3g $Na_2HPO_4 \bullet 2H_20$ (Merck), 4g KNO_3 (Merck), 1g $MgSO_4 \bullet 7H_20$ (Merck), 1g NaCl (Merck) per litre of tap water was prepared and autoclaved at 121 ºC for 20min. Ten ml of a 30 % aqueous solution of glucose was added per 60ml of solution. The suspension was poured onto an actively growing colony of *C. cladiosporiodes* on PDA plates and the spores were loosened using an etuleur. Conidia in the resulting suspension were counted with a hemacytometer, adjusted to 2×10^7 spores/ml and transferd to a 200ml spray bottle (Zainuri *et al*, 2001).

Developed chromatograms were sprayed with the spore suspensions of *C. cladosporioides* and subsequently incubated at 100% RH and 25 °C for 3–6 days. The presence of anti-fungal activity was recorded as zones of fungal inhibition where mycelial growth and sporulation was inhibited (Klarman and Stanford, 1968 , Terry *et al*, 2004). The Rf-values (Equation 3) of inhibition zone were determined by taking measurements from the line on which the compound was spotted (origin) to the distance the compound moved (middle of inhibition zone).

$$R_F = \frac{\text{Distance moved by sample spot from origin}}{\text{Distance moved by solvent front from origin}}$$ Equation 3

For each sample the experiment was repeated twice i.e. for the two independent plant experiments two samples were obtained per treatment and each sample was then used three times in this assay.

High performance liquid chromatography (HPLC)

The extract samples described above were diluted in a 1:1 ratio with distilled water and methanol (1:1). HPLC analyses were preformed on all the 48h treatment extracts by manually injecting 20 µl of the filtrate onto the HPLC column. The chromatographic system used was an Agilent 1100 Series consisting of a QuatPump high pressure pumps and a Phenomenex Luna 3u C18 (2) column. The diode array detector was attached to an analytical computer with a data storage system (ChemStation LC). The mobile phase consisted of water buffer (pH 2.6) and acetonitrile (Saarchem).

STATISTICAL ANALYSIS

Significant differences were determined using Fisher's protected least significant difference test, and are indicated on the graphs using letter notation. The comparison wise error rate was $P<0.05$. Standard error of the means are indicated as error bars on the graphs.

RESULTS

Total phenolic content

Tomato and lettuce shoot samples showed no increase in TPC for any of the treatments. However, significant increases in TPC were observed for both tomato (Figure 4.1) and lettuce (Figure 4.2) root samples for all treatments applied.

The tomato root samples (Figure 4.1) showed no significant increase or decrease in TPC for untreated plants over a 96h period. Treatment with fosetyl-al and PPG respectively resulted in the most significant increase in TPC, followed by caffeine and the combination of PPG and caffeine. The TPC of root samples treated with fosetyl-Al, PPG and caffeine respectively did not increase significantly, within these treatment groups, over a 96 h period. Root samples treated with a combination of PPG and caffeine showed a significant increase between 24h and 48h but no significant increase between 48h and 96h after treatment.

Lettuce root samples that were untreated and uninfected showed a significant increase in TPC over a 24h period, although not statistically significant between 24 and 96h (Figure 4.2). Untreated lettuce plants inoculated with *Pythium* showed no initial increase in TPC over the first 48h but showed a decreased between 48 and 96h. Potassium phosphonate treatment gave the highest TPC followed by the combination of PPG and caffeine, PPG and caffeine in decreasing order. PPG treated root samples showed a higher overall TPC for infected than uninfected plants. Uninfected root samples of PPG treated plants showed a significant increase in TPC over the first 48h

period and a subsequent significant decrease between 48 and 96h. No significant change in TPC was observed in the roots of infected lettuce plants treated with PPG over the 96h period. Root samples of caffeine treated plants showed a higher TPC for infected than uninfected plants. Root samples of plants treated with a combination of PPG and caffeine did not differ significantly in TPC between uninfected and infected plants. Uninfected and infected plants treated with a combination of PPG and caffeine resulted in no significant increase between a 24h and 48h period but subsequently increased between 48h and 96h. Root samples of plants treated with Potassium phosphonate did not significantly differ for uninfected and infected samples.

Thin-layer chromatography bio-autography

The data is presented in Table 4.1. The standards, introduced subsequently to HPLC identification, had the following R_F values: 0.64 for salicylic acid, 0.44 for ferulic acid and 0.22 for caffeic acid. Clear inhibition zones were present at R_F-values between 0.42 and 0.45 for all lettuce plants infected with *P.* F-group, independent of the treatment. Clear zones were also visible for uninfected plants treated with the combination of PPG and caffeine as well as plants treated with potassium phosphonate at similar R_F-values. None of the other samples included in this experiment formed clear inhibition zones.

High performance liquid chromatography (HPLC)

In the HPLC chromatogram it was observed that more smaller peaks at lower concentrations were present for untreated uninfected root and shoot samples than for

any of the infected and/or treated plants (which had fewer and lager peaks). All treated and/or infected samples were significantly different from the untreated uninfected control samples. Standards included in the HPLC data base allowed for the identification and calculation of the concentration of three of the major peaks in treated and/or infected plant extracts. The peaks that were identified included caffeic acid, salicylic acid and ferulic acid. The percentage dry weight of these compounds in root and shoot samples were calculated and is given in Figures 4.3 and 4.4.

HPLC analysis of tomato root and shoot samples (Figure 4.3) resulted in peaks that were identified as caffeic acid and salicylic acid for shoot samples and salicylic acid for root samples. Salicylic acid was present in tomato shoot samples treated with caffeine and fosetyl-al although the percentage of dry weight did not differ significantly between the two samples. Caffeic acid was present in all the treated shoot samples and the percentage of dry weight did not differ significantly between treatments. Salicylic acid was present in all tomato root samples but the fosetyl-al sample resulted in the largest amount in terms of percentage dry weight followed by PPG and the combination of PPG and caffeine which did not differ significantly from each other.

HPLC analysis of lettuce root and shoot samples resulted in peaks that were identified as caffeic acid, salicylic acid and ferulic acid for shoot and root samples (Figure 4.4). Root samples contained higher concentrations of these compounds when compared to shoot samples. In shoot samples salicylic acid was present in untreated infected, PPG uninfected, caffeine uninfected and infected potassium phosphonate samples, where the first three did not differ significantly from each other and the

potassium phosphonate sample gave a significantly higher concentration. Caffeic acid was present in all but the untreated uninfected sample, where none of the concentrations differed significantly. Ferulic acid was present in all infected samples as well as uninfected samples treated with a combination of PPG and caffeine and the uninfected samples treated with potassium phosphonate, but none of these concentrations were significantly different.

DISCUSSION

Phenolic compounds in plants constitute a major class of bio-active secondary plant metabolites with potentially anti-microbial activities. During this study our aim was to identify whether treatments with PPG, caffeine and the combination of the two compounds induces such compounds in tomato and lettuce plants.

The total phenolic content (TPC) was determined in tomato shoot and roots treated with fosetyl-al, PPG, caffeine and combinations of PPG and caffeine over a 96h period. TPC was also determined in shoots and roots of similarly treated lettuce plants, including a range of *Pythium* infected plants over a 96h period.

The shoot samples of both tomato and lettuce plants showed no increase in TPC with any of the treatments which could possibly be attributed to the treatments being applied as a root drench. Treatments with the fungicide standards (fosetyl-al and potassium phosphonate) resulted in the highest TPC levels in both tomato and lettuce roots, confirming that these compounds are indeed inducers of resistance mechanisms in plants. Tomato plants treated with PPG and lettuce plants treated with a

combination of PPG and caffeine respectively, resulted in the highest TPC of the experimental treatment compounds. These results indicate that the phenolic compounds in the PPG treatments (i) could have been accumulated in the roots, (ii) induced biochemical pathways in the plants to increase the production of phenolic compounds or (iii) contained precursors for the production of these phenolic compounds.

Bio-autography was used to determine whether any of the accumulated compounds in the tomato and lettuce roots where anti-fungal compounds. Bio-autography methods were developed for TLC where compounds are separated according to hydrophilicity and *C. cladosporioides* applied to the TLC plate, clear zones indicate inhibition (Narasimhachari and Ramachandran, 1967; Homma *et al*.,1989; Hamburger and Cordell, 1987). Direct spraying of conidial suspensions is an easy technique to use, giving the most reliable results for detection of fungitoxic compounds (Homans and Fuchs, 1970).

The data from the TLC-autography analysis (Table 4.1) showed that the R_f – value of the anti-fungal compounds corresponded to the R_f – value of the ferulic acid standard. The presence of ferulic acid in these samples was confirmed by means of HPLC analysis. HPLC analysis revealed the presence of salicylic acid and caffeic acid in specific root and shoot samples. Once again treatment with the synthetic fungicide standard resulted in the highest levels of phenolic acids per gram dry weight. The most significant observation was the high concentration of ferulic acid and caffeic acid present in uninfected lettuce roots treated with a combination of PPG and

caffeine (Figure 4.5), as most of the other increases in the levels of these compounds were associated with infection.

HPLC analysis detected these acids in the roots as well as the shoots whereas the Folin-Ciocalteu method did not pick up significant increases in the TPC of the shoot samples. A possible explanation for this could be that when the original extraction method for the shoot samples were decided, the Folin-Ciocalteu method was not as yet included in the protocol. The Folin-Ciocalteu method relies on the principle that the reaction will occur in an alkaline environment while extraction with ethyl acetate created an acidic environment. Efforts made to evaporate the ethyl acetate and re-dissolve the samples in methanol were unsuccessful.

Several studies of the phenol and polyphenol composition in different cultivars of lettuce have been performed and two main classes of products have been identified namely caffeic acid derivatives (Ke and Saltveit, 1988) and flavonols (Hermann, 1976). Caspersen (2000) also showed that under stress conditions ferulic acid, a precursor for lignin formation (Locher *et al*., 1994), accumulates in hydroponically grown lettuce. Thus the presence of these phenolic acids in our extracts is not suprising but what is of importance is the effect of the treatments on the levels of these compounds.

Yao (2004) identified six phenolic acids in *C. sinensis* using HPLC namely: gallic acid, iso-chlorogenic acid, chlorogenic acid, *p*-coumaric acid, courmarylquinic acid and hydroxyphelpropionic acid. Caffeic- and ferulic acids are produced in plants from phenylalanine and tyrosine via the shikimate pathway, forming *p*-coumaric acid,

which undergoes further hydroxylation to caffeic acid and subsequent O-methylation to ferulic acid as shown in Figure 4.6 (Pannalaa *et al.*, 1998). These compounds usually occur naturally as various conjugated forms resulting from enzymic hydroxylation, O-methylation, O-glycosylation or esterification of *p*-coumaric acid, principally as the quinic or carbohydrate esters, for example chlorogenic acid being an ester of caffeic- and quinic acid (Pruidze *et al*, 2003). From this we can deduce that by treating plants with PPG as a plant treatment, one would be adding valuable precursors for phenolic acids such as ferulic- and caffeic acid as well as esters of these acids that may be hydrolysed by process in the plants or surrounding environment.

Salicylic acid is a plant hormone involved in the basic metabolic pathway involved in resistance against plant pathogens and also in induced resistance. The HPLC analysis also showed an increase in the salicylic acid concentrations for treatments on tomato plants but no significant increase occurred in lettuce plants. Further experimental work will have to be done to confirm the up-regulation of salicylic acid in plants treated with PPG, caffeine or combinations thereof.

Full characterisation of the anti-fungal compounds in lettuce and tomato plants as well as the elucidation of pathways involved in their biosynthesis is still required. More detailed information will allow for precise definition of the role these anti-fungal compounds play in plant defence. In turn, such knowledge may enable strategies to enhance the levels of these compounds in plants (Joyce and Johnson, 1999). In addition, an increase in the concentration of these anti-fungal compounds, many of which are phenolics, may lead to increased health benefits for consumers (Törrönen and Määttä, 2002).

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Table 4.1

R_f-values of Salicylic acid, Ferulic acid and Caffeic acid standards as well as lettuce root extracts that gave clear inhibition zones with thin-layer chromatography bio-autography

Compound tested	Mean R_F-value	Standard deviation of the mean
Salicylic acid	0.64	0.01
Ferulic acid	0.44	0.01
Caffeic acid	0.22	0.02
IR 24 h	0.42	0.01
IR 48 h	0.44	0.03
IR 96 h	0.44	0.02
PPG_t IR 24 h	0.44	0.02
PPG_t IR 48 h	0.46	0.03
PPG_t IR 96 h	0.44	0.01
CAF_t IR 24 h	0.45	0.02
CAF_t IR 48 h	0.45	0.02
CAF_t IR 96 h	0.43	0.01
COM_t UN 24 h	0.43	0.00
COM_t UN 48 h	0.44	0.01
COM_t UN 96 h	0.43	0.00
COM_t IR 24 h	0.44	0.02
COM_t IR 48 h	0.43	0.00
COM_t IR 96 h	0.43	0.01
Potassium Phosphonate$_t$ UN 24 h	0.46	0.02
Potassium Phosphonate$_t$ UN 48 h	0.45	0.03

Potassium		
Phosphonate$_t$ UN 96 h	0.45	0.02
Potassium		
Phosphonate$_t$ IR 24 h	0.45	0.03
Potassium		
Phosphonate$_t$ IR 48 h	0.44	0.01
Potassium		
Phosphonate$_t$ IR 96 h	0.43	0.01

IR = Infected roots

UR = Uninfected roots

PPG_t = Polyphenon G treatment at 12.5 g.L-1

CAF_t = Caffeine treatment at 1.25 g.L-1

COM_t = Combination of Polyhenon G (12.5 g.L-1) and cafiiene (1.25 g.L-1)

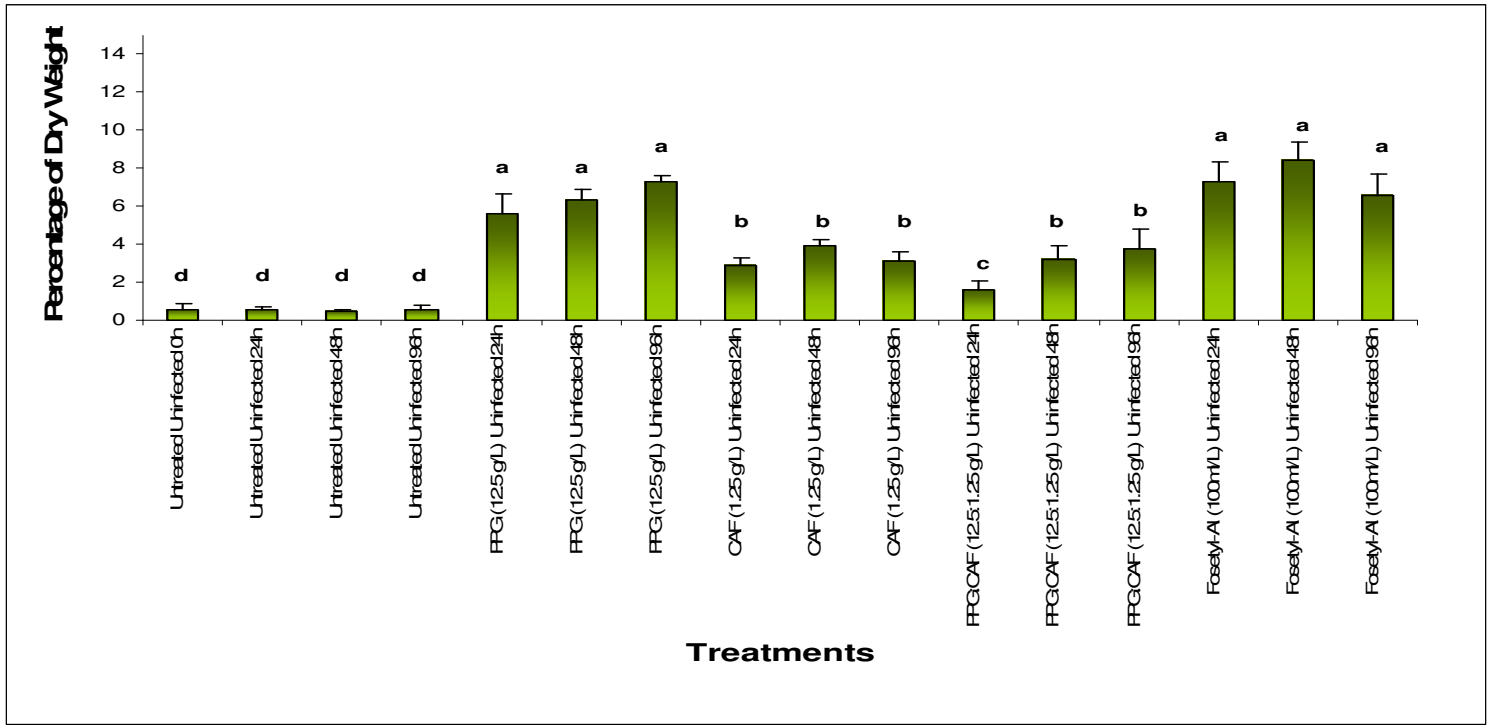

Figure 4.1

Percentage of dry weight of total phenolics in extracts of tomato roots treated with Polyphenon G (PPG), caffeine (CAF), the combination PPG:CAF and fosetyl-al over a 96h period. Columns represent the means. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$)

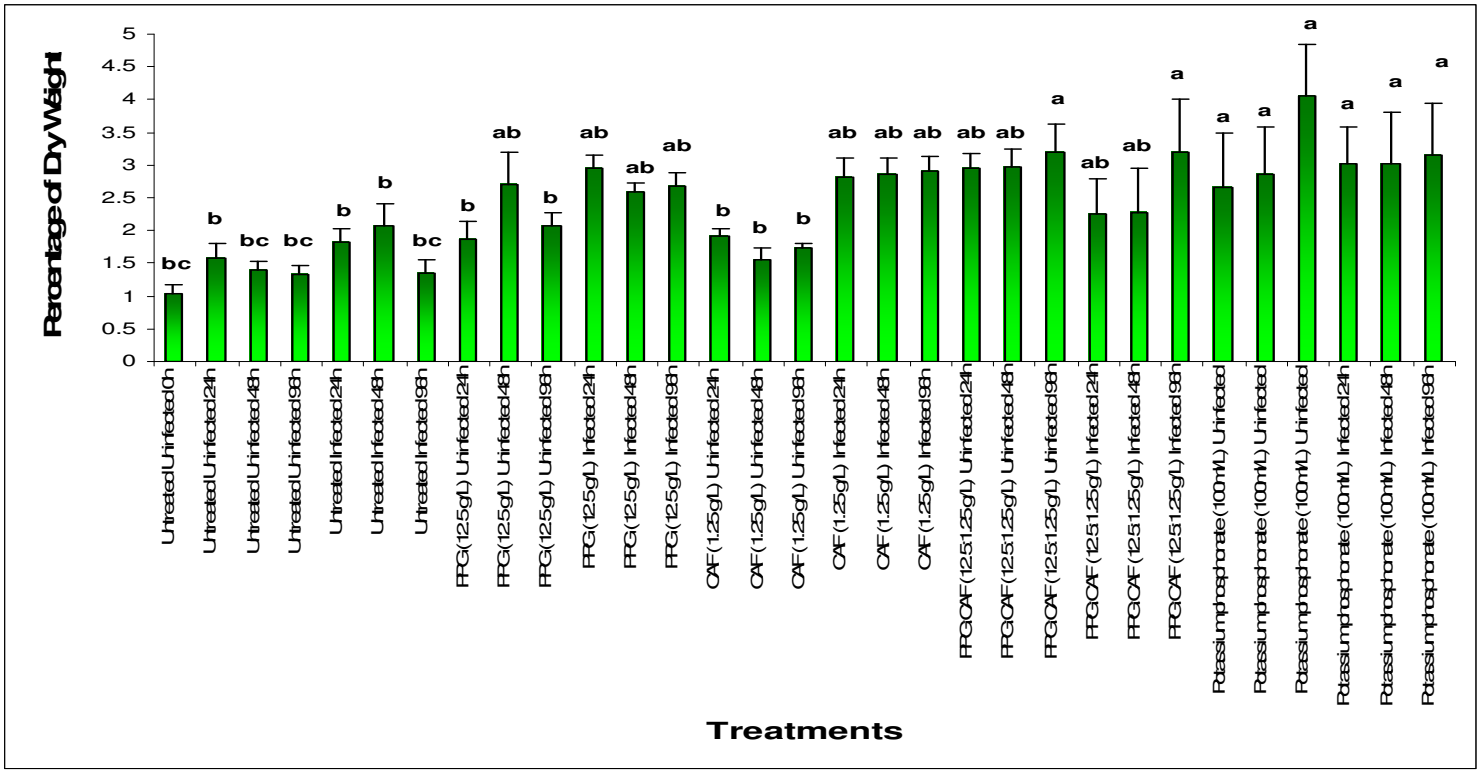

Figure 4.2

Percentage of dry weight of total phenolics in extracts of lettuce roots treated with Polyphenon G (PPG), caffeine (CAF), the combination PPG:CAF and potassium phosphonate over a 96h period. Columns represent the mean. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$)

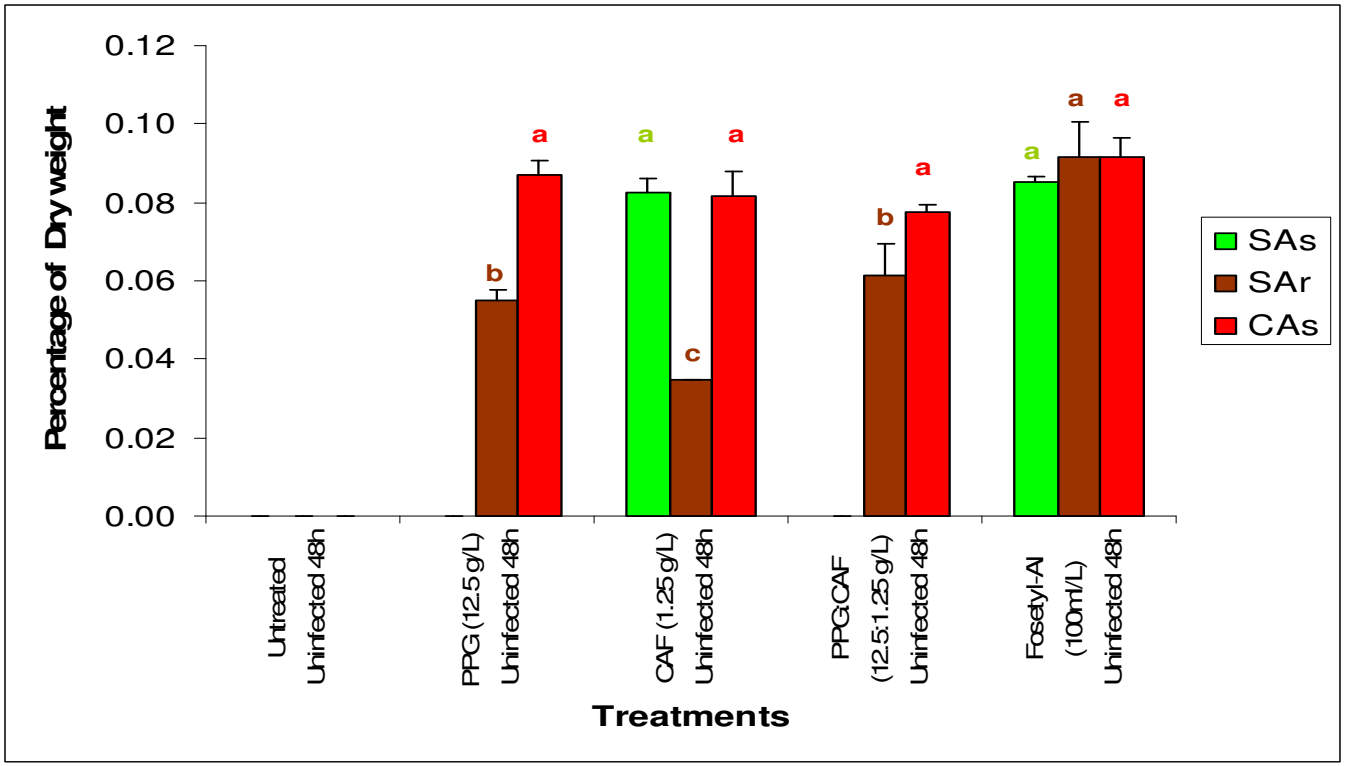

Figure 4.3

Percentage of dry weight of salicylic and caffeic acids in extracts of tomato roots and shoots treated with Polyphenon G (PPG), caffeine (CAF), the combination PPG:CAF and fosetyl-al over a 48h period. Columns represent the mean. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$) SAs = Salicylic acid in shoots. SAr= Salicylic acid in roots. CAs = Caffeic acid in shoots.

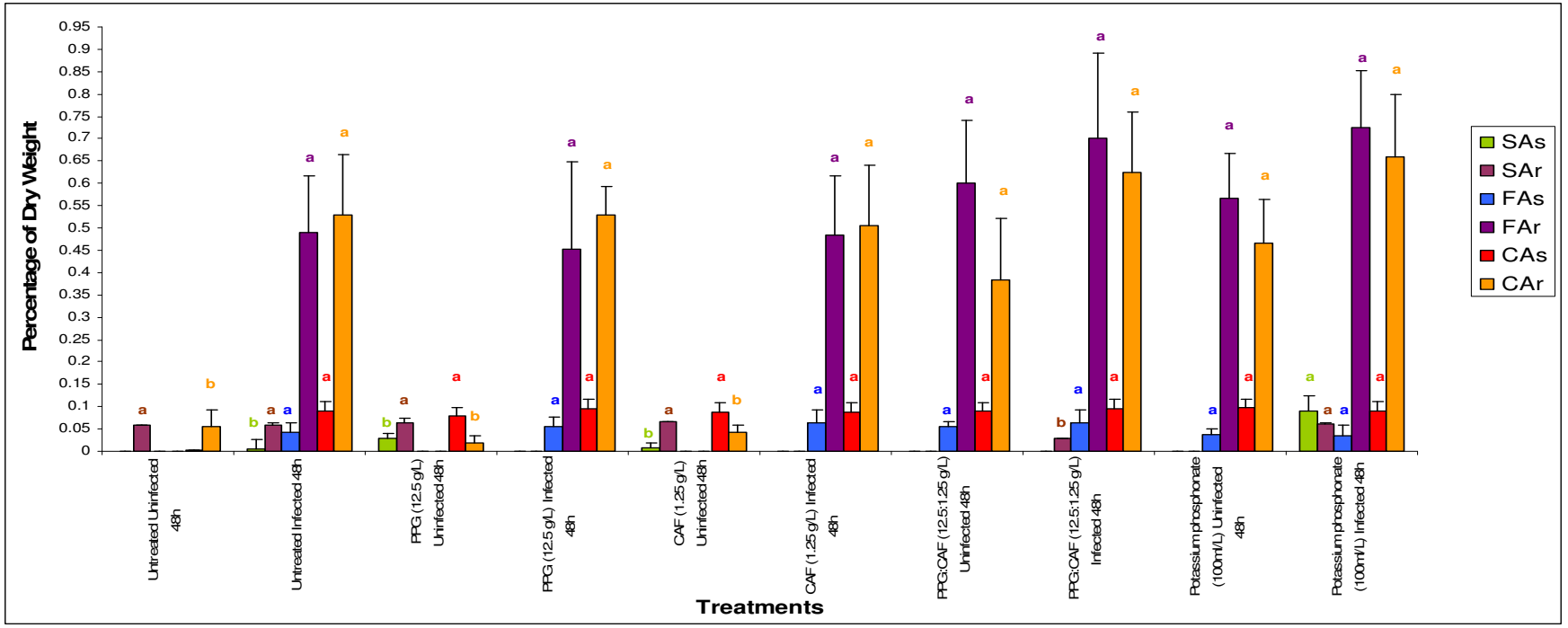

Figure 4.4

Percentage of dry weight of salicylic, ferulic and caffeic acids in extracts of lettuce roots and shoots treated with Polyphenon G (PPG), caffeine (CAF), the combination PPG:CAF and potassium phosphonate over a 48h period. Columns represent the mean. Error bars indicate the standard error of the mean. Treatments with a different letter notation are significantly different (calculated at $P < 0.05$)

SAs = Salicylic acid in shoots SAr = Salicylic acid in roots

FAs = Ferulic acid in shoots FAr = Ferulic acid in roots

CAs = Caffeic acid in shoots CAr = Caffeic acid in root

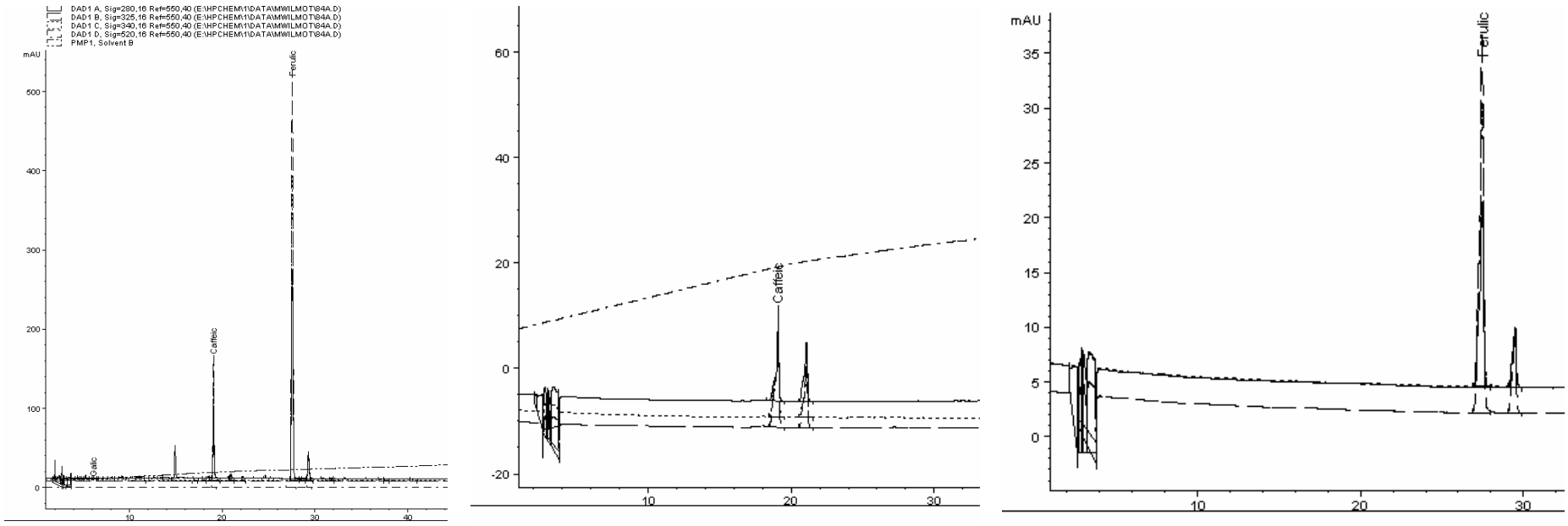

Figure 4.5

Reverse phase high preformance liquid chromatography chromatogram of (a) lettuce root sample 48h after being treated with a combination of 12.5 g.L-1 Polyphenon G and 1.25 g.L-1 caffeine (b) caffeic acid standard (c) ferulic acid standard

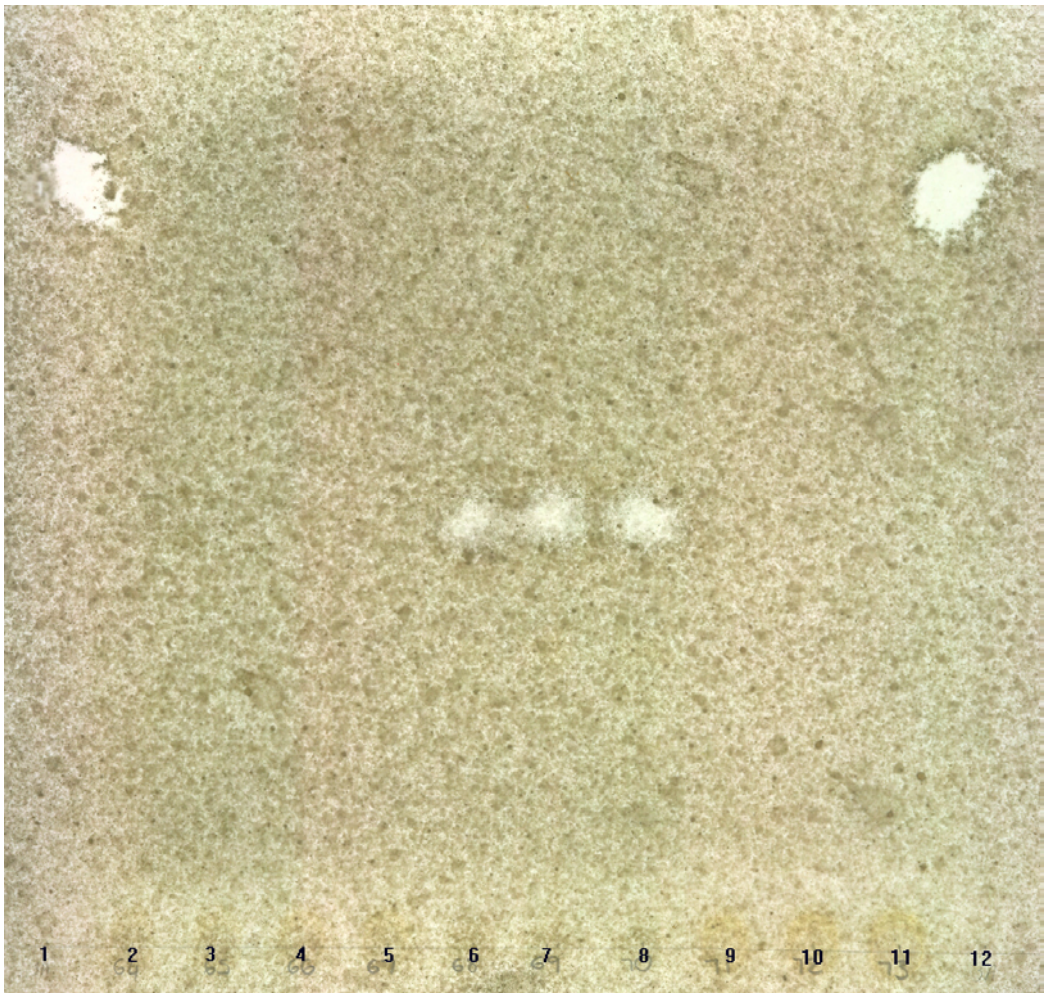

Figure 4.7a

Thin layer chromatography bio-assay plates preformed on lettuce root extracts.

Lanes 1 and 12: Salicylic acid

Lanes 2,3,4, and 5: Uninfected untreated extract samples harvested at 0, 24, 48 and 96 hours respectively

Lanes 6,7 and 8: Infected untreated extract samples harvested at 24, 48 and 96 hours respectively

Lanes 9, 10 and 11: PPG treated (12.5 $g.L^{-1}$) uninfected extract samples harvested at 24, 48 and 96 hours respectively

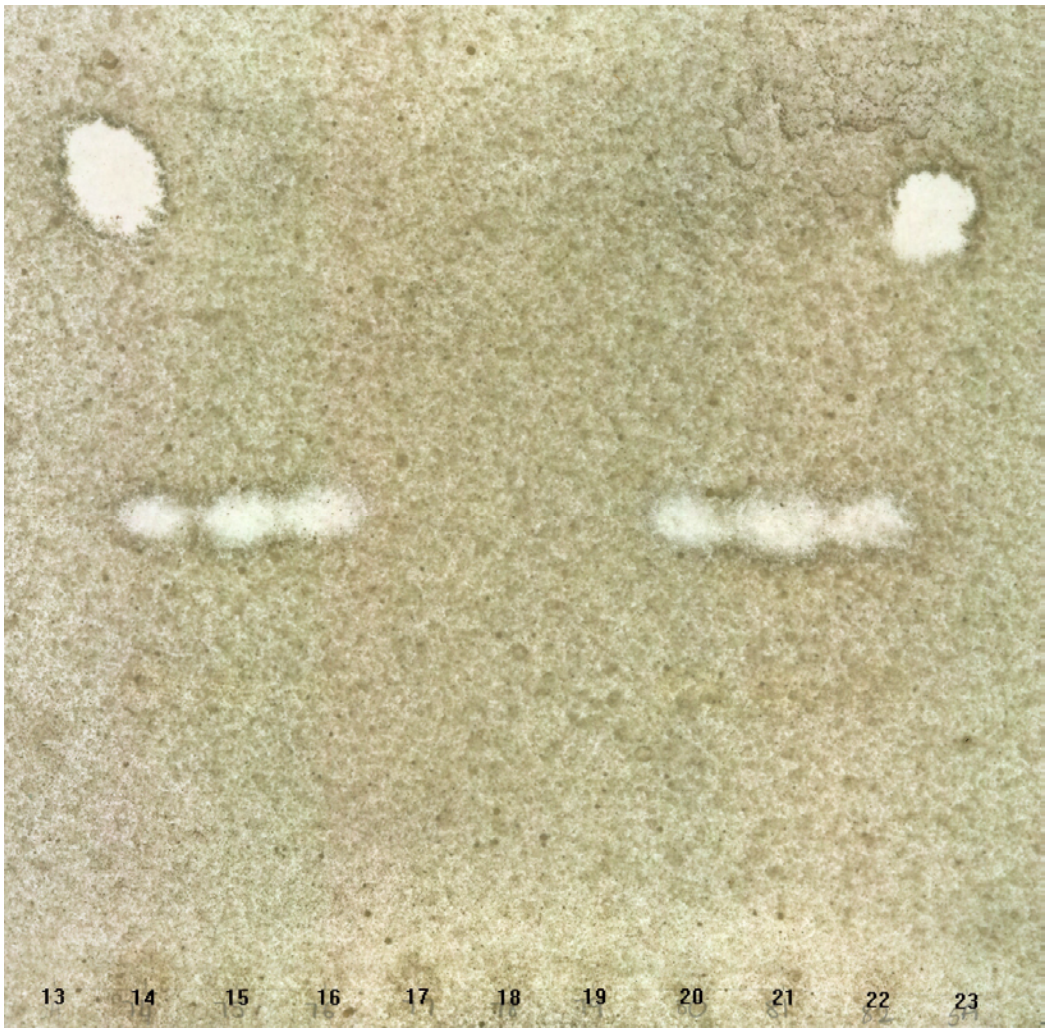

Figure 4.7b

Thin layer chromatography bio-assay plates preformed on lettuce root extracts.

Lanes 13 and 23: Salicylic acid

Lanes 14,15 and 16: PPG treated (12.5 $g.L^{-1}$) infected extract samples harvested at 0, 24, 48 and 96 hours respectively

Lanes 17,18 and 19: Caffeine treated (1.25 $g.L^{-1}$) uninfected extract samples harvested at 24, 48 and 96 hours respectively

Lanes 20,21,and 22: Caffeine treated (1.25 $g.L^{-1}$) infected extract samples harvested at 24, 48 and 96 hours respectively

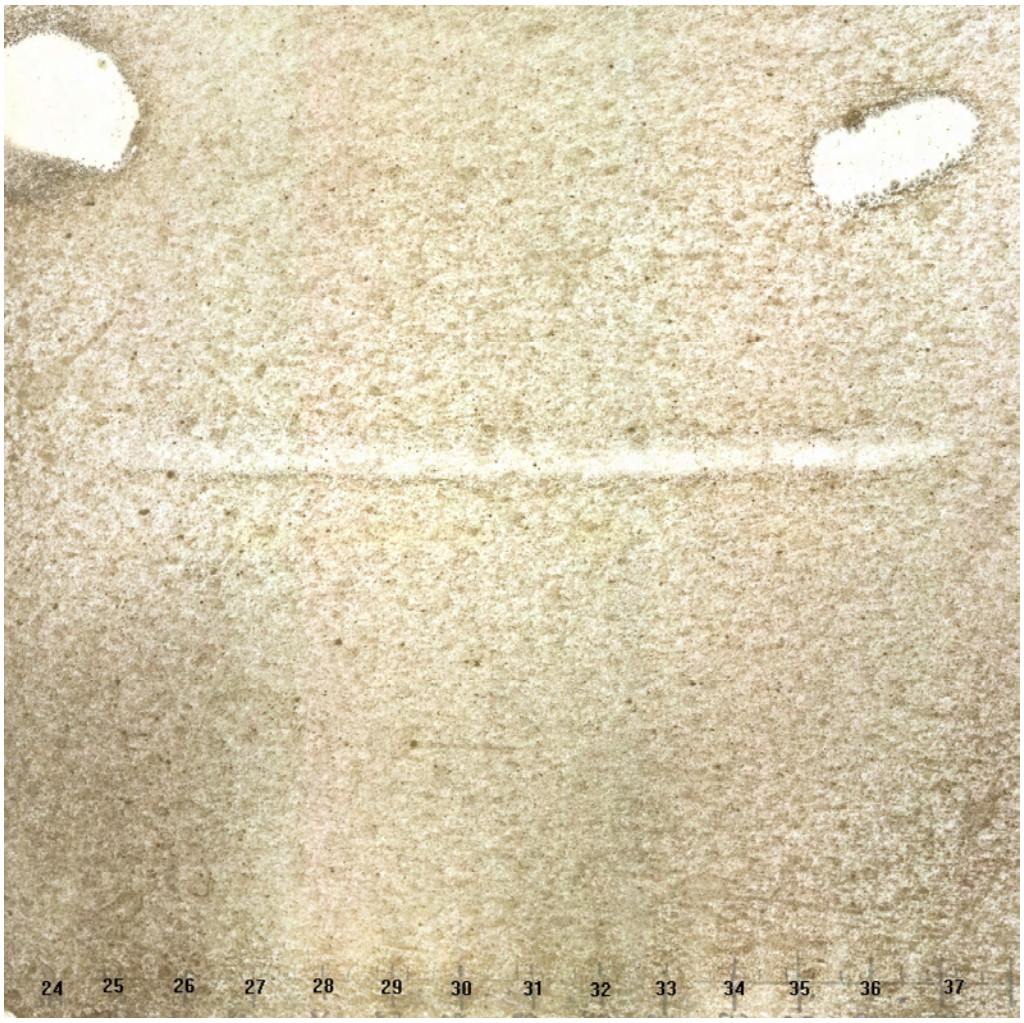

Figure 4.7c

Thin layer chromatography bio-assay plates preformed on lettuce root extracts.

Lanes 24 and 37: Salicylic acid

Lanes 25,26 and 27: Combination of PPG (12.5 $g.L^{-1}$) and caffeine (1.25 $g.L^{-1}$) treated uninfected extract samples harvested at 0, 24, 48 and 96 hours respectively

Lanes 28,29 and 30: Combination of PPG (12.5 $g.L^{-1}$) and caffeine (1.25 $g.L^{-1}$) treated infected extract samples harvested at 24, 48 and 96 hours respectively

Lanes 31,32 and 33: Potassium phosphonate treated (100$ml.L^{-1}$) uninfected extract samples harvested at 24, 48 and 96 hours respectively

Lanes 34,35 and 36: Potassium phosphonate treated (100$ml.L^{-1}$) infected extract samples harvested at 24, 48 and 96 hours respectively

CHAPTER 5

General Discussion

GENERAL DISCUSSION

In the light of a 20% yield loss due to plant pathogens (Oerke *et al*, 1994) and even greater losses when highly susceptible varieties are grown (Schwinn, 1992) the improvement of currently available fungicides and the development of new fungicides still remain of fundamental importance in world crop production.

The aim of the current study was to determine whether Polyphenon G (PPG), a concentrated extract from green tea (*Camellia sinesis*) and the individual compounds in PPG had activity against phytopathogenic fungi *in vitro* and *in vivo*, and to investigate the mode of action of the effective compounds.

The main findings of the *in vitro* study was that caffeine was the main anti-fungal compound tested, followed by EGCg, ECg, EGC, EC, Polyphenon G, green-, black- and rooibos tea extracts in decreasing order of activity. Theanine showed no anti-fungal effect. In some cases the Polyphenon G and caffeine combination had lower IC_{50} values than the individual compounds, indicating a synergistic effect. *Rhizopus stolonifer* (Ehrenb.:Fr) and *Penicillium expansum* (Link.) were the least sensitive to all the compounds, while *Phytophthora nicotianae* (Brenda de Haan), *Phytophthora capsici* (Leonian) and *Pythium* F-group were most sensitive to all the compounds. The fact that the Oomycetes (*Pythium* and *Phytophthora*) are difficult to control and the fungicides that do control these pathogens are becoming obsolete (Fontema *et al*, 2005). *Pythium* spp. is also problematic in hydroponic systems which makes the use of tea extracts an important possibility, which could indicate another niche where these compounds could be of economic importance. Based on our *in vitro*

results, it was decided to test PPG, caffeine and combinations of the two compounds under greenhouse conditions.

During greenhouse trials it was found that the combinations of PPG and caffeine gave the best overall results and effectively controlled five of the seven pathogens on their respective hosts. In some instances the test compounds performed better than the commercial fungicides at the recommended doses. The results also demonstrate that the effect of these compounds on fungi varies between species. Various application methods as well as the anti-microbial action of these compounds on other crops as well as varieties of the same crop types still has to be investigated. Field trials would be the next logical step in confirming the practical use of PPG and the combinations of PPG and caffeine in agriculture.

Phenolic compounds in plants constitute a major class of secondary plant metabolites with bioactive potential attributed to antioxidant and anti-microbial activities (Gulter, 1988). Free phenolic acids or derivatives present in ester or ether form, are found in varying quantities throughout plant tissues in response to characteristic synthesis patterns resulting from encounters with different forms of environment stress (Joyce, 1999). In the current study we aimed at identifying whether PPG and caffeine as well as combinations of these two compounds induced resistance in tomato and lettuce plants, and also shedding light on the mechanisms involved. The methods used in our study included determining total phenolic content by means of the Folin-Ciocalteu reagent, thin layer chromatography (TLC) bioautography to identify possible anti-microbial compounds and high performance liquid chromatography (HPLC) for identifying induced compounds based on standards included in the analysis.

We found that PPG, caffeine and combinations of PPG and caffeine increased the total phenolic concentration in both tomato and lettuce roots. Of the test compounds, PPG resulted in the highest phenol values in tomato roots and the combination of PPG and caffeine resulted in the highest phenol values in lettuce roots. TLC bioautography showed inhibition zones for all *Pythium* infected sample extracts, but interestingly also for the combination of PPG and caffeine in uninfected plants. These inhibition zones were identified as ferulic acid. HPLC analysis verified the presence of ferulic acid and also identified salicylic and caffeic acids in root and shoot extract samples. These results indicate that either the phenolic compounds in the treatments were accumulated in the roots or that the treatments induced *de novo* synthesis in the plants to increase the production of phenolic compounds or that the treatments caused induction of resistance in the plant. This study has shown that the combination of PPG and caffeine caused the accumulation of ferulic acid in the roots of uninfected lettuce plants similarly to fosetyl-al (a commercial plant activator). Future research could be aimed at optimising their effect.

Fungicides continue to be essential for the effective control of plant diseases. *In vivo* greenhouse screening and field trials are expected to remain the dominant methods for characterizing new compounds. Low toxicity to humans and wildlife, low environmental impact, low residues in food, and compatibility with integrated pest management programs are increasingly important considerations in the development of fungicides. The tea extracts assessed during the current study comply with the criteria and are therefore viable compounds for further development as crop protection agents.

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Inhibition of Phytopathogenic Fungi on Selected Vegetable Crops by Catechins, Caffeine, Theanine and Extracts of *Camellia sinensis*

By

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Degree : M.Sc. Plant Pathology

RESUMÉ

The aim of our study was to determine whether Polyphenon G (PPG, a concentrated *Camellia sinensis* extract) and the individual compounds in PPG had activity against phytopathogenic fungi *in vitro* and *in vivo.*

The present study reports on the sensitivity of twenty different phytopathogenic fungal species to extracts from black-, green- and rooibos tea extracts, concentrated green tea extract (Polyphenon G), caffeine, theanine, epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC), and epicatechin (EC), and Polyphenon G combined with caffeine. The inhibition of fungal growth by the compounds was as follows (in decreasing order): caffeine > EGCg ≥ ECg > EGC ≥ EC > Polyphenon G > green tea extracts ≥ black tea extracts > rooibos tea extracts ≥ theanine. In some cases the Polyphenon G and caffeine combination reduced the IC_{50} values for both the compounds, indicating a synergistic effect. *Phytophthora nicotianae* and *P. capsici* were most sensitive to all the compounds, while *Rhizopus stolonifer* and *Penicillium expansum* were least sensitive.

PPG and caffeine was subsequently tested individually and in combination in a greenhouse trial against seven pathogens on four crops. The combinations of Polyphenon G and caffeine gave the best overall results and effectively controlled *Fusarium solani* on cucumber, *P. capsici* and *Sclerotium rolfsii* on tomato, *Sclerotinia sclerotiorum* and *Pythium* F-group on lettuce. PPG individually significantly inhibited the growth of *Sphaerotheca fuliginea* of zucchini squash plants.

In efforts to determine the mode of action of PPG, caffeine and the combination thereof, methods used in our study included determining total phenolic content by means of the Folin-Ciocalteu reagent, thin layer chromatography (TLC) bioautography to identify possible anti-microbial compounds and high performance liquid chromatography (HPLC) for identifying induced compounds based on standards included in the analysis. Results showed that ferulic, salicylic and caffeic acids increased in uninfected lettuce plants treated with a combination of PPG and caffeine. These results indicate that either the phenolic compounds in the treatments were accumulated in the roots or that the treatments induced de novo synthesis in the plants to increase the production of phenolic compounds or that the treatments caused induction of resistance in the plant.

The results of the current study demonstrate the potential for tea (*C. sinensis*) extracts to be developed as effective crop protection agents against a range of plant diseases on a variety of crops.