Enhancing biocontrol activity of *Colletotrichum coccodes*

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ABSTRACT

Resistance responses of *Abutilon theophrasti* were investigated to determine defense mechanisms of the weed against *Colletotrichum coccodes* and to verify if some chemical suppression of the resistance mechanism could be exploited to enhance the virulence. Induced resistance in *A. theophrasti* has been confirmed in treatments with *C. coccodes*, benzothiadiazole, bentazon, and acifluorfen. Induction of peroxidase and phenylalanine ammonia lyase (PAL) activities in the leaves that did not contact with the inducing agents was observed after the localized stresses to the first leaf or the root of the plant with those agents. α-Amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, oxalic acid, and analogues of oxalic acid and mannose were tested to enhance *C. coccodes* virulence. However, the compounds did not enhance *C. coccodes* virulence or affect *A. theophrasti* growth. Strong antifungal effects, poor inhibitory effects on plant defense mechanisms, or minor dependence of *A. theophrasti* on the defense mechanisms that the chemicals affected could be reasons. The efficacy of *C. coccodes* increased in the presence of 0.25 kg a.i. ha^{-1} bentazon more than when *C. coccodes* was applied alone, while the effect of glyphosate was minimal. Peroxidase activity was strongly induced by the treatment of *C. coccodes* and increased over time. PAL and activation of peroxidase was inhibited in the presence of bentazon, suggesting the synergy effect by bentazon is probably due to the suppression on the two defense-related enzymes. In conclusion, *A. theophrasti* exploits various biochemical and morphological types of defense mechanisms against *C. coccodes* infection. However, the activation of the defense responses can be suppressed or by-passed in an integrated weed management system.

RÉSUMÉ

Les réponses de résistance d'*Abutilon theophrasti* ont été observées après une variété de stress accompagnés de *Colletotrichum coccodes* afin de déterminer les mécanismes de résistance de la mauvaise herbe ainsi que pour vérifier si la suppression certains mécanismes de résistance pouvaient être exploités pour en améliorer la virulence. La résistance induite de *A. theophrasti* a été confirmée dans tous les traitements avec *C. coccodes,* benzothiadiazole (BTH), bentazon, et acifluorfen. Les activités de la peroxydase et de la phénylalanine amonia lyase (PAL) ont aussi été accrues dans les plantes induites. L'acideα-amino-oxy acétique (AOA), le 2-déoxy-D-glucose(DDG), le mannose, l'acide oxalique, les analogues de l'acide oxalique et du mannose ont été testés pour améliorer la virulence de *C. coccodes*. Cependant, les essais n'ont pas amélioré la virulence de *C. coccodes* ni affecté la croissance de *A. theophrasti*. Les raisons pourraient–être: de forts effets antifongiques, de faibles effets inhibiteurs sur les mécanismes de défense de la plante, ou une dépendance mineure de *A. theophrasti* sur les mécanismes de défense affectés par les traitements chimiques. 0.25 kg i. a. ha^{-1} de bentazon a augmenté l'efficacité de *C. coccodes*. L'activité de la peroxydase a été fortement induite par le traitement avec *C. coccodes* et son activité augmenta dans le temps. L'augmentation des activités de la peroxydase et de PAL a été inhibée en la présence de bentazon. En conclusion, *A. theophrasti* exploite des mécanismes de défense biochimiques et morphologiques variés. Toutefois, l'expression des réponses de défense peut être supprimée par un système intégré de gestion des mauvaises herbes.

PREFACE

Resistance mechanisms of *Abutilon theophrasti* against *Colletotrichum coccodes* were examined in this thesis. The thesis begins with abstracts in English and French, followed by a table of contents. Chapter 1 provides a comprehensive literature review of the research subject and an outline of the specific thesis objectives.

The main body of this thesis is comprised of the next three chapters, each of which is one complete manuscript. The chapters are linked by connecting texts that provide logical bridges preceding and following each manuscript.

Chapter 2 investigates induced resistance of *Abutilon theophrasti* to *Colletotrichum coccodes* and its effect on biocontrol efficacy of *C. coccodes*. Chapter 3 investigates effects of selected chemicals to enhance virulence of *C. coccodes*. In chapter 4, plant defense-related enzymes including peroxidase and phenylalanine ammonia lyase are investigated.

A general conclusion is presented in Chapter 5, followed by the description of the main contributions to knowledge of this research and the appendices.

STATEMENT FROM THESIS OFFICE

This thesis consists of a collection of papers of which the candidate is the lead author. The structure for this thesis is based on the following statement in "Guidelines for Thesis Preparation" from the Faculty of Graduate Studies and Research, McGill University:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.) The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents; a brief abstract in both English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary; a thorough bibliography; Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS

The candidate performed the development, execution and management of all experiments. The candidate was the primary author of the manuscripts. Dr. A.K. Watson, Dr. T. Paulitz, and Dr. S. Jabaji-Hare were co-authors of the manuscripts.

Dr. A.K. Watson was a supervisor and provided supervisory guidance on the design of the experiments and academic advices throughout the research period, and helped in manuscript preparation.

Dr. T. Paulitz provided advice on the design of the experiments and on the analysis of the data, and reviews of the manuscripts.

Dr. S. Jabaji-Hare was the candidate's committee member. She provided advice on the design of all experiments.

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Chapter 1. General Introduction

1.1. Weed control

Weeds are a major biological problem in most crops by decreasing crop yield. Since the beginning of agriculture, man has struggled to eliminate weeds from arable lands (Huffaker 1957). Traditionally, man has used weed control methods such as crop competition, crop rotation, biological, mechanical, manual, chemicals, and a combination of these methods (McWhorter and Chandler 1982). A significant rise in crop production took place with the development of two kinds of auxin-type herbicides. The phenoxyacetic acids, MCPA and 2,4-D, were independently discovered in secret in the UK and the USA in 1941 during World War II as potential chemical warfare agents (Cobb 1992). 2,4-D has been marketed by the American Chemical Paint Company as "Weedone" since 1945, and MCPA has been marketed as "Agroxone" by ICI since 1946. These chemicals have been used to control broadleaf weeds in narrowleaf crops. These two auxin-type herbicides are considered as the first truly selective and non-toxic organic herbicides. During the past 50 years, countless chemicals and their analogues have been developed for commercial purposes.

Although crop yield has greatly increased with the use of chemical herbicides, herbicide inputs increased in parallel with increasing crop yield (LeBaron 1990). Labor shortages and rising labor costs resulting from industrialization have stimulated an increased reliance on chemical herbicides (Watson 1999). The evolution of herbicide resistance resulting from too much reliance on herbicides is now a widespread problem in areas where herbicides are used intensively (Moss and Rubin 1993). Since the first report of a formerly susceptible weed population of *Senecio vulgaris* (common groundsel) that

acquired resistance to triazines (Ryan 1970), there has been a rapid increase in the incidence of herbicide resistance worldwide.

Recently, the tremendous improvement of genetic engineering techniques introduced methods to transfer resistant genes from certain organisms to crops. A well-known example is glyphosate-resistant plants, which are also known as Roundup-Ready crops. Glyphosate is a non-selective and post-emergence herbicide inhibiting 5-enoyl-pyruvyl shikimic acid 3-phosphate (EPSP) synthase (Cobb 1992). It is widely used in the field where the total control of vegetation is required. Glyphosate resistant genes were introduced into many crops including tobacco, cotton, sugar beet, oil seed rape, soybean and wheat. Many other resistance genes to herbicides such as bromoxynil, propanil, atrazine, 2,4-D, glufosinate, and sulphonylurea have been isolated and transferred from microorganisms or plants to crops (Cobb 1992). In the USA, 29 million hectares were planted with soybean in 1999, and half of this area was planted with genetically modified herbicide-resistant seeds (Abelson and Hines 1999). The rapid adoption in the market is probably due to the potential benefits such as insurance against pests, management and labor savings, reductions in equipment outlays associated with no-tillage production systems, the wide spectrum of weed control, the flexibility of herbicide application timing, fewer types and fewer applications of herbicides, and reduction of soil erosion resulting from herbicide-input. This very successful story makes some scientists expect that the use of biotechnology in agricultural area will lead us to "a plant revolution" or "a third technological revolution" by replacing conventional breeding and weed control methods (Abelson 1998, Abelson and Hines 1999).

However, there is controversy on the use of genetically modified herbicide-resistant crops. The potential dangers of genetically modified crops may include genetic

pollution, allergy to inserted proteins, and other ecological and health impacts. The genes conferring herbicide resistance of crops may be transferred from genetically modified crops to wild relatives or traditional crops near the farmlands where transgenic crops grow, thus creating weedy crops or superweeds that are invasive plants with the potential to lower crop yields and disrupt natural ecosystems (Ferber 1999). The sequential use of broad-spectrum herbicides may lead to undesirable ecological impacts by total weed removal. In the UK, the effects of the introduction of an herbicide-resistant sugarbeet on the population dynamics of an annual weed, *Chenopodium album,* has been modeled. This weed, which occurs worldwide, is an important source of food for farmland birds. A single herbicide application can bring high mortality of weeds in the herbicide resistant sugarbeet field. Such high mortality leads to dramatic reductions in weed densities, consequently resulting in loss of food resources for birds. This model may be applied broadly to many kinds of birds (Watkinson *et al.* 2000).

Patent protection on genetically modified herbicide resistant crops may facilitate genetic erosion by inhibiting farmers from re-using, sharing and storing seeds. The spread of transgenic crops threatens crop genetic diversity by simplifying cropping systems thus promoting genetic erosion. In addition, the use of herbicide resistant crops does not simply give much greater weed control efficacy than the conventional method. The efficacy of glyphosate is affected by leaf stages and other herbicides (Vangessel *et al.* 2000). Sequential postemergence herbicide applications or soil-applied herbicides followed by postemergence herbicides are usually more economically effective than single postemergence herbicide applications in glufosinate and glyphosate resistant soybean (Culpepper *et al.* 2000). The main reason for the rapid adoption of genetically modified crops by farmers appears to be that biotechnology made their life easier because

adoption of genetically modified crops allows farmers to simplify weed control methods. An informal poll revealed that farmers growing genetically modified soybeans choose the crops not because weed management with this crop is more effective, but because it is simpler than the conventional method (Firbank and Forcella 2000).

With increasing concern about the herbicide resistance in weedy plant species and the effect of pesticides and genetically modified crops on the ecosystem and humans, the desire to prevent herbicide resistance and to find alternatives of chemical herbicides has been increased (Buhler 1999, Moss and Rubin 1993, Wyse 1992). Reduced tillage systems, increasing farm size, and economic pressure are hindering crop producers from using the various weed control options. In addition, weed populations continue to adapt to weed control practices through herbicide resistance. Limited crop choices are one of the other factors that reduce crop rotation and intensify the selection pressure on weed communities. Integrated weed management (IWM) is one possible way to solve these weed management problems (Buhler 1999). IWM is defined as the integration of effective, environmentally safe, and sociologically acceptable control tactics that reduce weed interference below the economic injury level, and involves herbicide mixture, using synergists, safeners and crop rotations (Powles *et al.* 1997, Thill *et al.* 1991). Pathogens of weeds could be a solution that is economically and environmentally sustainable (Watson 1999).

1.2. Biological control of weeds

Biological control of weeds is defined as the deliberate use of natural enemies to suppress the growth or reduce the population of a weed species (Huffacker 1957, Wapshere 1982, Watson 1993). The methods for the exploitation of natural enemies to

control weeds can be identified in two classes, the classical approach and the inundative or augmentative approach (bioherbicides or mycoherbicides). The classical approach is based on the introduction of natural enemies from the geographic origin of the exotic plants that were introduced into a new region and became weedy in the absence of their natural enemies (Templeton 1982, Watson 1993). The rust pathogen, *Puccinia chondrillina* Bubak & Syd. on *Chondrilla juncea*, the moth, *Cactoblastis cactorum* on prickly pear (*Opuntia vulgaris*), and the leaf beetle, *Chrysolina quadrigemina* on St. Johnswort (*Hypericum perforatum*) are successful and dramatic examples of introduction of natural enemies into a new region (Burdon *et al.* 1981, Huffacker 1957).

Bioherbicides exploit indigenous pathogens. The pathogen is applied to target weeds using techniques and methodologies similar to those used with chemical herbicides (Watson 1993). The concept of mycoherbicides was first introduced by Daniel *et al.* (1973), who demonstrated that an endemic pathogen might destroy its weed host by artificially applying a massive dose of inoculum at a particularly susceptible stage of weed growth (Charudattan 1991). The active ingredient in a bioherbicide is a living microorganism and is applied in inundative doses of propagules (Auld and Morin 1995). Thus, for a bioherbicide to be used successfully, it must be possible to produce abundant and durable inoculum by an artificial manner (mass production). The pathogen must be genetically stable and specific to the target weed and must be able to infect and kill the weed in environments of reasonably wide latitude (Charudattan 1991).

The first commercially developed bioherbicide was DeVine™, a liquid formulation of *Phytophthora palmivora* (Butler) Butler. DeVine™ was made for the control of stranglervine [*Morrenia odorata* (H. & A.) Lindl. # MONOD] in Florida citrus

groves, and was registered in 1981 (Kenney 1986). DeVine™ is still available from Abbott Laboratories on pre-order basis (Watson 1999). Collego™, a dry powdered formulation of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. f. sp. *aeschynomene*, is a selective postemergent mycoherbicide for control of northern jointvetch [*Aeschynomene virginica* (L.) B.S.P. # 2 AESVI] in rice (*Oryza sativa* L.) and soybean [*Glycine max* (L.) Merr.] in the southeastern United States (Bowers 1986). Collego™ was registered in 1982 (Bowers 1986), and marketed from 1982 to 1992 (Watson 1999). Collego™ was re-registered in 1997, and was used on about 5,000 ha in 1998 (Watson 1999). BioMal®, a dry formulation of *Colletotrichum gloeosporioides* f. sp. *malvae*, was registered in Canada for the control of round leaved mallow (*Malva pusilla* Sm.) in flax (*Linium usitatissimum* L.), lentils (*Lens esculenta Moench*), and wheat (*Triticum aestivum* L.) in 1992 (Mortensen 1998). This product has never been on the market because of high production costs (Watson 1999). Although another product, Lubao (*Colletotrichum gloeosporioides* f. sp. *cuscutae*) has been used in China since 1966 for the control of parasitic dodder (*Cuscuta chinensis* Lam. and *C. australis* R. Br.) in soybeans, the present status of Lubao is unknown (Watson 1999). Recently, Camperico (*Xanthomonas campestris* pv. *poae*) is being developed for control of annual bluegrass (*Poa annua* L.) in turfgrass (Imaizumi *et al.* 1997).

There are many reasons to use bioherbicides, such as resistance to the chemical herbicide, parasitic weeds, and environmental limitations in urban area (Gressel *et al.* 1996). The use of indigenous, naturally occurring weed pathogens can reduce chemical inputs and provide viable, economic, and effective weed control components within integrated weed management programs (Watson 1999).

1.3. *Colletotrichum coccodes* as a bioherbicide

Colletotrichum is one of the important genera of plant pathogenic fungi causing diseases of cereals and grasses, legumes, vegetables and perennial crops (Sutton 1992). Taxonomy is mostly based on morphological and cultural criteria, especially conidial characteristics, presence of setae and sclerotia and shape of appressoria. Conidia may be cylindrical or elliptical. There are differences in the surface carbohydrates of conidia of *Colletotrichum* species that correlate closely with morphology, host specificity and mode of infection (Mills *et al.* 1992, O'Connell *et al.* 1992). The difficulties in taxonomy have proliferated the number of the species. At least 11 acknowledged generic synonyms have been described and almost 900 species are described in *Colletotrichum*. There is no unanimity in describing cultural features from isolates although *Colletotrichum* has been identified on the basis of the combination with conidial and appressorial morphology and cultural characters. The accepted taxa were decreased to 39 species by Sutton (1992). The unclear identification system led to the effort to establish a new system with taxonomic biochemical markers, such as lectin, and molecular biology techniques, such as sequence comparisons of rDNA of *Colletotrichum* species. Monoclonal antibodies are also used to differentiate between *Colletotrichum* taxonomy species.

Colletotrichum coccodes (Wallr.) Hughes has a wide host range causing anthracnose primarily on potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.). *C. coccodes* can exist as a moderately competitive saprophyte on decaying host roots and weed hosts (Dillard 1992). Identification of *C. coccodes* is based on the morphological characteristics in the similar way with other *Colletotrichum* species. It has aseptate conidia with obtuse ends from unicellular hyaline conidiophores within

asexual acervuli. Following sporulation, a large aggregate composed of several small sclerotia is formed from a stroma which is a mass of tightly interwoven thick-walled hyphal cells (Tu 1980). Conidia of *C. coccodes* survive about three weeks and its sclerotia survive for 84 weeks in greenhouse soil (Blakeman and Hornby 1966). Sclerotia play an important role in the winter survival in nature since its conidia have a short life (Tu 1980). *C. coccodes* survives less on the soil surface than in the soil because of greater fluctuation in environmental conditions (Dillard and Cobb 1998).

Many *Colletotrichum* species have been considered or commercialized as bioherbicides. *C. gloeosporioides* (Penz.) Penz. and Sacc. f.sp. *aeschynomene* and *C. gloeosporioides* (Penz.) Sacc. f.sp. *cuscutae* have been successfully commercialized to control northern jointvetch and dodder, respectively. *C. orbiculare* (Berk. And Mont.) von Arx, DAR 48942 and *C. gloeosporioides* f.sp. *malvae* had been patented to control spiny cocklebur and round-leaved mallow, respectively, but not commercialized (Templeton 1992). *C. coccodes* has been currently studied as a potential bioherbicide for velvetleaf (*Abutilon theophrasti* Medik.) (Gotlieb *et al.* 1987, Wymore *et al.* 1988). *C. coccodes,* isolated from velvetleaf leaves, is highly specific for velvetleaf and has excellent potential as a bioherbicide (Gotlieb *et al.* 1987). It causes gray-brown foliar lesions, and black and sunken stem lesions on infected velvetleaf. Velvetleaf is killed when inoculated at a young age (Wymore *et al.* 1988). The biomass of velvetleaf was reduced by 89% compared to control at the two- to three-leaf stages (Gotlieb *et al.* 1987). When *C. coccodes* is inoculated at later growth stages, although infected velvetleaf plants are stunted and development is delayed, they continue to grow, shedding the diseased leaves (Wymore *et al.* 1988). Infection by *C. coccodes* plays an important role in plant competitive interactions (DiTommaso and Watson 1995, DiTommaso *et al.* 1996). The

impact of inoculation of *C. coccodes* is high on seed yield and height hierarchy of velvetleaf when the plant competes with soybean (*Glycine max* [L.] Merr.), while the influence of inoculation is slight in pure stands (DiTommaso *et al.* 1996, DiTommaso and Watson 1997).

Mass production has been optimized in liquid culture (Yu *et al.* 1997, 1998) and enhancement of *C. coccodes* virulence has been attempted in a tank-mix with chemicals and other organism (Fernando *et al.* 1994, Fernando *et al.* 1996, Wymore *et al.* 1987). Co-inoculation of phylloplane *Pseudomonas* spp. with *C. coccodes* enhanced the disease due to phylloplane competition for carbon, nitrogen, and iron (Fernando *et al.* 1994, Fernando *et al.* 1996). The plant growth regulator thidiaz sulphonylurea uron acted synergistically when applied in combination with *C. coccodes* (Hodgson *et al.* 1989, Wymore *et al.* 1987, Wymore and Watson 1989).

1.4. Pathogenic strategy of *Colletotrichum*

Generally for fungal pathogens to infect plant tissues, certain steps are essential: 1) attachment to the plant species, 2) germination on the plant surface and formation of infection structures, 3) penetration of the host, and 4) colonization of the host tissue (Schafer 1994). Attachment of conidia to host plant cuticle involving passive interactions by preformed proteins and active protein synthesis after landing on the leaf surface is critical for the initiation of disease in fungal-plant interactions (Perfect *et al.* 1999). Appressoria melanization of *C. coccodes* begins within 4h and infection vesicles are present after 22 hours on tomato foliage after inoculation (Byrne *et al.* 1997). Appressoria that are differentiated from germ tubes produce penetration pegs that pierce the cuticle and the epidermal cell wall (Morin *et al.* 1996).

Host penetration may depend on appressorial turgor pressure which is impossible without a firm adhesion to the plant surface (Schafer 1994). Fungi produce a variety of dark pigments known as melanins. Melanin is deposited in a layer of the cell wall close on the plasma membrane (Perfect *et al.* 1999). Melanization is considered to be related to appressorial turgor pressure and is required for mechanical penetration (Mendgen *et al.* 1996). The role of melanin seems to provide a semi-permeable layer inside the appressorium (Schafer 1994). This layer mediates the build-up of hydrostatic pressure that allows the fungus to puncture the plant epidermis mechanically.

After penetration, the penetration hyphae accumulate components of a cytoskeleton in the tip and secrete cell wall degrading enzymes to penetrate the plant cell wall (Mendgen *et al.* 1996). Plant pathogens produce many types of cell wall degrading enzymes and these enzymes play a major role in the infection process and in the development of symptoms (Wijesundera *et al.* 1989). The infectious hyphae form secondary hyphae that spread intercellularly and intracellularly within the host tissue (Morin *et al.* 1996). A considerable range of enzymes have been identified in culture filtrates of *C. coccodes* including pectin methyl esterase, endopolygalacturonase, endo pectin transeliminase, endo pectate transeliminase, α-amylase, β-amylase, cellulase, β-glucosidase (Davet 1976).

During colonization of plant tissues, fungal pathogens exhibit two main modes of nutrition; a) biotrophy where nutrients are obtained from living host cells and b) necrotrophy where nutrients are obtained from dead host cells (Thrower 1966). Both of these nutritional strategies are exhibited by *Colletotrichum*. *Colletotrichum* utilizes two main infection strategy; 1) intracellular hemibiotrophic colonization and 2) subcuticular

intramural colonization (Bailey *et al.* 1992). The initial stages of infection such as attachment of conidia, germination, production of germ tube, formation of appressoria, and penetration through cuticle are very similar for both groups of pathogens. Following penetration, intracellular hemibiotrophic pathogens grow within the cell lumen without penetrating the host protoplast. After colonizing one or more host cells, the infection hypha branches into secondary necrotrophic hyphae. These fungi which initially feed on living host cells before switching to necrotrophy are considered to be hemibiotrophic or facultative biotrophs (Perfect *et al.* 1999). Subcuticular intramural pathogens develop beneath the cuticle by forming an intramural network of hyphae, before spreading rapidly inter- and intra-cellularly and killing the tissue (Bailey *et al.* 1992).

1.5. Plant defense response against fungal attack

Once a plant pathogen arrives at the plant surface, plants defend themselves actively against pathogen attacks with an arsenal of defense mechanisms as well as passive or pre-existing defense mechanisms. These defense mechanisms combine two characteristics, structural characteristics that act as physical barriers to obstruct the pathogen from gaining entrance and spreading through the plants, and biochemical reactions that take place in the cells and tissues of the plant (Agrios 1997).

Passive defense mechanisms involve structural barriers, such as a waxy cuticle, or strategically pre-existing antimicrobial compounds to prevent colonization of the tissue (Hutcheson 1998, Osborne 1996). Preformed compounds such as juglone, arbutin, and other phenolics or their glycosides play an important role in the resistance of plants to many microorganisms (Paxton and Groth 1994). Many of these compounds are accumulated in significant quantities in normal plant tissues and toxic to microorganisms.

Phenolic compounds are often conjugated with sugars and serve as biosynthetic precursors of the more toxic phytoalexins which are small antimicrobial molecules made by the plant in response to microbial attack (Paxton and Groth 1994, Scafer 1994). The concentration of phenolic compounds is affected and induced by the fungal infection and associated with the hypersensitive response (Benhamou and Bélanger 1998). Phenolic compounds also are components of lignin in plant tissues (Paxton and Groth 1994). Lignification may play an important role in plant defense response to fungal attack (Vance *et al.* 1980). It is thought lignin is formed as a response to microbial penetration and mechanical damage.

Active defense response of plants can be classified as following three separate classes including programmed cell death (PCD) in infected plant cells, elicitation of the adjacent cells in the vicinity of the infected site responding to diffusible signal molecules, and systemically acquired resistance (Hutcheson 1998). This elicitation of defense mechanisms can be brought about under various abiotic or biotic conditions (Benhamou and Bélanger 1998, Mahé *et al.* 1993, Mucharromah and Kuc 1991). The hypersensitive response (HR) is a macroscopic manifestation of pathogen-induced PCD (Hutcheson 1998). HR is genetically controlled and coordinately regulated with other defense-related biochemical events typically seen during the resistant response (Greenberg 1997). Although the mechanism of the HR is not fully understood, HR is associated with several events, including the accumulation of phytoalexin, lignification, increased peroxidase activity which is necessary for lignin biosynthesis and cross-linking of cell wall proteins, and the expression of genes encoding the pathogenesis-related (PR) proteins (Malamy and Klessig 1992).

Plants that have undergone a resistance response including the HR in their infected tissues attain immunity to many other pathogens in other tissues that have not been exposed to pathogens (Hutcheson 1998). This immunity is called systemic acquired resistance (SAR), and may require salicylic acid (SA) as a signal molecule for its induction. The adjacent cells surrounding the initial infection site recognize the signal molecules and plants acquire resistance that is hormonally induced throughout the plant.

In order to elicit resistance mechanism of whole tissues that are not infected by pathogens, a signal must pass from the infection site to the distal tissues during the induction of SAR (Klessig and Malamy 1994). This transmissible signal molecule, that was first hypothesized by Ross in 1966 and demonstrated in grafting experiments by Guedes *et al.* (1980), can activate resistance mechanisms in plants at low concentrations (Enyedi *et al.* 1992). SA is one of the numerous phenolic compounds, defined as a compound containing an aromatic ring with hydroxyl group or its derivative, found in plants (Klessig and Malamy 1994). SA is believed to be a signal, which naturally occurs and is produced by plants during the resistance responses. SA is synthesized from cinnamic acid that is catalyzed from phenylalanine by phenylalanine ammonia lyase (PAL) (Klessig and Malamy 1994).

Phytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms, whereas phytoanticipins are present in plants before challenge by microorganisms (Paxton 1981, Van Etten *et al.* 1994). Phytoalexins accumulate at infection sites and inhibit the growth of fungal and bacterial pathogens after contact with biotic and abiotic factors (Favaron 1988, Rowell and Bailey 1983, Stolle 1988). The effect of phytoalexins depends on their

quality as well as quantity, and these qualifications vary with the pathogen, plant, inoculated organ or tissue age of the plant, and environmental factors (Kuc 1995).

Pathogenesis-related proteins (PRs) play an essential role in the inhibition of growth of fungi during colonization (Lozovaya *et al.* 1998). PRs, described in the 1970s in tobacco leaves infected with TMV, were initially defined as acid-soluble, protease-resistant, acidic proteins localized in the extracellular space (Sticher *et al.* 1997). This restrictive definition has been changed to plant proteins that accumulate after pathogen attack or related situation including microbial pathogens, nematodes, insects, or herbivores, treatment with certain chemicals, or other types of stresses (Appel *et al.* 1995, Dann *et al.* 1996, Kastner *et al.* 1998, Lafitte *et al.* 1993, Münch-Garhoff *et al.* 1997, Van Loon *et al.* 1994).

Most PRs accumulate in the extracellular space or in the vacuole (Sticher *et al.* 1997). In both compatible and incompatible interactions in wheat, PRs are located in the host plasmalemma and in the domain of the host cell wall near the plasmalemma of the mesophyll cells, but higher concentrations of the enzyme are located in infected resistant wheat leaves than in infected susceptible ones (Hu and Rijkenberg 1998). In particular, the enzyme is also detected in the secondary thickening of xylem vessels and in the walls of guard cells, epidermal cells and phloem elements. Acidic PRs typically accumulate in the cell wall and basic ones in the vacuole, though not for all plant species (Walton 1997, Sticher *et al.* 1997). The vacuolar PRs seem to exert an effect on the defense reaction after decompartmentalization of a cell, whereas the extracellular PRs are directly in contact with the pathogen penetrating the tissue (Sticher *et al.* 1997).

To date, 11 families of PRs have been identified (Sticher *et al.* 1997). PRs are constitutively expressed in the plant organs at low concentration and the activity

increased after infection associated with reduced lesion formation (Anguelova *et al.* 1999, Dann *et al.* 1996, Siegrist and Kauss 1990, Van Pelt-Heerschap and Smit-Bakker 1999). This increase of enzyme activity is time dependent and requires gene expression (Boudart *et al.* 1998, Daugrois, *et al.* 1992, Kastner *et al.* 1998, Lafitte *et al.* 1993). It implies that the early recognition of the fungus or the early accumulation of the PRs play an important role in the defense response (Anguelova 1999, Kastner *et al.* 1998). The genetically engineered transgenic plants that have genes encoding PRs showed increased resistance to infection with fungi (Bliffeld *et al.* 1999, Sticher *et al.* 1997).

Callose and lignin are produced at the point of attempted invasion in the response to fungal penetration (Aist 1976, Paxton and Groth 1994). Papillae have been observed in potential or actual penetration sites of plants during fungal penetration (Aist 1976, Mould 1991, Skou 1982). Callose has been reported to be a primary ingredient of papillae although they contain many other materials such as lignin, phenols, cellulose, protein, pectin, suberin, gums, and silicon (Aist 1976). These wall-like depositions are located between the plasmalemma and cell wall and associated with penetration resistance (Aist 1976, Lyngkjær 1997). Callose, a polysaccharide consisting primarily 1,3-β-linked glucose, is rapidly deposited by plant cells in response to mechanical perturbation or fungal attack (Stanghellini *et al.* 1993). Penetration resistance is associated with rapid deposition of large callose-containing appositions in the epidermal cell wall (Lyngkjær 1997, Stanghellini 1993).

Application of 2-deoxy-glucose (DDG) or mannose results in increasing susceptibility to pathogen attacks (Bayles 1990, Lyngkjær 1997, Stanghellini 1993). This suppression effect is shown as an increase in penetration efficiency and delayed papillae

formation (Bayles *et al.* 1990, Lyngkjær 1997, Stanghellini 1993). Encasement of the haustoria with heavy deposits of callose-like materials may interrupt the flow of nutrients from the invaded host cells (Cohen *et al.* 1989). This nutrient deprivation that results from callose deposition may be the basis of resistance to the pathogen (Cohen *et al.* 1989, Stanghellini 1993).

Phenylalanine ammonia-lyase (PAL) plays a pivotal role in the biosynthesis of lignin (Strack 1997). Lignin is one of the most abundant biopolymers on earth and can be formed as a response or as a resistance mechanism to most microorganisms (Vance *et al.* 1980). Lignification is not restricted to incompatible interactions in gene for gene systems but is also observed in nonhost resistance (Sticher *et al.* 1997). Lignin is always associated with wall polysaccharides (Strack 1997). Incorporation of lignin into a plant cell wall is bound to strengthen it mechanically and to make it more resistant to degradation by enzymes secreted by an invading pathogen. The precursors of lignin are also toxic to pathogens. Lignified papillae and cell walls could constitute a barrier preventing nutrients uptake of pathogen and, therefore, help to starve a pathogen (Sticher *et al.* 1997).

1.6. Velvetleaf (*Abutilon theophrasti*)

Velvetleaf (*Abutilon theophrasti* Medik.) is a quantitative short day plant and an annual weed that originated in China (Oliver 1979, Roeth 1987, Spencer 1984). It was introduced into North America presumably before 1750 as a fiber crop from England or India (Roeth 1987, Spencer 1984). But it was not utilized economically and became a problematic weed because of weedy characteristics such as seed dormancy, ability to germinate from deep in the soil, and tolerance for many herbicides (Spencer 1984). It is a

major weed in soybean, maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and sorghum (*Sorghum bicolor* [L.] Moench) in the United States and eastern Canada (Spencer 1984, Warwick and Black 1988). Velvetleaf is difficult to control because it has excellent competitive ability such as rapid growth rate, prolific seed production, prolonged seed dormancy, and allelopathic effects (Warwick and Black 1988). Its seeds may remain viable for 50 years or more when stored dry or in the soil (Warwick and Black 1988). Moreover, since only 5-15% of the seeds in the soil germinate in a year, effective control of this weed may be more difficult (Roeth 1987, Warwick and Black 1988). Generally, there are 35 to 45 seeds per capsule and 700 to 17,000 seeds per plant (Warwick and Black 1988). In non-competitive field conditions, maximum height and ground cover occur at 10 weeks and maximum capsule production at 13 weeks after emergence. This deleterious weed can tolerate triazine herbicides (Ritter 1986). An enhanced capacity to detoxify the herbicide results from glutathione conjugation by glutathione *S*-transferase (Anderson and Gronwald 1991, Stowe and DiTomaso 1989).

A few attempts have been tried to control velvetleaf by biological methods. The scentless plant bug, *Niesthrea louisianica*, reduced viable seed production by 98 to 99% in comparison with insect-free control plants (Patterson *et al.* 1987). The pathogen, *Verticillium dahliae* had been suggested as a biocontrol agent of velvetleaf (Green and Wiley 1987). *Fusarium lateritium* was effective in controlling velvetleaf in the field when it was applied by postemergence foliar applications or preemergence applications (Boyette and Walker 1985). A foliar pathogen of velvetleaf, *C. coccodes*, showed excellent efficacy of controlling the weed (Gotlieb *et al.* 1987).

1.7. Thesis objectives

The main objective of this study was to investigate the enhancement of virulence of *C. coccodes* to *A. theophrasti* and to clarify the physiological defense responses of velvetleaf to *C. coccodes*. Therefore, the thesis consisted of the following detailed objectives:

(1) To verify if induced resistance to *C. coccodes* occurs in velvetleaf.

(2) To determine whether induced resistance may limit the biocontrol efficacy of *C. coccodes* to velvetleaf.

(3) To determine the level of reduction in efficacy of the bioherbicide.

(4) To screen various chemicals as virulence enhancers of *C. coccodes.*

(5) To verify the effect of selected chemicals on *C. coccodes* and velvetleaf.

(6) To evaluate the efficacy of the selected chemicals for velvetleaf control.

(7) To verify the involvement of defense-related enzymes in the defense response of *A. theophrasti* to infection by *C. coccodes*

(8) To determine the effects of chemical virulence enhancers of *C. coccodes* on the enzyme responses.

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CONNECTING TEXT

The success of biological weed control using pathogens is inevitably dependent on the susceptibility of the weed host. A bioherbicide exploits indigenous pathogens and enhances its level of disease in the field. This increases the possibility that induced resistance may affect the efficacy of the bioherbicide. As of now, the evidence that plants show resistance to bioherbicides has not been reported although research on the development of bioherbicides has been widely conducted. In this chapter, induced resistance in *Abutilon theophrasti* to *Colletotrichum coccodes* was verified and quantified. The candidate was the primary author of this chapter. Dr. A.K. Watson, Dr. T. Paulitz, and Dr. S. Jabaji-Hare were co-authors.

Chapter 2. Induced Resistance as a Constraint to Biological Weed Control

2.1. Abstract

The impact of induced resistance of *Abutilon theophrasti* to *Colletotrichum coccodes* on the biocontrol efficacy of *C. coccodes* was determined. *C. coccodes,* benzothiadiazole (BTH), bentazon, and acifluorfen were applied to the first leaf or to the root of *A. theophrasti* to induce resistance of the plant against *C. coccodes* infection. The size of lesion caused by the challenge treatment of *C. coccodes* on the third leaf in induced-plants was significantly reduced compared to lesion diameter in non-induced plants when there was a two-day interval between inducing treatments with 10 ppm BTH, 200 μM acifluorfen, and 8 mM bentazon and challenge treatment with *C. coccodes*. Decrease in lesion size in *C. coccodes*-induced plants was large at a two-day interval, but not statistically significant. A minimum of two days seemed to be required for a signal molecule to be transduced systemically from the locally stressed site to other plant parts. The effect of reducing the efficacy of *C. coccodes* by BTH was strong and persisted for a week after the BTH treatment. Resistance induced by the herbicides was transient as it rapidly disappeared shortly after it occurred. Peroxidase and phenylalanine ammonia lyase (PAL) activities in the second or third leaves were greatly induced by the inducing treatments with the agents. This study demonstrates that resistance in *A. theophrasti* can be induced by the localized stresses. However, adverse effects to the biological control agent could be prevented in an integrated weed management system by arranging the application method and timing, since the induced resistance in *A. theophrasti* against *C. coccodes* was transient and disappeared rapidly.

2.2. Introduction

Velvetleaf (*Abutilon theophrasti* Medik.) is a major weed in soybean [*Glycine max* (L.) Merr.], maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and sorghum (*Sorghum bicolor* [L.] Moench) in the United States and eastern Canada (Spencer 1984, Warwick and Black 1988). It has deleterious weedy characteristics such as seed dormancy, ability to germinate from deep in the soil, and tolerance for many herbicides (Spencer 1984). Triazine resistance in velvetleaf was first reported in USA, and it is a result of increased rate of herbicide detoxification (Ritter 1986, Stowe and DiTomaso 1989). The enhanced capacity to detoxify the herbicide results from glutathione conjugation by glutathione S-transferase (Anderson and Gronwald 1991). A few attempts have been tried to control velvetleaf biologically including the scentless plant bug, *Niesthrea louisianica*, and pathogens, *Fusarium lateritium, Verticillium dahliae*, and *Colletotrichum coccodes* (Boyette and Walker 1985, Gotlieb *et al.* 1987, Green and Wiley 1987, Patterson *et al.* 1987). *Colletotrichum coccodes* isolated from foliar lesions on velvetleaf caused severe foliage blight when it was applied at the cotyledonary to two-leaf stage indicating very good bioherbicide potential of this weed pathogen (Gotlieb *et al.* 1987). However, when *C. coccodes* was applied at later growth stages, plants continue to grow after shedding infected leaves.

The success of biocontrol of weeds using pathogens is inevitably dependent on the susceptibility of the weed host. Bioherbicides exploit indigenous pathogens and enhance disease level in the field by inundative application of pathogen propagules. Disease is a function of the interaction between a plant host and a pathogen resulting in adverse changes of the host in its form and function thus leading to partial impairment or plant

death. Whether the interaction between the two occurs or not is genetically determined. Disease occurs only when the pathogen overcomes the plant's preformed or induced defense mechanism. The plant resistance mechanisms can be enhanced by pathogen invasions or chemical applications (Sticher *et al.* 1997). Interest in induced resistance has increased sharply as a mechanism to improve resistance of crops to disease since it is non- or less-toxic than chemical pesticides and provides a broad range of protection. Although it has not received much attention in the literature on biological weed control, induced resistance is considered as a constraint to bioherbicide development.

Plants that have undergone a resistance response in infected tissues attain immunity systemically in non-exposed tissues to many other pathogens as well. This type of induced resistance is termed systemic acquired resistance (SAR). SAR is defined as a state of enhanced defensive capacity, and characterized by the accumulation of salicylic acid and pathogenisis-related (PR) proteins. Salicylic acid accumulates locally near the infection site and is systemically transmitted to other tissues at low levels. Exogenously applied natural signal compounds can induce resistance in a plant (Van Loon *et al.* 1998, Pieterse and Van Loon 1999). *S*-methyl benzo [1,2,3]thiadiazole-7-carbothiate (BTH) that can induce plant resistance mechanisms has been successfully commercialized as Bion™. It can protect crops from a variety of pathogens including *Magnaporthe grisea, Sclerotinia sclerotiorum*, and *Pythium ultimum* (Oostendorp *et al.* 2001). 3-allyoxy-1,2-benzisothiazole-1,1-dioxide (PBZ), commercialized as Oryzemate®, is a chemical inducer of defense mechanisms in rice against rice blast by *Magnaporthe grisea*. PBZ has also been registered against bacterial spot on cucumber, bacterial rot and spot on lettuce, black rot on cabbage, bacterial soft rot on Chinese cabbage and bacterial spot on sweet

pepper (Yamaguchi 1998). Resistance can be induced not only by biotic stimulation but also by herbicides. Herbicide-induced resistance has been reported in melon, cucumber, and tobacco (Cohen *et al.* 1996, Starratt and Lazarovits 1999, Strobel and Kuc 1995). In some cases, the plants generating SAR induced by pathogens or herbicides show cross-resistance to the pathogens and the chemicals. In cucumber and tobacco systems, resistance was systemically induced by pathogens to pathogens, by chemicals to pathogen, by pathogens to chemicals, and by chemicals to chemicals (Strobel and Kuc 1995).

Induced resistance has been widely reported in crops, while the reports on weeds are few. Three applications of *C. coccodes* generally resulted in less severe disease symptoms and resulted in the smallest decreases in *A. theophrasti* growth (DiTommaso and Watson 1995). A subsequent application of the foliar pathogen, *Phomopsis convolvulus,* on the perennial weed, *Convolvulus arvensis,* caused limited disease symptoms in comparison to the extent of disease observed after the first inoculation (Morin *et al.* 1989). Although *Pseudomonas syringae* pv. *phaseolicola* (Psp) showed potential to control kudzu (*Pueraria lobata*) in greenhouse experiments, the field efficacy was rated low. The second spray treatment of Psp did not enhance disease levels. Water-soaked symptoms were slower to develop after the second spray application than the first, possibly due to induced resistance (Zidack and Backman 1996). *Cassia obtusifolia* L. (sicklepod) showed induced-resistance three days after inducing treatment by *Alternaria cassiae* (Casst®), a potential bioherbicides to sicklepod, and phytoalexins were suspected as major defense arsenals (Weete 1992, Sharon and Gressel 1991). Bioherbicides exploit indigenous and locally occurring pathogens, thus increasing the possibility that induced

resistance may affect the efficacy of bioherbicide (Zidack 2000). The objectives of this study were 1) to verify if induced resistance to *Colletotrichum coccodes* occurs in velvetleaf, 2) to determine whether it may limit the biocontrol efficacy of *C. coccodes* to velvetleaf, and if so, 3) to determine level of reduction in efficacy of the bioherbicide.

2.3. Materials and Methods

2.3.1. Plant production

A. theophrasti seeds were collected from an agricultural field population at the Emile A. Lods Agronomy Research Centre of McGill University, Ste-Anne-de-Bellevue, QC in fall 1999 and stored at room temperature in plastic bags. Seeds were dipped into boiling water for 10 seconds to break dormancy, then placed onto distilled water saturated filter paper (P8, Fisher Scientific, Nepean, Ontario) in 9 cm diameter Petri dishes and incubated in the dark for 48 hours. Germinating seeds were sown in 10 cm top-diameter plastic pots (three seeds per pot) in a commercial potting medium (Promix BX, Premier Brands, Inc., NY, USA) and the pots were placed on a controlled environment bench (Conviron®, Winnipeg, MB) with 24/18°C day/night, 300 μmol $m^{-2}s^{-1}$ fluorescent light for 14 hours per day. The plants were watered daily and fertilized with 50 ml of 20-20-20 $N\text{-}P_2O_5\text{-}K_2O$ (1.25 $g{\cdot}L^{-1}$) per pot. Plants were at the three-leaf stage at the time of treatment.

2.3.2. Inoculum production

A stock culture of *C. coccodes* (DAOM 182826 deposited in the Biosystematics Research Institute, Ottawa, ON) was isolated from diseased velvetleaf and maintained on

potato dextrose agar (PDA, DIFCO Laboratories, Detroit) slants at 3°C under mineral oil. A small piece of the culture was placed onto the middle of a PDA plate and kept in the dark at 22°C (± 2°C) for one week. Mycelial plugs were removed from the edge of the PDA plates and transferred to a modified Richard's solution [10 $g \cdot L^{-1}$ of sucrose, 10 $g \cdot L^{-1}$ of KNO_3, 5.0 $g \cdot L^{-1}$ of KH_2PO_4, 2.5 $g \cdot L^{-1}$ of $MgSO_47H_2O$, 0.02 $g \cdot L^{-1}$ $FeCl_36H_2O$, 150 ml V-8 juice (Cambell Soup Company Inc.) and distilled water to 1 L] in Erlenmeyer flasks. Cultures were incubated for seven days on a rotary shaker at 200 rpm at room temperature [22°C (± 2°C)]. Cultures were filtered with four layers of cheesecloth. Conidia obtained from the liquid culture were used for inoculation. The inoculum density was adjusted using a haemocytometer.

2.3.3. Chemicals

S-methyl benzo[1,2,3]thiadiazole-7-carbothiate (Bion™, BTH) formulated as 50% active ingredient (a.i.) in a wettable granular form, was obtained from Novartis Crop Protection, Toronto, Canada. Bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide, Basagran™, BASF] formulated 48 % a.i. and acifluorfen (5[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoic acid, Blazer™, BASF) formulated 24 % a.i. were obtained from the herbicide inventory at the Emile A. Lods Agronomy Research Centre of McGill University.

2.3.4. Induction of resistance

Plants were grown as described above. Plants were selectively thinned to a final density of one per pot for uniformity a week before the experimental treatment. In order

to induce the resistance in *A. theophrasti*, *C. coccodes*, BTH, bentazon, and acifluorfen were used. Distilled water was applied to the control plants. *C. coccodes* was inoculated on the upper surface of the first true leaf (Figure 2.1). Ten 5-μL droplets of *C. coccodes* suspension (10^7 conidia·mL^{-1}) were applied with a pipette on the surface of the first true leaf. Conidia were obtained from the liquid culture as described above. The plants were then incubated in a dark dew chamber for 24 hours at 22°C, and 100 % relative humidity (RH). The herbicides, bentazon and acifluorfen, were diluted in distilled water and applied as droplets with a pipette. Ten 5-μL droplets of 8 and 16 mM bentazon solution were applied to the upper surface of the first leaf. Acifluorfen was applied at 200 and 400 μM in the same manner as the bentazon treatments. BTH was applied to roots as a soil drench. Fifty ml of 1 or 10 ppm BTH were applied per pot as a soil drench with distilled water. Each treatment consisted of four replicates and the experiments were replicated twice.

2.3.5. Assessment of induced resistance to *C. coccodes*

In order to assess the occurrence of induced resistance in *A. theophrasti* caused by the inducing agents, *C. coccodes* was applied as a challenge treatment to the upper surface of the third leaf one, two, four, and seven days after the inducing treatments (Figure 2.1). This time intervals between the inducing and challenge treatments were designed to assess time required for expression of induced resistance. Twenty 5-μL droplets of spore suspension (10^6 conidia·mL^{-1}) were applied to each third leaf in all plants and incubated in a dark dew chamber for 24 hours as described above. After incubation, the plants were returned to the controlled environment growth bench. In order

to assess the degree of induced resistance occurred by each inducing agent against *C. coccodes,* lesion diameter caused by the challenge treatment was measured seven days later.

2.3.6. Enzyme extraction

Enzyme extraction was carried out as described by Nickerson *et al.* (1993) and with a few modifications. Plants were grown as described above. Plants were selectively thinned to a final density of two per pot for uniformity a week before the experimental treatment. *C. coccodes*, BTH, bentazon, and acifluorfen were applied were applied to the first leaf or to the root of *A. theophrasti* to induce the resistance as described above. The second and third leaves, which did not contact with the inducing agents, were excised from six plants 1, 2, 4, and 7 days after the treatments and pooled for extraction. The leaves were weighed and stored in –80°C. The samples were ground to a dry powder with mortar and pestle in the presence of 20 % (w/w) polyvinylpyrrolidone. The powder was transferred to a conical polypropylene tube and 7 ml of buffer solution was added for each gram of fresh weight of plant tissue. 50 mM sodium acetate buffer (pH 5.0) for peroxidase analysis and 100 mM sodium borate buffer (pH 8.8) containing 14 mM 2-mercaptoethanol for PAL analysis were used as buffer solutions. The leaf suspension was incubated on ice for 30 minutes and vortexed vigorously at 10 minutes intervals. The suspension was filtered through cheesecloth and the filtrate was centrifuged at 10,000 g for 10 min using an SS-34 rotor in a Sorvall RC5B centrifuge (Dupont) at 4°C. The supernatant was immediately used for the enzyme assay or stored at –80°C.

2.3.7. Enzyme activity studies

Peroxidase analysis was carried out as described by Nickerson *et al.* (1993) with a few modifications. Peroxidase activity was measured at 470 nm in 3 ml of the reaction mixture consisting of 0.1 ml of the enzyme extract, 0.3 % guaiacol (v/v), 0.3 % H_2O_2 (v/v), and 50 mM sodium acetate buffer for 60 seconds. Each enzyme extract was assayed three times to achieve an average value for the sample.

PAL analysis was carried out as described by Edwards and Kessmann (1992) with a few modifications. 0.1 ml of the extract was incubated at 40°C with 0.9 ml 12.1 mM L-phenylalanine in 100 mM sodium borate buffer (pH 8.8) for 60 minutes. PAL activity was measured spectrophotometrically at 290 nm. Each enzyme extract was assayed three times to achieve an average value for the sample. The protein content of the extract was determined by the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford 1976).

2.3.8. Data analyses

Experiments were carried out in a completely randomized design. Analyses of variance (ANOVA) were carried out using SAS (SAS 6.02, SAS Institute Inc, Cary, NC). When necessary, appropriate transformations of the values were performed to normalize data and stabilize the variance throughout the data range prior to ANOVA. Data were separated with Fisher's least significant difference test ($P < 0.05$).

2.4. Results

2.4.1. Resistance induced by *Colletotrichum coccodes*

When *C. coccodes* was challenge-treated to the third leaf one day after the inducing treatment with *C. coccodes*, lesions caused by the challenge treatment were larger than lesions in the water-treated plants (Table 2.1). Lesion diameter decreased when there was a two-day time interval between the inducing and challenge treatments. Although the decrease in lesion diameter at the two-day interval between inducing and challenge treatments was not statistically significant, lesion diameter was reduced by 26 % in the induced-plants as compared to non-induced plants. The effect of decreasing lesion diameter weakened over time. At the four-day interval, lesion diameter was reduced by 9 % and by the seven day interval there was no effect on lesion size.

2.4.2. Resistance induced by benzothiadiazole

Ten ppm BTH treatment significantly reduced the diameter of lesions caused by the challenge treatments with *C. coccodes* when *C. coccodes* was applied later than two days after the inducing BTH treatment (Table 2.2). Decrease of lesion diameter was not observed when 1 ppm BTH was applied at any time point. The level of the decrease of lesion diameter in the 10 ppm BTH treatment was constantly lower when there was more than two days of time interval between inducing and challenge treatments. Ten ppm BTH treatment reduced lesion diameter up to 45 % at a four-day time interval between the inducing and challenge treatments. Decrease of lesion diameter did not occur when *C. coccodes* was applied one day after the soil drench of BTH.

2.4.3. Herbicide-induced resistance

Decrease of lesion diameter was observed when there was a two-day interval between the inducing treatment and the challenge treatment with acifluorfen (Table 2.3). There was no decrease in lesion diameter at the one-day time interval and the reduction observed in the acifluorfen-induced plants at the two-day interval weakened over time, and it disappeared by the seven-day interval.

The level of reduction in lesion diameter in the bentazon-treated plants was low compared to other chemical-induced plants (Table 2.4). Treatment of 8 mM bentazon significantly reduced lesion diameter caused by *C. coccodes* at the two-day interval between induction and challenge treatments. As with the acifluorfen-induced plants, the significant reduction observed in 8 mM bentazon-treated plants at the two-day interval between inducing and challenge treatments disappeared over time.

2.4.4. Enzyme responses when *A. theophrasti* was induced systemically by the application of *C. coccodes*, BTH, bentazon and acifluorfen

The enzyme activity was measured in the second and third leaves after inducing treatments to the first leaf with *C. coccodes*, bentazon, and acifluorfen, or to the root with BTH. Localized treatments of these agents systemically induced the activity of peroxidase in the second and third leaves that did not contact with the inducing agents (Figure 2.2). Peroxidase activity was greatly induced by treatments of *C. coccodes*, acifluorfen, and bentazon four days after the treatments compared to non-induced plants. Peroxidase was activated at a similar level over time by *C. coccodes* and acifluorfen, while it was lower in bentazon-treated plants. Peroxidase activity was highly induced two days after BTH treatment. Ten ppm BTH treatment resulted in a sharp increase in peroxidase activity over

time. Peroxidase activity induced by BTH was about two-fold higher than the control, seven days after the BTH treatment. Induction of peroxidase activity was also observed when the herbicides acifluorfen and bentazon were applied, but the induction rates were much lower than in the BTH treatment.

The localized treatments with *C. coccodes,* BTH, acifluorfen, and bentazon also resulted in higher PAL activity in the plant tissue that did not contact with the inducing agents at a four-day interval between inducing and challenge treatments (Figure 2.3). Induction of PAL activity was not observed one day after treatment and the earliest induction of PAL activity was observed in the acifluorfen-treated plants two days after treatment. PAL activity was high in all treatments four days after the treatments and the differences between PAL activities in non-induced and induced plants were the largest. Difference between PAL activities in the non-induced plants and induced plants decreased seven days after the inducing treatments. At seven days after the inducing treatments, there was no difference in PAL activity in *C. coccodes*-treated plants compared to non-induced plants. PAL activity in the control (non-induced) plants increased progressively as plants grew.

2.5. Discussion

A bioherbicide exploits indigenous and locally occurring pathogens and it is the application of inundative doses of propagules to the target weed (Auld and Morin 1995). The endemic organism always exists and causes non-lethal levels of disease in the surrounding environment (Zidack 2000). Thus, an endemic pathogen must be augmented by an inundative treatment to achieve effective weed suppression. These characteristics may bring more chances to infect a target weed consistently even though it is not lethal,

but sufficient to induce resistance to a bioherbicide applied in the field. Three applications of *C. coccodes* generally resulted in less severe disease symptoms and the smallest decreases in velvetleaf growth, possibly because the earlier treatment had induced resistance of velvetleaf to *C. coccodes* (DiTommaso and Watson 1995).

Decrease in lesion diameter in *C. coccodes*-induced plants was so weak and transient that it only appeared at a two-day interval between inducing and challenge treatments, and the decrease was not statistically significant. However, enhanced activities of peroxidase and PAL in *C. coccodes*-induced plants suggest that defense-related mechanisms in *A. theophrasti* may be induced by *C. coccodes* infection. Systemic induction of peroxidase and PAL activities are the common reactions in the process of induced defense responses to pathogen attack (Madi and Katan 1998, Stadnik and Buchenauer 2000). PAL is prominently involved in many plant defense responses, such as cell wall strengthening and phytoalexin secretion, by catalyzing phenylalanine to cinnamic acid in the entry point of phenylpropanoid pathway that produces phenolic compounds (Strack 1997). While PAL is an important enzyme affecting the defense response by regulating production of phenolic compounds at the key position in phenylpropanoid pathway, peroxidase is associated with structural defense responses by catalyzing oxidative cross-linking of lignin and hydroxyproline-rich glycoproteins (Hoson 2000). Induction of PAL and peroxidase after treatments of *C. coccodes* and the chemicals indicates those chemicals can induce resistance of *A. theophrasti* and the enzymes are deeply involved in defense mechanisms. Peroxidase and PAL activities were much higher in all induced plants four days after treatment compared to non-induced plants.

BTH significantly induced resistance to *C. coccodes* when there is more than two days of time interval between the inducing and challenge treatments. Once induced resistance occurred, it persisted long enough to suppress infection of *C. coccodes,* even a week after the BTH treatment. Peroxidase and PAL activities were most strongly induced by BTH, and the enhanced level of the enzyme activities persisted longer compared to other treatments. These results confirm that this plant activator effectively induces resistance in *A. theophrasti* and the resistance induced by BTH can reduce the biocontrol efficacy of *C. coccodes*. However, 1 ppm BTH treatment was not effective to induce resistance of *A. theophrasti*, thus there must be a threshold concentration of BTH when in contact with plant roots.

Induction of resistance appeared two days after the inducing treatment, indicating that it takes minimum two days for a signal molecule to transmit and systemically activate resistance in other plant tissue. A sequential reaction takes place in a plant from a leaf initially attacked by the pathogen to other plant tissues when a plant activates induced resistance. The initial recognition of the pathogen by the infected plant tissue is followed by increase of signal molecules such as salicylic acid, the central role of which in induced-resistance signaling pathway is believed to be pivotal (Metraux 2001). Persistence of resistance at the induced level in a plant varies with the plant-microorganism system. Induced resistance of sicklepod caused by *Alternaria cassiae* appeared within one day and persisted for about nine days, while resistance of cucumber induced by *Pseudomonas lachrymans* appeared four days after inducing inoculation and reduced disease by *Colletotrichum lagenarium* for 37 days (Caruso and Kuc 1979, Weete 1992). In the *A. theophrasti-C. coccodes* system, a slight reduction of lesion diameter was observed only at a two-day time interval, and it rapidly disappeared.

The physiological change with aging might also affect the time-dependent expression of induced resistance in *A. theophrasti*. Resistance to pathogens commonly increases with physiological age of plants, and degree of the induction can be affected by plant age (Reuveni 1998, Weete 1992). Inoculation of older plants showed that resistance, independent of induced resistance, developed during aging in sicklepod (Weete 1992). Reduction of lesion number on the leaves of sicklepod caused by induced resistance to *Alternaria cassiae* was less in the older leaves than the younger leaves when the first leaf was induced, and significant reduction of lesion number appeared up to the fourth leaf of plants with the degree of resistance considerably less on the fifth leaf. When challenge inoculation was applied to the third leaf of *A. theophrasti* seven days after the inducing treatments, the physiological status of the plant was different from the plant challenge-inoculated two days after inducing treatment, as observed in the PAL analysis. PAL activity in non-induced plants was progressively higher as the plant aged, and induced resistance of *A. theophrasti* was weaker when the time interval between inducing and challenge treatments was longer.

The peroxidizing herbicides, bentazon and acifluorfen, also induced resistance of *A. theophrasti* in a time-dependent manner as with BTH or *C. coccodes* treatments. Bentazon and acifluorfen are widely used for controlling broadleaf weeds in soybean and corn fields (Ahrens 1994). Both of these chemicals cause peroxidation of the lipid membrane due to the production of active oxygen species. Active oxygen species such as superoxide contribute to the coordinated activation of programmed cell death and induction of hypersensitive response at the site of lesion formation (Greenberg 1997). Other herbicides also induce disease resistance. Chloroacetamide herbicides, such as acetochlor, inhibit biosyntheses of fatty acids and lipids, gibberellins, and flavonoids

(Fuerst 1987). Dinitroaniline herbicides, such as trifluralin, interfere with microtubule structure and function by binding tubulin, the major microtubule protein (Vaughn and Lehnen 1991). These herbicides induced resistance of melon to *Fusarium* and tomato and eggplant to *Fusarium* and *Verticillium* species (Cohen *et al.* 1996, Grinstein *et al.*1984, Starratt and Lazarovits 1999). Acetochlor had little or no effect on growth rate or sporulation of the pathogen in culture (Cohen *et al.*1996). However, the promotion of necrosis by an herbicide is not always sufficient for the induction of systemic disease resistance. Acetochlor did not induce resistance in tomato seedlings, but was the most effective agent to Fusarium wilt in melons (Bolter *et al.*1993). Acifluorfen was an active agent to induce resistance to *Colletotrichum lagenarium* in cucumber, but this agent failed to trigger SAR to tobacco mosaic virus (TMV) and the blue mold fungus, *Peronospora tabacina,* in tobacco (Strobel and Kuc 1995).

Enzyme activities induced by the chemicals were stronger than those by *C. coccodes* overall. PAL activity in *C. coccodes*-inoculated plants was not different from non-inoculated plants at a seven-day interval, while the enzyme activities in the chemical-treated plants were still higher than in non-inoculated plants. This weaker induction of PAL activity in *C. coccodes*-inoculated plants may explain why the decrease in lesion diameter in *C. coccodes*-inoculated plants was lower than in the chemical-treated plants, and why the resistance induced by *C. coccodes* was more transient and unstable. In general, induced resistance is not very dramatic, and chemical induction of resistance is often greater than biological induction. The chemical activator may induce different patterns of host genes than pathogens (Molina *et al.* 1999, Schweizer *et al.* 1999). It is premature to say the chemicals and *C. coccodes* induce resistance of *A. theophrasti* in different manners, however, the results herein indicate that the level of resistance in *A.*

theophrasti was different depending on a type of inducer and the timing of the inducing and challenge treatments.

Induced resistance may reduce the efficacy of biocontrol agents using pathogens. Resistance of *A. theophrasti* induced by *C. coccodes*, BTH, acifluorfen, and bentazon has been confirmed in all treatments. Although the degrees of induced resistance vary depending on the inducing agents, significant reductions in lesion diameter in induced-plants indicate that induced resistance can be a biological constraint to a bioherbicide. Nevertheless, the effect of induced resistance on the efficacy of *C. coccodes* as a bioherbicide appears to be dependent on the type of weed management system this agent is integrated in, the application method, and the application schedule since the degree and persistence of induced resistance varied with the type of inducing agent and timing. Resistance of *A. theophrasti* may be induced by the biocontrol agent itself or by other chemical control agents. However, the harmful effect to a biological control agent can be expected and prevented in an integrated weed management system by arranging the application timing and method since resistance induced by the herbicides or *C. coccodes* was rather transient and it disappeared rapidly.

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Table 2.1. The effect of induced resistance by *Colletotrichum coccodes* on lesion size caused by the challenge treatment with *C. coccodes* on the third leaf in various time intervals between inducing and challenging treatments.

Time interval (DAI [a])	Lesion diameter (mm) caused by challenge treatment with *C. coccodes* at seven DAC [b]	
	Induced with	
	Water	*C. coccodes*
One	2.91 ± 0.28 [c] a	3.28 ± 0.12 a
Two	2.31 ± 0.14 a	1.71 ± 0.17 a
Four	2.23 ± 0.27 a	1.96 ± 0.14 a
Seven	2.43 ± 0.23 a	2.35 ± 0.37 a

[a] DAI = days after inducing treatment.

[b] DAC = days after challenge treatment.

[c] Data are presented with means ± standard errors. Means are separated by Fisher's least significance difference test to present the difference among the levels of the factors. Means with the same letter within a row are not significantly different at 5% level.

Table 2.2. The effect of induced resistance by benzothiadiazole (BTH) on lesion size caused by the challenge treatment with *Colletotrichum coccodes* on the third leaf in various time intervals between inducing and challenging treatments.

Time interval (DAI [a])	Lesion diameter (mm) caused by challenge treatment with *C. coccodes* at seven DAC [b]		
	Induced with		
	Water	BTH 1 ppm	BTH 10 ppm
One	2.21 ± 0.23 [c] a	2.34 ± 0.13 a	2.53 ± 0.15 a
Two	2.25 ± 0.12 a	2.28 ± 0.27 a	1.40 ± 0.10 b
Four	2.11 ± 0.16 a	2.03 ± 0.23 a	1.16 ± 0.12 b
Seven	2.47 ± 0.18 a	2.62 ± 0.19 a	1.67 ± 0.15 b

[a] DAI = days after inducing treatment.

[b] DAC = days after challenge treatment.

[c] Data are presented with means ± standard errors. Means are separated by Fisher's least significance difference test to present the difference among the levels of the factors. Means with the same letter within a row are not significantly different at 5% level.

Table 2.3. The effect of induced resistance by acifluorfen on lesion size caused by the challenge treatment with *Colletotrichum coccodes* on the third leaf in various time intervals between inducing and challenging treatments.

Time interval (DAI [a])	Lesion diameter (mm) caused by challenge treatment with *C. coccodes* at seven DAC [b]		
	Induced with		
	Water	Acifluorfen 200 μM	Acifluorfen 400 μM
One	2.42 ± 0.26 [c] a	2.35 ± 0.24 a	2.46 ± 0.27 a
Two	2.70 ± 0.20 a	1.70 ± 0.17 b	2.22 ± 0.32 ab
Four	2.86 ± 0.40 a	2.63 ± 0.18 a	2.22 ± 0.50 a
Seven	2.46 ± 0.17 a	2.50 ± 0.15 a	2.39 ± 0.17 a

[a] DAI = days after inducing treatment.

[b] DAC = days after challenge treatment.

[c] Data are presented with means ± standard errors. Means are separated by Fisher's least significance difference test to present the difference among the levels of the factors. Means with the same letter within a row are not significantly different at 5% level.

Table 2.4. The effect of induced resistance by bentazon on lesion size caused by the challenge treatment with *Colletotrichum coccodes* on the third leaf in various time intervals between inducing and challenging treatments.

Time interval (DAI [a])	Lesion diameter (mm) caused by challenge treatment with *C. coccodes* at seven DAC [b]		
	Induced with		
	Water	Bentazon 8 mM	Bentazon 16 mM
One	2.36 ± 0.13 [c] a	2.08 ± 0.08 a	2.11 ± 0.13 a
Two	2.35 ± 0.10 a	1.93 ± 0.16 b	2.24 ± 0.14 ab
Four	1.96 ± 0.18 a	1.68 ± 0.18 a	2.06 ± 0.36 a
Seven	2.35 ± 0.17 a	2.10 ± 0.21 a	2.54 ± 0.16 a

[a] DAI = days after inducing treatment.

[b] DAC = days after challenge treatment.

[c] Data are presented with means ± standard errors. Means are separated by Fisher's least significance difference test to present the difference among the levels of the factors. Means with the same letter within a row are not significantly different at 5% level.

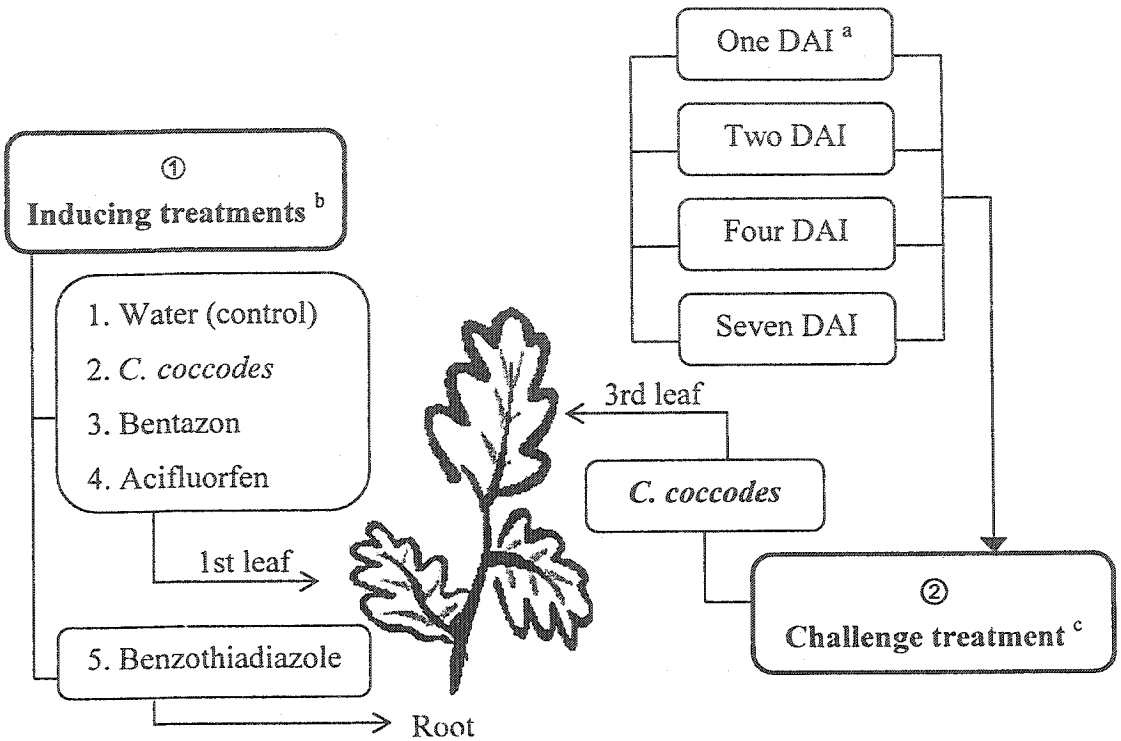

Figure 2.1. Diagram of inducing and challenge treatments.

[a] DAI= days after inducing treatment.

[b] Inducing treatment is treatment of *C. coccodes*, benzothiadiazole, bentazon, or acifluorfen to the first leaf or to the root of *A. theophrasti* for the purpose of enhancing the degree of resistance in the plant against pathogen attack. Distilled water was applied to the control plants.

[c] Challenge treatment is treatment of *C. coccodes* to the third leaf for the purpose of assessing the occurrence, persistence, and degree of the induced resistance caused by the previous inducing-treatments by the agents described above.

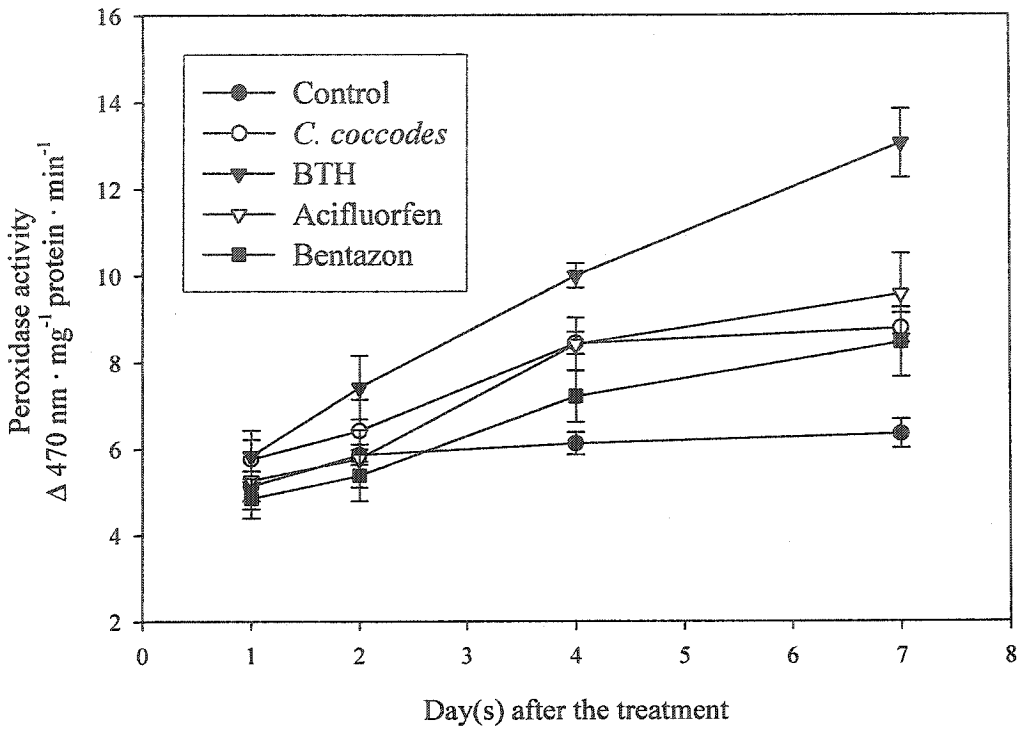

Figure 2.2. Level of peroxidase activity in the second and third leaves of *Abutilon theophrasti* after inducing-treatments with *Colletotrichum coccodes*, BTH, bentazon, or acifluorfen.

C. coccodes (10^7conidia·mL^{-1}), 200 μM acifluorfen, and 8 mM bentazon were applied to the first leaf of velvetleaf by using a micropipette. Ten ppm of BTH was applied by a soil drench.

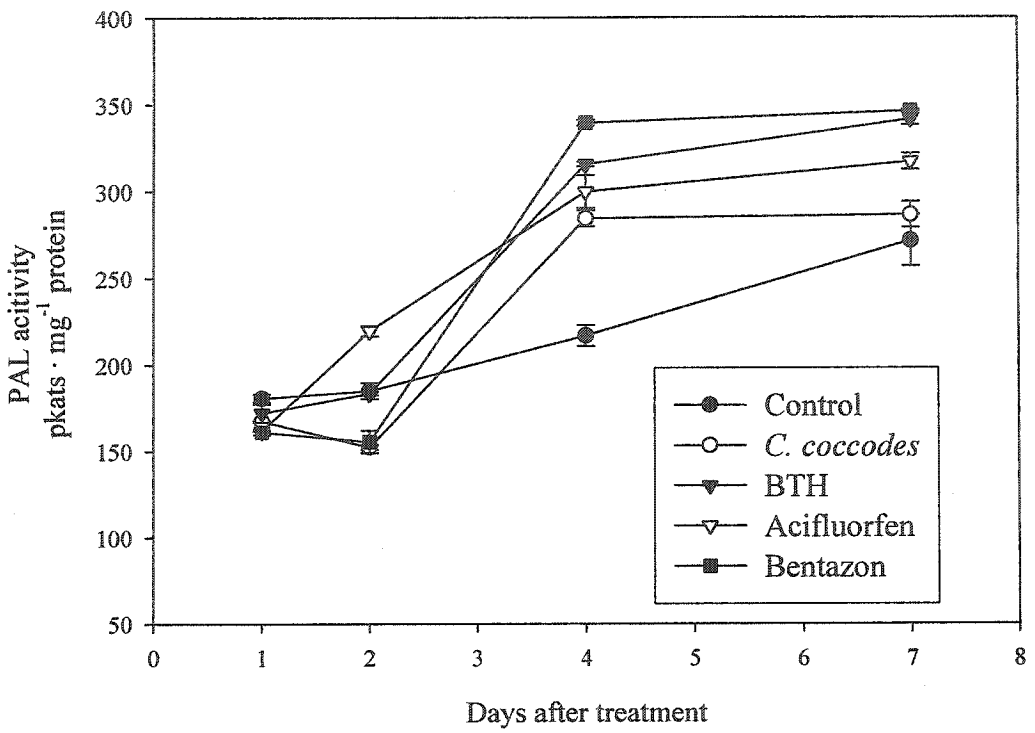

Figure 2.3. Level of PAL activity in the second and third leaves of *Abutilon theophrasti* after inducing-treatments with *Colletotrichum coccodes*, BTH, bentazon, or acifluorfen. *C. coccodes* (10^7conidia·mL^{-1}), 200 μM acifluorfen, and 8 mM bentazon were applied to the first leaf of velvetleaf by using a micropipette. Ten ppm of BTH was applied by a soil drench.

CONNECTING TEXT

In chapter 2, induced resistance in *A. theophrasti* against *C. coccodes* was investigated. The results revealed that induced resistance by localized stresses could reduce biocontrol efficacy of *C. coccodes*, but it could be avoided in an integrated weed management system by arranging the application method and timing. However, weak virulence of *C. coccodes* hinders further studies on the pathogen. Enhanced virulence of *C. coccodes* is preferable for better biological weed control system. Although enhancement of efficacy of a potential bioherbicide can be achieved in many different manners, the inhibition of a plant defense mechanism may be ideal to increase efficacy of a bioherbicide by suppressing the plant defense mechanisms. In this chapter, several chemicals reported to interfere with plant metabolisms were tested for the enhancement of *C. coccodes* virulence to *A. theophrasti* and their effects were evaluated. The candidate was the primary author of this chapter. Dr. A.K. Watson, Dr. T. Paulitz, and Dr. S. Jabaji-Hare were co-authors.

Chapter 3. Enhancement of *Colletotrichum coccodes* Virulence by Inhibitors of Plant Defense Mechanisms

3.1. Abstract

α-Amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, and oxalic acid were examined as possible agents to enhance the efficacy of *Colletotrichum coccodes*, as a biological control agent of *Abutilon theophrasti*. Vegetative growth of *C. coccodes* on PDA was inhibited by the presence of 0.1 % DDG and AOA, while mannose did not affect the radial growth of *C. coccodes*. More *C. coccodes* conidia were produced in modified Richard's medium mixed with AOA and mannose than in the control. Conidia production was inhibited in the medium with 0.1% DDG. AOA, DDG, mannose, and oxalic acid also significantly inhibited conidia germination and appressoria formation of the fungus. However, despite the inhibitory effects of the chemicals on the growth of the fungus, the virulence of *C. coccodes* was significantly enhanced when *C. coccodes* was applied after mannose and oxalic acid were vacuum-infiltrated through the leaf cuticle. Based on the hypothesis that more lipophilic analogues of the chemicals might readily penetrate the leaf cuticle, the (1-amino-ethyl)-phosphonic aciddiisopropyl ester of oxalic acid (ester OA), oxalic acid diammonium salt (ethanedioic acid), and 4,6-di-O-methyl-D-mannose (methyl mannose) were tested to determine if these chemicals would enhance the efficacy of *C. coccodes*. However, none of the compounds resulted in more severe disease on velvetleaf and there was no impact on the growth of velvetleaf. This may be due to strong antifungal effects, possible functional change resulting from structural change, size of the chemicals, or lack of inhibitory effect on plant defense

mechanisms of the tested chemicals. It is also possible that *A. theophrasti* depends little on the defense mechanisms that the chemicals can affect. Comprehensive understanding of the defense mechanism of *A. theophrasti* to the biocontrol agent is required to obtain better weed suppression.

3.2. Introduction

Colletotrichum coccodes has been studied as a potential bioherbicide for the control of velvetleaf, *Abutilon theophrasti* Medik. (Gotlieb *et al.* 1987, Wymore *et al.* 1988). It can severely damage, or even kill velvetleaf when it is applied at the cotyledonary to one-leaf stage by causing severe foliage blight. However, when *C. coccodes* is applied at later growth stages, plants continue to grow after shedding infected leaves. The poor aggressiveness of this pathogen has hindered its practical use and commercialization. Many attempts have been made to enhance the virulence of this pathogen such as a tank-mix application with a plant growth regulator, thidiazuron, bacteria, *Pseudomonas* spp., and several herbicides (Fernando *et al.* 1994, 1996, Wymore *et al.* 1987). Virulence enhancement can also be achieved in other ways. If plant disease defense mechanisms are suppressed, virulence enhancement may be maximized. When a plant is attacked by a pathogen, the plant defends itself actively by activating defense mechanisms in combination with preexisting defense mechanisms. These defense responses can be structural such as callose formation or lignification, biochemical reactions such as release of pathogenesis-related proteins or induction of other kinds of disease defenses related to metabolic processes. If these defense responses were blocked or suppressed by other factors, virulence of the biological control agent should concomitantly be enhanced.

Sugar analogues such as 2-deoxy-D-glucose (DDG) and mannose have inhibitory effects on energy-yielding metabolism and polysaccharide synthesis as well as glycosylation of glycoproteins (Datema and Schwarz 1979, Moore 1981). The application of these chemicals resulted in increased penetration efficiency by a fungal pathogen and a decrease in the frequency of callose containing papillae in barley (Bayles *et al.* 1990, Lyngkjer *et al.* 1997). Both compounds probably affect plant metabolic processes associated with structural defense responses such as callose formation and accumulation of phenolic compounds, by directly interfering with metabolic enzymes or reducing host cell metabolic activity resulting from phosphate sequestration.

α-Amino-oxy acetic acid (AOA) inhibits phenylalanine ammonia lyase (PAL), one of the key enzymes catalyzing the change of phenylalanine to cinnamic acid at the initial step in the biosynthesis of lignin precursors. AOA application suppressed a defense response of oats to *Erysiphe graminis,* by decreasing autofluorescent responses of the plant (Carver *et al.* 1991, 1992). PAL belongs to the class of carbon-nitrogen lyases (C-N cleavage) and catalyzes a non-oxidative deamination of phenylalanine to form the first secondary phenylpropane structure. AOA is generally inhibitory to transamination in various other metabolisms. These compounds may contribute to lowering the free energy of activation for the product elimination process (Strack 1997).

Oxalic acid is a product of the glyoxylate bypass of the tricarboxylic acid cycle (Maxwell and Bateman 1968). It plays an important role in the process of pathogenesis by *Sclerotinia sclerotiorum, Sclerotium rolfsii*, and *Endothia parasitica* (Bateman and Beer 1965, Havir and Anagnostakis 1985, Maxwell and Lumsden 1970, Noyes and Hancock 1981, Tu 1985). It affects phenolic metabolism of the host tissues by inhibiting

polyphenoloxidase (Magro *et al.* 1984). Oxalic acid production and secretion of pectic enzymes result in a synergy between oxalic acid and pectolytic enzyme by lowering the pH in favor of pectolytic enzyme (Bateman and Beer 1965, Maxwell and Lumsden 1970, Magro *et al.* 1988). Oxalic acid precipitates calcium from the middle lamellae to form calcium oxalate crystals, leaving pectic materials more susceptible to enzymatic degradation, and rendering the tissue more susceptible to a fungus. Plant tissues containing high calcium content with high calcium pectate level may be more difficult to macerate than tissues with calcium deficiency (Bateman and Beer 1965).

All these chemicals may have difficulty penetrating the cuticular waxes of leaves since they are hydrophilic polar compounds. Polar compounds are too hydrophilic to pass through leaf cuticles while nonpolar compounds can readily enter and pass through the cuticle. Cuticular penetration was an important issue during the development of phenoxyalkanoic acid herbicides, such as 2,4-D, and most commercial formulations of phenoxyalkanoic acids are in the forms of salt or ester (Crafts 1956, Loos 1975). Lowering the polarity of herbicide solutions with the addition of acid salts or with the use of ammonium salts increases their toxicity. Analogues of chemicals inhibitory to plant defense mechanisms can be modified to ester or salt forms and their lipid solubility may be increased. If chemicals inhibiting plant disease defense mechanisms can readily enter leaf cuticles and suppress the defense mechanisms, they may synergistically act to enhance the efficacy of the biocontrol agent. Enhancement of efficacy of a potential bioherbicide may be attempted and achieved in many different ways. Inhibition of plant defense mechanism may be ideal to increase the efficacy of a biocontrol agent against a plant by suppressing the plant defense mechanisms. The objectives of this study were; a) to screen various chemicals as virulence enhancers of *C. coccodes*, b) to verify the effect

of selected chemicals on *C. coccodes* and velvetleaf, and c) to evaluate the efficacy of the selected chemicals for velvetleaf control.

3.3. Materials and Methods

3.3.1. Plant production

A. theophrasti seeds were collected from the agricultural field population at the Emile A. Lods Agronomy Research Centre of McGill University, Ste-Anne-de-Bellevue, QC in fall 1999 and stored at room temperature in plastic bags. Seeds were dipped into boiling water for 10 seconds to break dormancy, then placed onto distilled water saturated filter paper (P8, Fisher Scientific, Nepean, Ontario) in 9 cm diameter Petri dishes and incubated in the dark for 48 hours. These germinating seeds were sown in 10 cm top-diameter plastic pots (three seeds per pot) in a commercial potting medium (Promix BX, Premier Brands, Inc., NY, USA) and the pots were placed on a controlled environment bench (Conviron®, Winnipeg, MB) with 24/18°C day/night, 300 μmol $m^{-2}s^{-1}$ fluorescent light for 14 hours per day. The plants were watered daily and fertilized with 50 ml of 20-20-20 N-P_2O_5-K_2O (1.25 g·L^{-1}) per pot. Plants were at the three-leaf stage at the time of treatment.

3.3.2. Inoculum production

A stock culture of *C. coccodes* (DAOM 182826 deposited in the Biosystematics Research Institute, Ottawa, ON) was isolated from diseased velvetleaf and maintained on potato dextrose agar (PDA, DIFCO Laboratories, Detroit) slants at 3°C under mineral oil. A small piece of the culture was placed onto the middle of a PDA plate and kept in the

dark at 22°C (± 2°C) for one week. Mycelial plugs were removed from the edge of the PDA plates and transferred to a modified Richard's solution [10 $g \cdot L^{-1}$ of sucrose, 10 $g \cdot L^{-1}$ of KNO_3, 5.0 $g \cdot L^{-1}$ of KH_2PO_4, 2.5 $g \cdot L^{-1}$ of $MgSO_47H_2O$, 0.02 $g \cdot L^{-1}$ $FeCl_36H_2O$, 150 ml V-8 juice (Cambell Soup Company Inc.) and distilled water to 1 L] in Erlenmeyer flasks. Cultures were incubated for seven days on a rotary shaker at 200 rpm at room temperature [22°C (± 2°C)]. Cultures were filtered through four layers of cheesecloth. The filtrate was centrifuged at 6,000 g for 15 minutes and the conidia pellet was resuspended in distilled water. The inoculum density was adjusted using a haemocytometer.

3.3.3. Chemical materials

The following chemicals were evaluated as potential virulence enhancers: α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, and oxalic acid. The following analogues of oxalic acid and mannose were tested; 4,6-di-O-methyl-D-mannose (methyl mannose), [(1-amino-ethyl)-phosphonic acid diisopropyl ester, compound with oxalic acid (ester OA)] and [oxalic acid diammonium salt (ethanedioic acid, salt OA)]. These chemicals were the only analogues of both oxalic acid and mannose commercially available when the experiment was being carried out. All chemical materials were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

3.3.4. The effect of chemicals on the radial growth of *C. coccodes*

The effects of chemicals on the radial growth of *C. coccodes* were determined on half-strength PDA (19.5 $g \cdot L^{-1}$). AOA, DDG, and mannose were dissolved with autoclaved distilled water and sterilized by membrane filtration and added to the PDA

medium. The media were agitated to mix the chemicals well before the PDA was solidified. The PDA was dispensed into 9 cm diameter Petri dishes. The final concentration of the chemicals in the PDA was adjusted to 0.01 and 0.1 % solution. A piece of agar was taken from the margin of the starter culture of *C. coccodes* by using a 5 mm-diameter cork borer and transferred onto the center of the chemical-treated PDA plates. The *C. coccodes*-inoculated plates were kept in the dark at room temperature and colony diameter was measured every second day for 10 days. The procedures were carried under the sterilized condition. Each treatment was replicated seven times.

3.3.5. The effect of chemicals on the conidia production of *C. coccodes*

The effect of chemicals on the conidia production of *C. coccodes* was determined by counting the conidia numbers after culturing *C. coccodes* in MR's medium for seven days. *C. coccodes* was cultured in 100 ml of MR's medium in the presence of chemicals in 250 ml Erlenmeyer flasks as described above. AOA, DDG, and mannose were sterilized by membrane filtration and added to the MR's medium. The final concentrations of the chemicals in the media were adjusted to 0.01 and 0.1 % solution. The cultures were placed on the rotary shaker with the speed of 200 rpm at the room temperature for seven days. Four replicates of cultures were used for each chemical at each concentration. Conidia were filtrated with four layers of cheesecloth and the number of conidia in each replicate was counted eight times with a haemocytometer.

3.3.6. The effect of chemicals on germination and appressoria formation of *C. coccodes*

The effect of the chemicals on the growth of *C. coccodes* was determined by counting germinated conidia and appressoria of *C. coccodes* applied with the chemicals

on cellophane membranes. Cellophane membranes were cut into 1 cm^2 pieces and autoclaved for 20 minutes in distilled water. The membranes were then placed on a 1 % water agar amended with AOA, DDG, mannose, and oxalic acid at the final concentration of 0.01 and 0.1 %. Five μL of conidia suspension (10^6 conidia·mL^{-1}) was applied on the cellophane membrane. Each treatment consisted of three replicates. The plates were covered and incubated in the dark with moist paper towels in an enclosed plastic box. The cellophane membranes were carefully transferred from the agar plate onto the glass slides after 16 hours and stained with a drop of 1 % cotton blue. Germinated conidia and conidia that formed appressoria were counted on one spot in the slides under the bright field microscope (Olympus). A minimum of 100 conidia were observed in each replicate and expressed as the percentage of germination and appressoria formation, calibrated by dividing the number of observed conidia by the number of germinated conidia and by dividing the number of germinated conidia by the number of conidia that formed appressoria.

3.3.7. The effect of chemicals on the virulence of *C. coccodes* on *A. theophrasti*

The effect of chemicals on the virulence of *C. coccodes* was evaluated in two ways; by measuring disease development that resulted from *C. coccodes* suspended in chemical solutions and from *C. coccodes* application after the chemicals were vacuum-infiltrated. Plant materials were prepared as described above and selectively thinned to a final density of one plant per pot for uniformity a week before the experimental treatment. AOA, DDG, mannose, and oxalic acid were diluted to 0.01 and 0.1 % in distilled water and *C. coccodes* conidia were suspended into the solutions to the final concentration of

10^6 conidia·mL^{-1}. Ten 5-μL droplets of conidia suspension in chemical solutions were applied with a pipette on the surface of the third leaf. Distilled water was applied to the control plants. Four plants were replicated for each treatment. Following the application, the inoculated plants were placed in a dark dew chamber at 100 % relative humidity (RH) for 24 hours and then transferred to a controlled environment bench. Lesion number and diameter per leaf were measured seven days after inoculation, and expressed as lesion area per leaf. For vacuum infiltration, the chemicals were diluted to 0.01 and 0.1 % in distilled water. The chemical suspensions were sprayed onto the surface of the third leaf by using an artist's airbrush, then, gently vacuum-infiltrated into the leaves. Distilled water was infiltrated into leaves of the control plants. Ten 5-μL droplets of conidia suspension (10^6 conidia·mL^{-1}) were dropped with a pipette onto the leaves. Four plants were used for each treatment. Plants were placed in a dark dew chamber at 100 % RH for 24 hours and then transferred to a controlled environment bench. Disease development was measured seven days after inoculation as described above.

3.3.8. The effect of analogues of mannose and oxalic acid on the growth of velvetleaf and the efficacy of *C. coccodes*

Mannose, methyl mannose, ester-OA, and ethanedioic acid (salt-OA) were used to test their effect on velvetleaf growth and the efficacy of *C. coccodes.* Plant materials were prepared as described previously and selectively thinned to a final density of two plants per pot for uniformity one week before the experimental treatment. In order to determine whether the chemicals affected the growth of velvetleaf, the chemical solutions were applied to velvetleaf plants without *C. coccodes*. The chemicals were diluted in distilled

water to a final concentration of 0.001, 0.01, and 0.1 % and 50 ml of the solutions per m^{-2} were applied to plants using an artist's airbrush. Distilled water was applied to the control plants. Six plants were replicated in each treatment and the experiment was replicated twice. To determine the effect of the chemicals on the efficacy of *C. coccodes*, *C. coccodes* was suspended in 50 ml of 0.001, 0.01, and 0.1 % of the chemical solutions and applied to plants at rate of 10^8 conidia·m^{-2} using an artist's airbrush. Distilled water was applied to the control plants. Eight plants were used in each treatment and the experiment was replicated twice. Following the application, the potted plants were placed in a dew chamber at 100% relative humidity and 24 °C for 24 hours, then returned to a growth bench with 24/18°C day/night and 300 μmol $m^{-2}s^{-1}$ fluorescent light for 14 hour per day. The plants were harvested ten days after the application by cutting at the cotyledonary scar, drying at 70 °C for 72 hours and weighing the above ground biomass.

3.3.9. Data analyses

The effect of chemicals on the radial growth of *C. coccodes* was determined by linear regression. Data from other experiments were analyzed by using a general linear model (GLM) procedure of SAS (SAS 6.02, SAS Institute Inc, Cary, NC). Means were separated by Duncan's Multiple Range Test ($P < 0.05$).

3.4. Results

3.4.1. The effect of chemicals on the radial growth of *C. coccodes*

Vegetative growth of *C. coccodes* was not inhibited by the presence of mannose at any concentration (Figure 3.1). The growth of *C. coccodes* was inhibited slightly by AOA

and DDG at 0.01 %, where the slope coefficients of AOA and DDG were 6.8. The inhibitory effects of AOA and DDG became stronger at 0.1 %. The growth of *C. coccodes* was inhibited by DDG more strongly than AOA at this concentration.

3.4.2. The effect of chemicals on conidia production of *C. coccodes*

Conidia production of *C. coccodes* was significantly increased in the MR's medium mixed with AOA and mannose compared to the control (Table 3.1). Sporulation seemed to be induced by the presence of the chemicals, although conidia production was inhibited by the presence of 0.1 % DDG.

3.4.3. The effect of chemicals on the initial infection process of *C. coccodes*

Both conidia germination and appressoria formation of *C. coccodes* were inhibited by the treatment with chemicals (Table 3.2). DDG and AOA seemed to inhibit the germination and appressoria formation more strongly at 0.1 % than 0.01 %, although a statistically significant difference was not attained in all treatments. DDG was the strongest inhibitor of germination and appressoria formation among the tested chemicals. Increased inhibition at higher concentrations was not observed with mannose or oxalic acid.

3.4.4. The effect of chemicals on the virulence of *C. coccodes*

When *C. coccodes* conidia were applied in chemical mixtures, mannose was the only chemical that did not inhibit the virulence of *C. coccodes* (Table 3.3). AOA, DDG, and oxalic acid significantly reduced lesion area caused by *C. coccodes* to less than 32 %

of the control. Mannose at 0.1 % enhanced the virulence of *C. coccodes* increasing lesion area by 24%.

When the chemicals were infiltrated into the leaf cuticle by vacuum-infiltration, most chemicals, except 0.1 % of AOA and DDG, enhanced the virulence of *C. coccodes* (Table 3.3). Both mannose and oxalic acid at both concentrations enhanced virulence of *C. coccodes,* resulting in much larger lesion size caused by *C. coccodes*.

3.4.5. The effect of analogues of mannose and oxalic acid on the efficacy of *C. coccodes* to control velvetleaf

The analogues of oxalic acid and mannose did not affect the growth of *A. theophrasti* (Tables 3.4 and 3.5). *C. coccodes* treatment alone significantly reduced the growth of *A. theophrasti* (Tables 3.6 and 3.7). However, the analogues of oxalic acid and mannose did not enhance the efficacy of *C. coccodes*. Oxalic acid and ester-OA at high concentrations inhibited the efficacy of *C. coccodes*. The mannose or methyl mannose treatment did not affect the efficacy of *C. coccodes*.

3.5. Discussion

The inhibitory effect of sugar analogues such as DDG and mannose on plant metabolism occurs mainly by the inhibition of energy-yielding metabolism and polysaccharide synthesis and the interference with glycosylation of glycoproteins (Moore 1981, Herold and Lewis 1977). AOA inhibits the production of PAL, an enzyme that catalyzes the conversion of phenylalanine to cinnamic acid in the initial step of the biosynthesis of lignin precursors (Carver *et al.* 1992). A high level of oxalic acid may render the tissue more susceptible to a fungus, *Sclerotinia sclerotiorum,* as oxalic acid

precipitates calcium from the middle lamellae to form calcium oxalate crystals, leaving pectic materials more susceptible to enzymatic degradation, or by lowering the pH in favor of the pectolytic enzyme (Magro *et al.* 1984). These chemicals may increase the virulence of *C. coccodes* by inhibiting plant metabolism related to plant defense mechanism. However, the chemicals may also decrease the virulence by inhibiting fungal metabolism.

DDG has been reported to have an inhibitory effect on most filamentous fungi and yeasts (Moore 1981). DDG caused severe injuries ranging from cell wall disruption to cytoplasm disintegration of *Botrytis cinerea, Penicillium expansum,* and *Rhizopus stolonifer* (El-Ghaouth *et al.* 1997). 0.1 % DDG strongly inhibited colony growth of *C. coccodes*. The inhibitory effect of mannose on colony growth of *C. coccodes* was not as strong as other chemical treatments even though the reports on adverse effect of sugar analogues to fungi included mannose along with DDG (Moore 1981). In the presence of AOA, the result of the radial growth was contrary to that of the conidia production. The radial growth of *C. coccodes* was inhibited by 0.01 and 0.1 % AOA, while conidia production was increased in the presence of both concentrations of AOA. Mannose also increased conidia production in the MR's medium. Reproduction is associated with the decline or cessation of vegetative growth, and a number of factors interact to induce the shift from vegetative growth to sporulation. The physical and nutritional requirements for sporulation are usually more precise or more restricted than those permitting vegetative growth. Inhibition of vegetative growth by AOA and mannose must have turned the fungal metabolism pathway from vegetative growth to sporulation.

The chemicals also inhibited the initial infection process of *C. coccodes* by reducing the number of germinated conidia and the number of appressoria formed from

germinated conidia. The inhibitory effect on conidia germination and appressoria formation was stronger than the effect observed on colony growth and conidia production. This may be due to the lack of nutrients to the fungi during the examination of the effect of chemicals to initial infection process. The examination of effects of chemicals on the initial infection process of *C. coccodes* had been carried without supplying nutrients. The inhibitory effect of sugar analogues can be reversed by sucrose, glucose, or fructose. The reversal effects are probably due to competition at the uptake sites between the utilizable and toxic sugars (Herold and Lewis 1977). Conversely, the adverse effect might become stronger under the condition without any nutrient supply. When the fungus absorbs only toxic chemicals with water, its metabolism would be more suppressed.

Inhibitory effects on the growth of *C. coccodes* were found with all chemical treatments. Even so, the inhibitory effect of the chemicals may increase the efficacy of *C. coccodes* because it is hard to define the effect of the chemicals on the interaction between two organisms through the observation on the effects of the chemicals to one organism only. The inhibitory effect of the chemicals to suppress disease defense mechanisms of a host might overcome the inhibitory effect on the growth of *C. coccodes*. For this reason, *C. coccodes* was applied to velvetleaf in mixture with the chemicals to examine the effect of the chemicals on *C. coccodes* and velvetleaf interaction.

Lesion area increased when *C. coccodes* was applied in a mixture with 0.1 % mannose. The other chemicals reduced the virulence of *C. coccodes.* When the chemicals are absorbed into the leaf tissues by vacuum infiltration, the potential of enhancing effect was observed in most chemicals. Except in the higher concentration of AOA and DDG treatments, virulence of *C. coccodes* was enhanced in all other treatments. One reason for

this enhancement of virulence in the treatment of vacuum infiltration of chemicals, but not in the treatment of a mixture of chemicals and *C. coccodes*, might be the reduction in contact time with chemical solutions in the trial. Another important reason might be that all chemicals are lipophobic, thus only inhibiting the penetration of chemicals into the leaf cuticle, which is mostly lipid. If the chemicals easily penetrate the leaf cuticle, the effect of these chemicals may become very beneficial to the efficacy of *C. coccodes.*

Based on the hypothesis that the salt form or ester form of the compounds may enhance the virulence of *C. coccodes* as they can penetrate the leaf cuticle, analogues of oxalic acid and mannose were tested. Ethanedioic acid (oxalic acid with ammonium salt) and ester-OA at 0.001, 0.01, and 0.1 % were applied with *C. coccodes*. For mannose, methyl mannose was tested at the same concentrations as above. It was expected that the analogues of the compounds would be absorbed through the leaf cuticle and have a negative impact on the plant defense mechanism. However, none of the trials resulted in more severe disease to velvetleaf and no impact on the growth of velvetleaf was found. Several reasons may be attributed for this result. The analogues of the chemicals might not have been lipophilic enough to be absorbed. Since there was a limited choice of analogues of the chemicals on the market, only two kinds of analogues for oxalic acid and one analogue of mannose were tested. Thus, it may be inappropriate to conclude that the analogues of the chemical do not properly penetrate the leaf cuticle and do not affect the defense response of velvetleaf. Another reason is probably the functional change of the chemical analogues resulting from a structural change. Although a structural analogue acts as a functional analogue in many cases, it cannot be stereotyped that a structural analogue of a chemical has the same function. In addition, the size of the chemicals should be considered. The more branches are added to a compound, the bigger and the

heavier the molecules become. Although the lipid solubility of a chemical is improved, the larger size of chemical must then become a limiting factor to penetrate the leaf cuticle.

When *A. theophrasti* is applied with *C. coccodes*, the disease symptoms generally occur two or three days later. Although *C. coccodes* is applied with inundative doses of inoculum, it does not kill velvetleaf but gives severe damage to the plants, unless the plant is infected at the cotyledonary stage (Wymore *et al.* 1988). *A. theophrasti* manages to grow and reproduce, although it allows *C. coccodes* to develop and multiply in it. The apical meristem of the plants is not infected by *C. coccodes* since the leaf primordia and young leaves surround it, so that it keeps growing after the infection of *C. coccodes*. The plant sheds the infected leaves and boosts up the recovery ability, without spending the nutrients and energy to fight with the fungus at infection sites. Considering that the impact of *A. theophrasti* in the competition with soybean is high on height hierarchy for the light source, this strategy is likely to be very effective to survive against the infection by *C. coccodes* in nature (DiTommaso and Watson 1997). It is possible that *A. theophrasti* depends little on the defense mechanisms that the chemicals can affect. The main defense mechanism of *A. theophrasti* may be to avoid severe disease by abscising the infected leaves and compensating the loss of leaves by growing faster. It has not been clearly determined how *A. theophrasti* mainly defends itself against infection by *C. coccodes.* Virulence enhancement of a biocontrol agent by suppressing the defense responses of a host weed may be an ideal way to achieve a better biological weed control system. In particular, it would be the best way for the host-specific but weak pathogens like *C. coccodes*. However, the comprehensive understanding on the defense mechanisms of the plant should proceed first and it would facilitate achieving the goal to obtain an enhanced biological control system.

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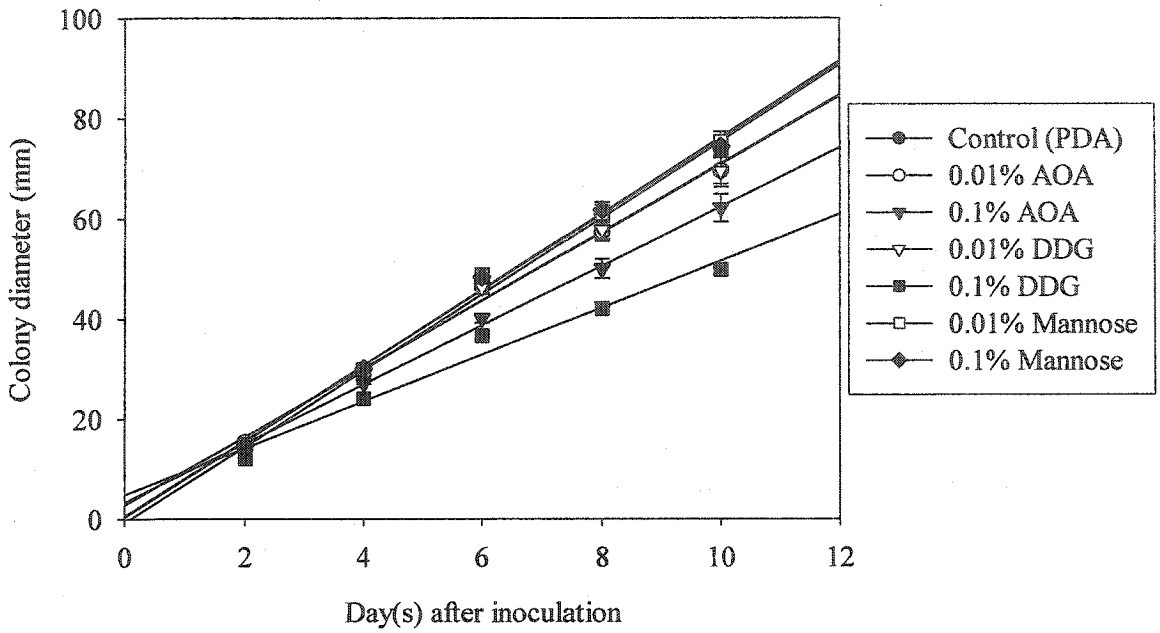

Figure 3.1. Effects of α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), and mannose on the radial growth of *Colletotrichum coccodes*.

Bars indicate standard error.

Control; $y = -0.76 + 7.6x$, $r^2 = 0.979$

0.01 % AOA; $y = 2.87 + 6.8x$, $r^2 = 0.944$

0.1 % AOA; $y = 3.29 + 5.9x$, $r^2 = 0.934$

0.01 % DDG; $y = 2.79 + 6.8x$, $r^2 = 0.946$

0.1 % DDG; $y = 4.83 + 4.7x$, $r^2 = 0.950$

0.01 % Mannose; $y = 0.34 + 7.6x$, $r^2 = 0.958$

0.1 % Mannose; $y = 0.50 + 7.6x$, $r^2 = 0.984$

Table 3.1. Effects of α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), and mannose on conidia production of *Colletotrichum coccodes*.

Treatment	Conidia number [a] ($\times 10^6$ /ml)
Control	11.44 ± 0.4 c [b]
0.01 % AOA	18.41 ± 1.2 a
0.1 % AOA	18.07 ± 1.5 a
0.01 % DDG	9.51 ± 1.0 c
0.1 % DDG	2.46 ± 0.3 d
0.01 % Mannose	15.04 ± 0.9 b
0.1 % Mannose	17.33 ± 0.7 ab

[a] Conidia number counted after seven days of growth in modified Richard solution with standard error.

[b] Means with the same letter are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

Table 3.2. Effects of α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, and oxalic acid (OA) on conidia germination and appressoria formation of *Colletotrichum coccodes.*

Treatment	Conidia germination[a] (%)	Appressoria formation[b] (%)
Control	93.7 ± 4.5 a [c]	64.4 ± 7.1 a
0.01 % AOA	70.4 ± 4.6 ab	44.8 ± 7.6 ab
0.1 % AOA	46.2 ± 13.9 bc	21.3 ± 8.4 bc
0.01 % DDG	46.3 ± 6.6 bc	21.0 ± 0.3 bc
0.1 % DDG	19.3 ± 4.5 d	4.3 ± 3.0 c
0.01 % Mannose	46.3 ± 11.0 bc	38.5 ± 15.4 b
0.1 % Mannose	41.1 ± 6.0 cd	33.8 ± 10.0 b
0.01 % OA	40.1 ± 2.5 cd	31.1 ± 6.8 b
0.1 % OA	46.8 ± 9.8 bc	31.3 ± 6.1 b

[a] Mean percentage of germinated conidia from all conidia counted, with standard error.

[b] Mean percentage of conidia that formed appressoria from all germinated, conidia with standard error.

[c] Means with the same letter within a column are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

Table 3.3. Effects of α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, and oxalic acid (OA) on the virulence of *Colletotrichum coccodes.*

Treatment	Lesion area (mm^2) caused by *C. coccodes*	
	when chemicals were applied together with *C. coccodes*	when chemicals were vacuum infiltrated
Control	111.7 ± 8.3 b [a]	72.8 ± 15.5 bc
0.01 % AOA	35.7 ± 11.6 c	105.6 ± 4.4 ab
0.1 % AOA	24.7 ± 5.3 cd	57.7 ± 21.1 cd
0.01 % DDG	35.3 ± 3.0 c	122.9 ± 19.1 a
0.1 % DDG	3.1 ± 1.0 d	30.4 ± 6.7 d
0.01 % Mannose	102.4 ± 4.9 b	109.1 ± 13.7 ab
0.1 % Mannose	146.6 ± 14.3 a	119.7 ± 16.2 a
0.01 % OA	27.1 ± 17.1 cd	126.4 ± 12.3 a
0.1 % OA	13.7 ± 2.0 cd	110.3 ± 4.1 ab

[a] Means with the same letter within a column are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

Table 3.4. Effects of oxalic acid and its analogues on the growth of velvetleaf.

Treatment	Dry weight [a] (mg)
Control	516 ± 20.1
0.001 % OA[b]	568 ± 35.0
0.01 % OA	566 ± 29.6
0.1 % OA	540 ± 25.0
0.001 % Salt-OA[c]	499 ± 28.0
0.01 % Salt-OA	537 ± 23.3
0.1 % Salt-OA	551 ± 23.1
0.001 % Ester-OA[d]	503 ± 26.5
0.01 % Ester-OA	495 ± 24.9
0.1 % Ester-OA	537 ± 23.5

[a] Mean dry weight of velvetleaf with standard error. Means are not significantly different at 5 % level (P=0.408).

[b] Oxalic acid.

[c] [Oxalic acid diammonium salt (ethanedioic acid)].

[d] [(1-amino-ethyl)-phosphonic acid diisopropyl ester, compound with oxalic acid]

Table 3.5. Effects of mannose and methyl mannose on the growth of velvetleaf.

Treatment	Dry weight [a] (mg)
Control	443.7 ± 20.0
0.001 % Mannose	446.8 ± 17.5
0.01 % Mannose	420.9 ± 29.5
0.1 % Mannose	453.9 ± 22.0
0.001 % Methyl-mannose[b]	435.3 ± 25.2
0.01 % Methyl-mannose	405.8 ± 15.2
0.1 % Methyl-mannose	433.7 ± 20.8

[a] Mean dry weight of velvetleaf with standard error. Means are not significantly different at 5 % level (P=0.759).

[b] 4,6-di-O-methyl-D-mannose

Table 3.6. Effects of oxalic acid and its analogues on the efficacy of *Colletotrichum coccodes*.

Treatment	Dry weight [a] (mg)
Control	459 ± 23.0 a
Colletotrichum coccodes	298 ± 21.0 c
C. c[b] + 0.001 % OA[c]	370 ± 18.0 c
C. c + 0.01 % OA	288 ± 15.4 c
C. c + 0.1 % OA	311 ± 21.8 b
C. c + 0.001 % Salt-OA[d]	307 ± 21.1 c
C. c + 0.01 % Salt-OA	280 ± 14.4 c
C. c + 0.1 % Salt-OA	306 ± 13.8 c
C. c + 0.001 % Ester-OA[e]	363 ± 21.8 c
C. c + 0.01 % Ester-OA	314 ± 14.8 bc
C. c + 0.1 % Ester-OA	303 ± 16.3 b

[a] Mean dry weight of velvetleaf with standard error. Means with the same letter are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *Colletotrichum coccodes*

[c] Oxalic acid

[d] [Oxalic acid diammonium salt (ethanedioic acid)]

[e] [(1-Amino-ethyl)-phosphonic acid diisopropyl ester, compound with oxalic acid].

Table 3.7. Effects of mannose and methyl mannose on the efficacy of *Colletotrichum coccodes.*

Treatment	Dry weight [a] (mg)
Control	411.4 ± 12.0 a
Colletotrichum coccodes	245.6 ± 9.9 b
C. c[b]. + 0.001 % Mannose	253.1 ± 16.4 b
C. c. + 0.01 % Mannose	243.8 ± 10.8 b
C. c. + 0.1 % Mannose	238.8 ± 6.6 b
C. c. + 0.001 % Methyl-mannose[c]	266.9 ± 13.2 b
C. c. + 0.01 % Methyl-mannose	256.1 ± 13.4 b
C. c. + 0.1 % Methyl-mannose	252.3 ± 12.2 b

[a] Mean dry weight of velvetleaf with standard error. Means with the same letter are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *Colletotrichum coccodes.*

[c] 4,6-di-O-methyl-D-mannose.

CONNECTING TEXT

Enhancement of biocontrol efficacy of *C. coccodes* has been attempted in various ways. In chapter 3, several chemicals interfering with plant defense metabolisms and their analogues were tested. It is attractive to increase the efficacy of a biological control agent by inhibiting plant defense responses. However, up to now, a sublethal dose of bentazon was the most beneficial to *C. coccodes* out of all the trials for the virulence enhancement of *C. coccodes*. Comprehensive understanding of defense mechanisms in *A. theophrasti* will facilitate the achievement of the goal of obtaining a better biological control agent. To understand more about biochemical defense responses in *A. theophrasti* to *C. coccodes*, peroxidase and PAL in *A. theophrasti* were analyzed when *C. coccodes* was applied alone or with a mixture of bentazon, glyphosate, or ethanedioic acid. The candidate was the primary author of this chapter. Dr. A.K. Watson, Dr. T. Paulitz, and Dr. S. Jabaji-Hare were co-authors.

Chapter 4. Enzyme Responses of *Abutilon theophrasti* in an Enhanced Biocontrol System

4.1. Abstract

The efficacy of *C. coccodes* increased in the presence of 0.25 kg a.i. ha^{-1} bentazon in dry weight reduction of *A. theophrasti* more than when *C. coccodes* was applied alone, while the effect of glyphosate was minimal. Glyphosate did not have a significant effect on the growth of *A. theophrasti* at 0.2 kg a.i. ha^{-1} and the efficacy of *C. coccodes* was not affected by this rate of glyphosate. Increase of PAL activity was observed as plants grew. The activity of PAL was low at the early stages compared to the activity measured at later stages. PAL activity in *A. theophrasti* was not affected by infection with *C. coccodes*. PAL activity was significantly inhibited by the presence of bentazon five days after treatment and this inhibition remained until seven days after treatment. The inhibition was stronger when bentazon was applied alone than when bentazon was applied with *C. coccodes*. The treatment with glyphosate or the mixture of glyphosate and *C. coccodes* did not affect PAL activity. PAL activity was not affected by treatment of ethanedioic acid at any time. Increasing peroxidase activity was also observed as plants grew. Peroxidase activity was strongly induced by the treatment of *C. coccodes* and increased over time. A significant difference in peroxidase activity in the *C. coccodes* treatment compared to the control appeared three days after the treatment. Peroxidase activity was not induced by 0.25 kg a.i. ha^{-1} bentazon alone. However, when bentazon was applied in combination with *C. coccodes*, it prevented the activation of peroxidase caused by the infection of *C. coccodes*. Treatment with 0.2 kg a.i. ha^{-1} of glyphosate and ethanedioic acid did not affect peroxidase activity. These results suggest that these enzymes are

involved in the resistance mechanism of *A. theophrasti,* but they probably delay infection of *C. coccodes* rather than impede it. This further indicates that suppression of weed defense mechanisms can bring enhanced pathogen virulence thus rendering improved weed biocontrol system.

4.2. Introduction

Plants actively defend themselves against pathogen attacks, and early accumulation of phenolic compounds in infected sites is critical for plant defense mechanisms (Rey *et al.* 1996). Phenolic compounds, produced by the phenylpropanoid pathway, are major components of papillae and lignin as well as being directly toxic to microorganisms (Strack 1997). After the infection by *Phytophthora cinnamomi*, the concentration of total phenolics in the root segments of *Eucalyptus calophylla,* resistant to *P. cinnamomi*, was significantly greater than in roots of *E. marginata*, susceptible to the pathogen (Cahill and McComb 1992). Phenolic polymer and lignin deposition in hypocotyls from *Gossypium hirsutum* increased dramatically following contact with a protein-lipopolysaccharide elicitor from *Verticillium dahliae* (Smit and Dubery 1997). The resistant type of *G. hirsutum* responded to the elicitor by depositing phenolic polymer and lignin earlier and more than the susceptible type. Phenolic compounds are often conjugated with sugars and serve as biosynthetic precursors of the more toxic phytoalexins (Paxton and Groth 1994).

Phenylalanine ammonia lyase (PAL) is located at the starting point of the phenylpropanoid pathway, interfacing it with the shikimic acid pathway and regulating production of phenolics (Strack 1997). Induction of enzyme activity is commonly observed in resistance responses of plants pertaining to structural defense mechanisms,

and induction or inhibition of the enzymes correlated with the increase of resistance or susceptibility of plants (Stadnik and Buchenauer 2000). The significant induction of phenolics was accompanied by increased PAL activity in *E. calophylla* (Cahill and McComb 1992). Lesion length in the roots of *E. calophylla* was extended by treatment with a PAL inhibitor, amino-oxy acetic acid, which reduced lignin concentration in roots. A PAL inhibitor, α-amino oxy-β-phenylpropionic acid (AOPP), increased susceptibility in oats to penetration by *Erysiphe graminis* appressoria, and was correlated with the suppression of localized autofluorescent host cell responses to fungal germ tube contact (Carver *et al.* 1992).

Plant peroxidases are divided into two major groups, ascorbate peroxidases and classical secretory peroxidases, also referred to as guaiacol-type peroxidases (Hoson 2000). The guaiacol-type peroxidases are distinguished by their nonspecific use of phenolic derivatives and involvement in polymerizing reactions. Peroxidase is localized on the plasma membrane where it is involved in polymerization of monolignol (Takabe *et al.* 2001). Its role in plant defense mechanisms is mainly related to lignification to strengthen cell walls against pathogen attacks (Van Loon 1997). The protein-lipopolysaccharide elicitor from *V. dahliae* remarkably induced enzymes related to production of phenolic polymers including peroxidase, and the induction was followed by the increased deposition of phenolic compounds (Smit and Dubery 1997). Peroxidase activity in cucumber roots was systemically induced after bacterization with plant growth-promoting rhizobacteria *Pseudomonas* strains that suppressed cucumber root disease caused by *Pythium aphanidermatum* (Chen *et al.* 2000). Peroxidase might also be involved in resistance response of *A. theophrasti* to *C. coccodes* (Nickerson *et al.* 1993).

Peroxidase activity increased as plants grew, positively correlating with a higher resistance level in older *A. theophrasti* against *C. coccodes*. Peroxidase activity was slightly higher in *C. coccodes* infected plants than in control plants.

It has been three decades since the first report demonstrating an endemic pathogen could be used to control a weed and developed as a bioherbicide (Daniel *et al.* 1973). During the period, many microorganisms have been researched as biocontrol agents. However, only few pathogens have been commercialized. Poor aggressiveness of a pathogen in the field is one of the reasons that many potent biocontrol agents were not developed commercially. *Colletotrichum coccodes* may belong to this category. *C. coccodes,* isolated from *Abutilon theophrasti* (velvetleaf) is specific to velvetleaf (unpublished laboratory and field studies), but its weak virulence to velvetleaf has always hindered commercialization. There has been significant research to develop *C. coccodes* as a biological control agent, and to increase its efficacy. However, in spite of the intensive research on *C. coccodes* and *A. theophrasti*, the host defense response to *C. coccodes* is still unclear since the research had been primarily focused on the practical usage of the pathogen. Weakening or suppressing the host defense responses could enhance virulence of a pathogen. The objectives of this study were to verify the involvement of defense-related enzymes in the defense response of *A. theophrasti* to the infection of *C. coccodes,* and to determine effects of chemical virulence enhancers of *C. coccodes* on the enzyme responses.

4.3. Materials and Methods

4.3.1. Plant production

A. theophrasti seeds were collected from the agricultural field population at the Emile A. Lods Agronomy Research Centre of McGill University, Ste-Anne-de-Bellevue, QC in fall 1999 and stored at room temperature in plastic bags. Seeds were dipped into boiling water for 10 seconds to break dormancy, then placed onto distilled water saturated filter paper (P8, Fisher Scientific, Nepean, Ontario) in 9 cm diameter Petri dishes and incubated in the dark for 48 hours. These germinating seeds were sown in 10 cm top-diameter plastic pots (three seeds per pot) in a commercial potting medium (Promix BX, Premier Brands, Inc., NY, USA) and the pots were placed on a controlled environment bench (Conviron®, Winnipeg, MB) with 24/18°C day/night, 300 μmol $m^{-2}s^{-1}$ fluorescent light for 14 hours per day. The plants were watered daily and fertilized with 50 ml of 20-20-20 N-P_2O_5-K_2O (1.25 $g \cdot L^{-1}$) per pot. Plants were selectively thinned to a final density of two per pot for uniformity a week before the experimental treatment. Plants were at the three-leaf stage at the time of treatment.

4.3.2. Inoculum production

A stock culture of *C. coccodes* (DAOM 182826 deposited in the Biosystematics Research Institute, Ottawa, ON) was isolated from diseased velvetleaf and maintained on potato dextrose agar (PDA, DIFCO Laboratories, Detroit) slants at 3°C under mineral oil. A small piece of the culture was placed onto the middle of a PDA plate and kept in the dark at 22°C (± 2°C) for one week. Mycelial plugs were removed from the edge of the PDA plates and transferred to a modified Richard solution [10 $g \cdot L^{-1}$ of sucrose, 10 $g \cdot L^{-1}$

of KNO_3, 5.0 $g \cdot L^{-1}$ of KH_2PO_4, 2.5 $g \cdot L^{-1}$ of $MgSO_4 7H_2O$, 0.02 $g \cdot L^{-1}$ $FeCl_3 6H_2O$, 150 ml V-8 juice (Cambell Soup Company Inc.) and distilled water to 1 L] in Erlenmeyer flasks. Cultures were incubated for seven days on a rotary shaker at 200 rpm at room temperature [22°C (± 2°C]. Cultures were filtrated with four layers of cheesecloth. The filtrate was centrifuged at 6,000 g for 15 minutes and the conidia pellet was resuspended in distilled water. The inoculum density was adjusted using a haemocytometer.

4.3.3. Chemical material

Oxalic acid diammonium salt (ethanedioic acid, salt-OA), bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide, Basagran™, BASF] and glyphosate [N-(phosphonomethyl) glycine, Touchdown™, Novartis] were used to increase the efficacy of *C. coccodes*. Herbicides used for the experiment were obtained from the herbicide inventory for field research at the Emile A. Lods Agronomy Research Centre of McGill University. Ethanedioic acid was purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

4.3.4. *Colletotrichum coccodes* and chemical applications

The rates of herbicides were 0.25 kg a.i. (active ingredient) ha^{-1} for bentazon and 0.2 kg a.i. ha^{-1} for glyphosate. A 0.01 % solution of ethanedioic acid was used in this study. Each chemical was applied with 50 ml of distilled water per m^{-2} onto *A. theophrasti* and *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} using an artist's airbrush. *A. theophrasti* was treated with *C. coccodes* alone, each herbicide and ethanedioic acid alone, and a mixture of *C. coccodes* with each chemical. Distilled water

was applied to the control plants. The plants were incubated in a dark dew chamber for 24 hours at 22°C, and 100 % relative humidity (RH), and then replaced to the controlled environment bench.

4.3.5. Effect of herbicides on the efficacy of *C. coccodes*

In order to confirm the synergistic effects of the two herbicides, bentazon and glyphosate to *C. coccodes*, each herbicide alone, *C. coccodes* alone, and a mixture of *C. coccodes* plus each herbicide were applied to *A. theophrasti*. Plant samples were grown as described above and five pots were used for each treatment. The experiment was replicated twice. Plant shoots were harvested 10 days after the treatments. The samples were dried at 70°C for 72 hours and weighed.

4.3.6. Enzyme extraction

Enzyme extraction and analysis were carried out as described by Nickerson *et al.* (1993) and by Edwards and Kessmann (1992) with a few modifications. Depending on plant leaves available when samples were harvested, about 1 to 2 g of the second and third leaves were excised from six to eight plants 1, 3, 5, and 7 days after the treatments and pooled for extraction. The leaves were weighed and stored in –80°C. The samples were ground to a dry powder with mortar and pestle in the presence of 20 % (w/w) polyvinylpyrrolidone. The powder was transferred to a conical polypropylene tube and 7 ml of buffer solution was added for each gram of fresh weight of plant tissue. 50 mM sodium acetate buffer (pH 5.0) for peroxidase analysis and 100 mM sodium borate buffer (pH 8.8) containing 14 mM 2-mercaptoethanol for PAL analysis were used as buffer

solutions. The leaf suspension was incubated on ice for 30 minutes and vortexed vigorously at 10 minutes intervals. The suspension was filtered through cheesecloth and the filtrate was centrifuged at 10,000 g for 10 min using an SS-34 rotor in a Sorvall RC5B centrifuge (Dupont) at 4°C. The supernatant was immediately used for the enzyme assay or stored at –80°C.

4.3.7. Enzyme activity studies

PAL analysis was carried out as described by Edwards and Kessmann (1992) with a few modifications. 0.1 ml of the extract was incubated at 40°C with 0.9 ml 12.1 mM L-phenylalanine in 100 mM sodium borate buffer (pH 8.8) for 60 minutes. PAL activity was measured spectrophotometrically at 290 nm.

Peroxidase analysis was carried out as described by Nickerson *et al.* (1993) with a few modifications. Peroxidase activity was measured at 470 nm in 3 ml of the reaction mixture consisting of 0.1 ml of the enzyme extract, 0.3 % guaiacol (v/v), 0.3 % H_2O_2 (v/v), and 50 mM sodium acetate buffer for 60 seconds.

Each enzyme extract was assayed three times to achieve an average value for the sample. The enzyme analysis of the ethanedioic acid treatment was replicated twice and three times in the bentazon. The protein content of the extract was determined by the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford 1976).

4.3.8. Data Analyses

The effects of herbicides on the efficacy of *C. coccodes* were determined by using a general linear model (GLM) procedure by using SAS (SAS 6.02, SAS Institute Inc,

Cary, NC). Means were separated by Duncan's Multiple Range Test (DMRT) to present the difference among the levels of the factor. The characteristic of enzyme dynamics was expressed as activity over time, and the experiments were carried out in randomized complete block designs. Means of the enzymatic activity for each treatment were separated with a least significant difference at the 5 % level.

4.4. Results

4.4.1. Effect of herbicides on the efficacy of *C. coccodes*

The efficacy of *C. coccodes* increased in the presence of 0.25 kg a.i. ha^{-1} bentazon as expressed in greater dry weight reduction of *A. theophrasti* than when *C. coccodes* was applied alone, while the effect of glyphosate was minimal (Tables 4.1 and 4.2). One quarter kg a.i. ha^{-1} of bentazon and *C. coccodes* had the same effect on the dry weight of *A. theophrasti* when they were applied individually. When *C. coccodes* was applied in combination with bentazon, a synergistic reaction occurred. Glyphosate did not have a significant effect on the growth of *A. theophrasti* at 0.2 kg a.i. ha^{-1} and the efficacy of *C. coccodes* was not affected by this rate of glyphosate.

4.4.2. Effect of bentazon, glyphosate, and ethanedioic acid on PAL

Increase of PAL activity was observed as plants grew (Figures 4.1 to 4.3). The activity of PAL was low in the early stages compared to the activity measured at later stages. PAL activity was not induced in the infected leaves by *C. coccodes* at any time (Figure 4.1). PAL activity was significantly inhibited by the presence of bentazon at five days after treatment and the inhibition remained until seven days after treatment ($P=0.002$). The inhibition was stronger when bentazon was applied alone than when

bentazon was applied with *C. coccodes*. The treatment with glyphosate or the mixture of glyphosate and *C. coccodes* did not affect PAL activity (Figure 4.2). Although there was a slight inhibition of PAL three to five days after glyphosate treatment and a minor induction seven days after the treatment with *C. coccodes* mixed with glyphosate, statistically there was no difference among the treatments (P=0.372). PAL activity was not affected by the treatment with ethanedioic acid at any time during this study (Figure 4.3).

4.4.3. Effect of bentazon, glyphosate and ethanedioic acid on peroxidase

As PAL activity increased with plant age, peroxidase activity was also observed to increase as the plant aged (Figures 4.4 to 4.6). Peroxidase activity was strongly induced by the treatment with *C. coccodes* and increased over time. Three days after treatment the peroxidase activity in the *C. coccodes* treatment was significantly greater than the control, with a two-fold increase in peroxidase activity in the infected leaves. Bentazon, applied alone at the rate of 0.25 kg a.i. ha^{-1}, did not activate peroxidase (Figure 4.4). However, when bentazon was applied in combination with *C. coccodes*, it prevented the activation of peroxidase caused by *C. coccodes* alone. Glyphosate at 0.2 kg a.i. ha^{-1} did not affect peroxidase activity (Figure 4.5). When glyphosate was applied with *C. coccodes*, peroxidase activity was similar to the *C. coccodes* alone treatment. The 0.01 % ethanedioic acid treatment was similar to the glyphosate treatment. It did not induce peroxidase activity alone or inhibit peroxidase activity of *C. coccodes* (Figure 4.6).

4.5. Discussion

Induction of PAL activity was not detected in the infected leaves in *A. theophrasti*. Interestingly, PAL activity in *A. theophrasti* was reduced when bentazon was applied and the efficacy of *C. coccodes* was significantly enhanced by the presence of bentazon. Some herbicides that inhibit the photosynthetic electron transport system such as atrazine, diuron, metribuzin, and paraquat have been reported to reduce PAL activity, although their modes of action are unknown (Hoagland 1989, Hoagland and Duke 1983). Benzo-(1,2,3)-thiadiazole-7-carbothioic acid (BTH) induced systemic resistance in a susceptible wheat cultivar to *Blumeria graminis* by enhancing the localized autofluorescence at the penetration sites (Stadnik and Buchenauer 2000). Cell wall-bound phenolic compounds, coumaric and ferulic acids, were higher in BTH treated plants than in untreated plants. Inhibition of PAL by the AOPP treatment suppressed the resistance induced by BTH and reduced the localized accumulation of autofluorogenic compounds whose major components are phenolics. Inhibition of PAL by bentazon probably interfered with the synthesis of phenolic compounds to reinforce the cell wall of *A. theophrasti* against infection, thus enhancing the efficacy of *C. coccodes*.

While PAL indirectly influences structural defense responses early in secondary metabolism at the starting point of the phenylpropanoid pathway, peroxidase is a key enzyme in later stages of cell wall strengthening by polymerizing phenolic compounds. Peroxidase activity in *A. theophrasti* was significantly induced by *C. coccodes* by the application of *C. coccodes*, and increased over time, more than two-fold of the untreated plants at seven days after the treatment. It was reported that peroxidase might be involved in defense mechanisms in *A. theophrasti* (Nickerson *et al.* 1993). *C. coccodes* was applied at one to two leaf stage and slight induction of peroxidase occurred in *C. coccodes*-

infected plants between nine to thirteen days after the treatment (Nickerson *et al.* 1993). In this study, *C. coccodes* was applied at the three-leaf stage when *A. theophrasti* began to develop high resistance to *C. coccodes* and peroxidase activity was induced between one to three days after the treatment in this study. When *C. coccodes* is applied before one leaf stage, it gives serious damage to *A. theohphrasti*, but when *C. coccodes* is applied after the three-leaf stage the plant efficiently protect itself from the disease after shedding its infected leaves. Induction of peroxidase activity much stronger and occurred earlier in this study was than in the previous report on peroxidase induction in *A. theophrasti* at one leaf stage (Nickerson *et al.* 1993). This difference between the leaf stages indicates that higher resistance in older *A. theophrasti* may be closely related to earlier activation of the defense mechanism and peroxidase plays an important role in the defense mechanism of *A. theophrasti* against *C. coccodes*.

The enhanced efficacy of *C. coccodes* mediated by bentazon supports the peroxidase-involved structural defense mechanisms of *A. theophrasti*. When *A. theophrasti* was treated with bentazon alone, peroxidase activity level was the same as the untreated plants, showing that bentazon does not directly interfere with peroxidase activity. However, when *C. coccodes* was applied in combination with bentazon, the peroxidase activity remained at the same level as when bentazon was applied alone, not following the increasing activity over time as in the *C. coccodes* treatment. Bentazon is an inhibitor of electron flow at the D1 polypeptide in photosystem II. Inhibition of electron transport generates reactive oxygen species. These toxic oxygen species affect unsaturated membrane lipids, resulting in a chain reaction of lipid peroxidation, loss of photosynthetic activities, loss of membrane semipermeability, membrane leakage, and eventually, wilting and desiccation (Devine *et al.* 1993). Stagnant peroxidase activity in

the treatment of *C. coccodes* mixed with bentazon may indicate that disintegration of cell membranes or malfunctioning of the membrane transporting system caused by bentazon resulted in disruption of normal integrated metabolic processes of cell wall reinforcement across cell membranes, thus damaging the structural defense responses of *A. theophrasti* delaying the infection of *C. coccodes*. Therefore, the results further suggest that cell wall reinforcement is an important part of the defense response of *A. theophrasti*. Peroxidase is a cell membrane bound enzyme that is synthesized in the rough endoplasmic reticulum and transported to the Golgi apparatus where it is glycosylated (Takabe *et al.* 2001). Thereafter, the processed peroxidase is transported to the plasma membrane and localized there by fusion of the Golgi vesicles to the membrane. Monolignols are synthesized in the cytosol and pass through the plasma membrane via mechanisms that are still unknown, and localized at the boundary between the plasma membrane and the newly formed cell walls. Mature peroxidases, localized on the plasma membrane, then oxidize the monolignols in the presence of hydrogen peroxide. Although mechanism of monolignols transport and regulation is still unknown, the concentration, composition, and polymerization of monomers are highly organized (Whetten and Sederoff 1995). Disrupted cell membranes could interrupt the proper procedure of cell wall strengthening. The enhancement of efficacy of *C. coccodes* by bentazon is probably brought by the inhibition of PAL and by the activation of peroxidase causing a reduction of basic components to reinforce structural rigidity in response to the fungal attack.

The effect of glyphosate on the efficacy of *C. coccodes* was minimal compared to bentazon. Glyphosate or the mixture of glyphosate and *C. coccodes* did not affect PAL or peroxidase activity. Glyphosate is a nonselective and foliar-applied herbicide that inhibits enolpyruvylshikimate-3-phosphate (EPSP) synthase in the shikimate pathway supplying

phenylalanine to the phenylpropanoid pathway (Cobb 1992). EPSP inhibition leads to depletion of three essential aromatic amino acids, phenylalanine, tyrosine, and tryptophan. Many aromatic secondary plant products that are important in plant growth and development and in interactions with other organisms are produced through the shikimic pathway. About 20 percent of the carbon that is fixed by photosynthetic plants flows through this highly regulated pathway (Strack 1997). In some plants, PAL activity was induced by glyphosate, possibly resulting from decreased feedback regulation (Hoagland 1996, Hoagland 1989, Hoagland and Duke 1983, Duke and Hoagland 1978). However, the concentration of end products, anthocyanin and hydroxyphenol was significantly reduced by the treatment of glyphosate in spite of induction of PAL activity (Hoagland and Duke 1983). In this study, glyphosate did not significantly affect either the efficacy of *C. coccodes* or the activities of PAL and peroxidase in *A. theophrasti*. It is unclear why the enzymes did not respond to glyphosate, but the lack of enhancing effect of glyphosate on the efficacy of *C. coccodes* might simply result from this minimal effect on PAL and peroxidase activities.

Ethanedioic acid was used to enhance virulence of *C. coccodes,* but found to be ineffective (Chapter 3). However, its effect on the dry weight of *A. theophrasti* was slightly lower and lesions on the infected leaves in the treatment appeared slightly earlier than in the treatment of *C. coccodes* alone, although there was no significant difference. A slight difference in biomass could be a larger interaction at the enzyme level. Understanding possible interactions with this chemical and the enzymes involved may help to explain why ethanedioic acid failed to enhance virulence of *C. coccodes*. Unfortunately, no clue was apparent in the enzyme analyses, as ethanedioic acid did not

affect the activity of PAL or peroxidase. Change in enzyme activity was closely related with infection by *C. coccodes,* regardless of the presence of the chemical.

The activity of PAL was not induced by the application of *C. coccodes* in the infected leaves, while peroxidase activity was strongly induced by *C. coccodes* infection. Induction of peroxidase and PAL in the resistance response does not always correlate positively. Infiltration of *Penicillium janczewskii* conidia or its culture filtrates into cotyledonary leaves induced systemic resistance and protected both melon and cotton plants against *Rhizoctonia solani* (Madi and Katan 1998). Enhanced level of peroxidase activity was detected in the roots of cotton, and in the leaves and stems of melon treated with *P. janczewskii* or its infiltrate. Activities of PAL increased in melon plants, but not in cotton plants. Peroxidase induction without positive induction of PAL may explain the compatibility of *C. coccodes* to *A. theophrasti* and the resistance mechanism of *A. theophrasti*. Although *C. coccodes* is not herbicidal to *A. theophrasti* after the two-leaf stage, it still infects the host and causes damage. *A. theophrasti* manages to grow and reproduce, even though *C. coccodes* continues to cause foliar disease. The apical meristem, protected by leaf primordia and young leaves from the application of *C. coccodes,* grows rapidly and recovers from the infection. The morphology of some plant species allows them to produce healthy new growth while other parts are dying (Auld and Morin 1995).

A. theophrasti may only need to delay spread of *C. coccodes* to other plant tissue from the local infected sites until the new leaves substitute the function of the old infected leaves in this situation. Since *C. coccodes* rarely infects the stem of *A. theophrasti*, the plant efficiently avoids spread of the disease by shedding infected leaves and boosting the recovery ability, without spending the nutrients and energy to combat the fungus at

infection sites. Peroxidase induction without positive induction of PAL at the infection sites probably means that *A. theophrasti* has limited activation of defense metabolisms to infection by *C. coccodes* in the infected leaves. The new leaves will have enhanced defense mechanism systemically induced by the infection with *C. coccodes* after recovering from the loss. It had been demonstrated that the local infection by *C. coccodes* systemically induced resistance of *A. theophrasti* in the uninfected upper leaf tissues (Chapter 2). Activation of peroxidase and PAL was detected in the plants whose resistance was systemically induced by local infection with *C. coccodes*. Three applications of *C. coccodes* generally resulted in less severe disease symptoms and resulted in the smallest decreases in *A. theophrasti* growth (DiTommaso and Watson 1995). Moreover, *A. theophrasti* subjected to three inoculations showed enhanced growth compared to plants that had not received the third application.

There have been several attempts to enhance the virulence of *C. coccodes*. Application of *C. coccodes* tank-mixed with bentazon turned out to be most effective. The enhancement of efficacy of *C. coccodes* by bentazon results from inhibiting PAL and the activation of peroxidase. The inhibition of the enzymes suppresses the defense mechanism of *A. theophrasti*, thus delivering the enhanced biocontrol system of *A. theophrasti*. However, the morphology of *A. theophrasti* is probably the critical barrier to *C. coccodes*, backed by induced resistance in case the plant is infected. The results herein show that suppression of weed defense mechanisms can bring enhanced pathogen virulence thus improving biocontrol of weeds. Better understanding of the defense mechanisms interactions would provide enhanced weed control of a bioherbicide (Watson and Ahn 2001). However, the host-pathogen interaction and the resistance or defense mechanisms of the host have rarely been comprehensively studied in the scope of

biological weed control, although countless reports on the interactions have been published during the past several decades. More knowledge of weed defense mechanisms to bioherbicides would be a major asset in developing efficacious biocontrol systems.

4.6. Literature cited

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Table 4.1. Effect of bentazon on the efficacy of *Colletotrichum coccodes* .

Treatment	Dry Weight (mg)
Control	251 a[a]
C. coccodes[b]	210 b
Bentazon[c]	212 b
C. coccodes[b] + bentazon[c]	155 c

[a] Means sharing the same letter are not significantly different at the 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *C. coccodes* was applied at the rate of 10^9 conidia m^{-2}.

[c] Bentazon was applied at the rate of 0.25 kg a.i. (active ingredient) ha^{-1}.

Table 4.2. Effect of glyphosate on the efficacy of *Colletotrichum coccodes.*

Treatment	Dry Weight (mg)
Control	248 a[a]
C. coccodes[b]	194 b
Glyphosate	217 ab
C. coccodes[b] + glyphosate[c]	188 b

[a] Means sharing the same letter are not significantly different at the 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *C. coccodes* was applied at the rate of 10^9 conidia m^{-2}.

[c] Glyphosate was applied at the rate of 0.2 kg a.i. (active ingredient) ha^{-1}.

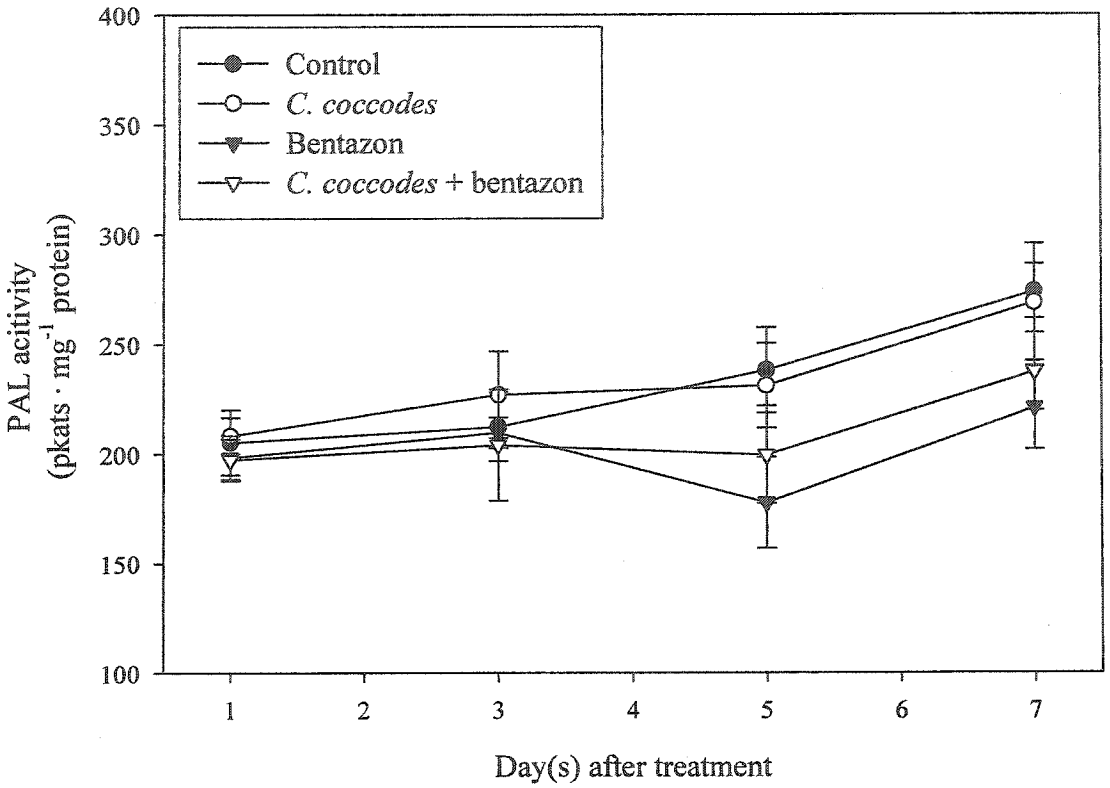

Figure 4.1. Effect of bentazon and *Colletotrichum coccodes* on phenylalanine ammonia lyase (PAL) activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and bentazon was applied at the rate of 0.25 kg a.i. (active ingredient) ha^{-1}.

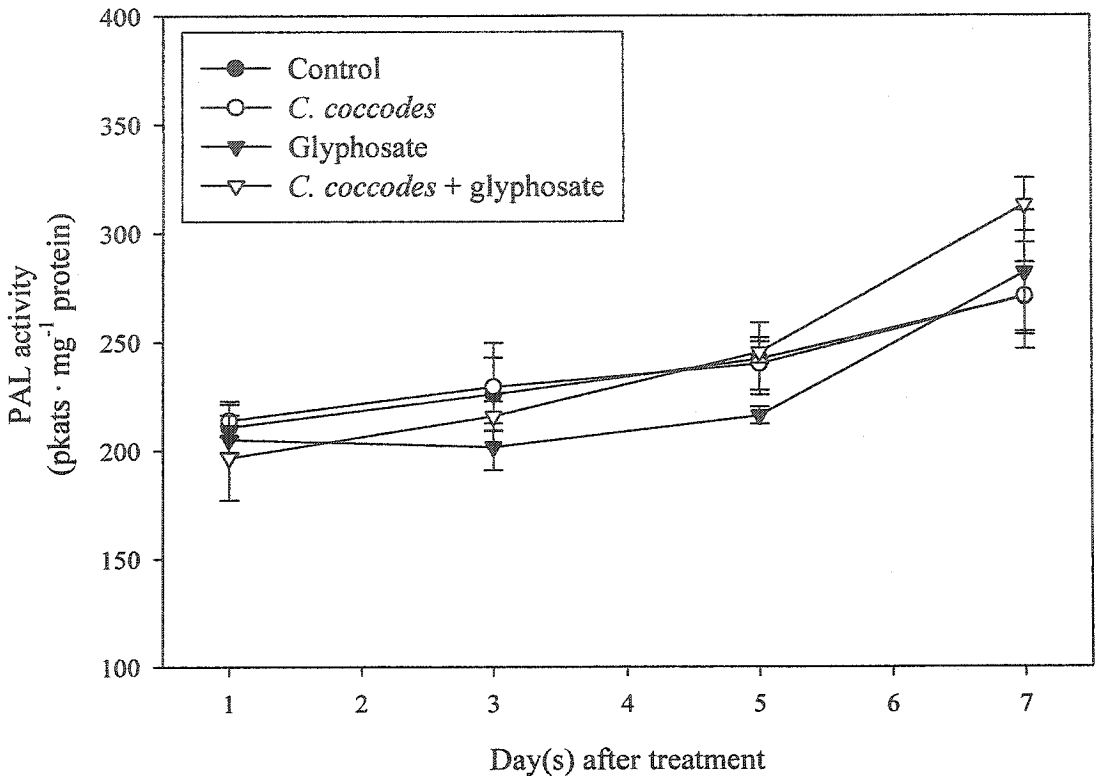

Figure 4.2. Effect of glyphosate and *Colletotrichum coccodes* on phenylalanine ammonia lyase (PAL) activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and glyphosate was applied at the rate of 0.2 kg a.i. (active ingredient) ha^{-1}.

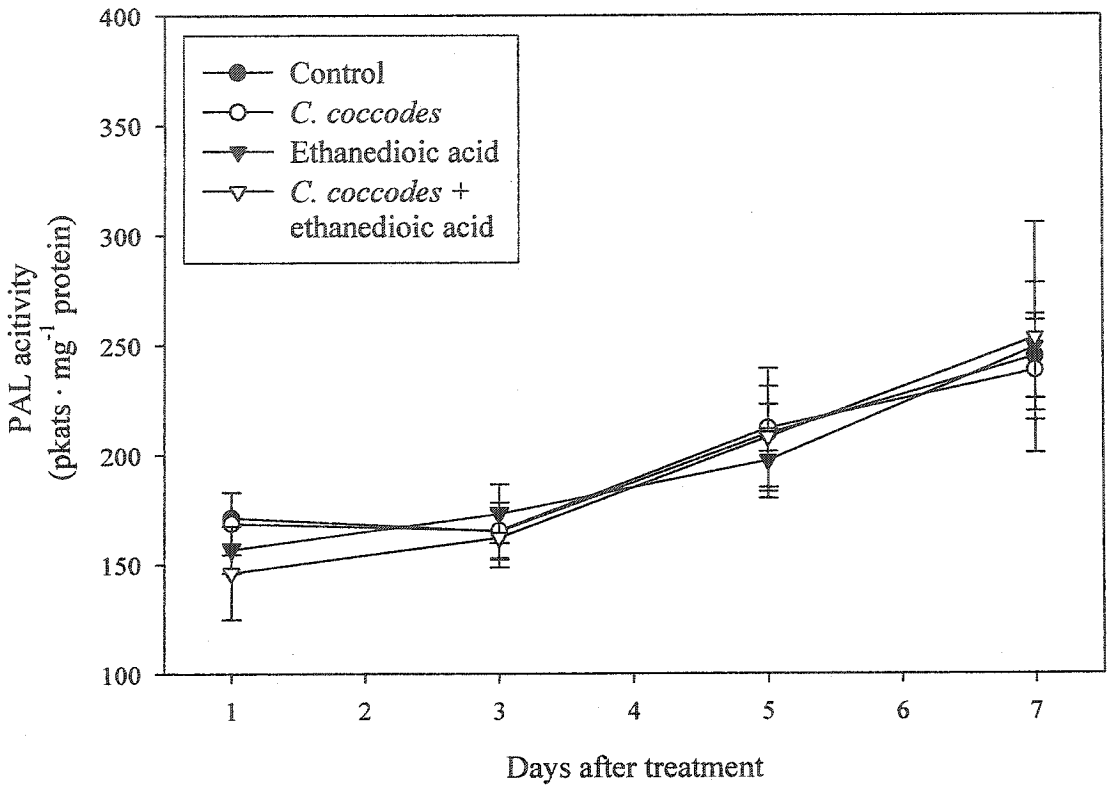

Figure 4.3. Effect of oxalic acid diammonium salt (ethanedioic acid) and *Colletotrichum coccodes* on phenylalanine ammonia (PAL) activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and 50 ml of 0.01 % ethanedioic acid solution was applied per m^{-2}.

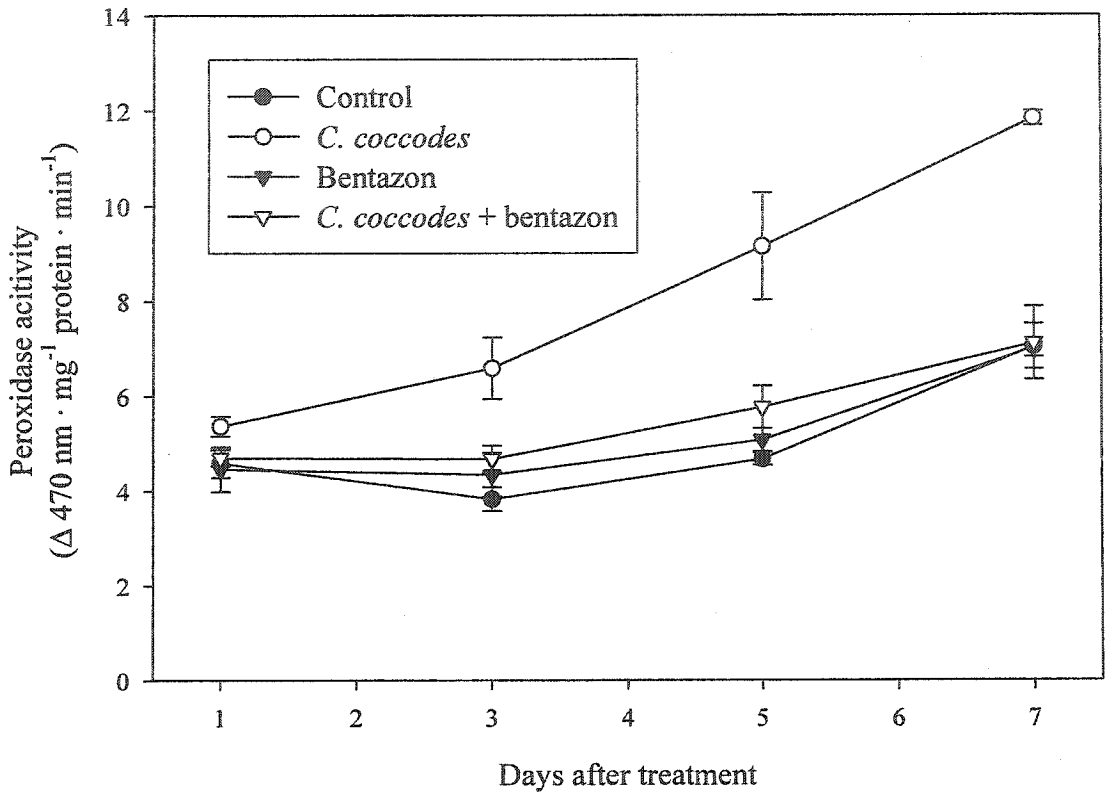

Figure 4.4. Effect of bentazon and *Colletotrichum coccodes* on peroxidase activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and bentazon was applied at the rate of 0.25 kg a.i. (active ingredient) ha^{-1}.

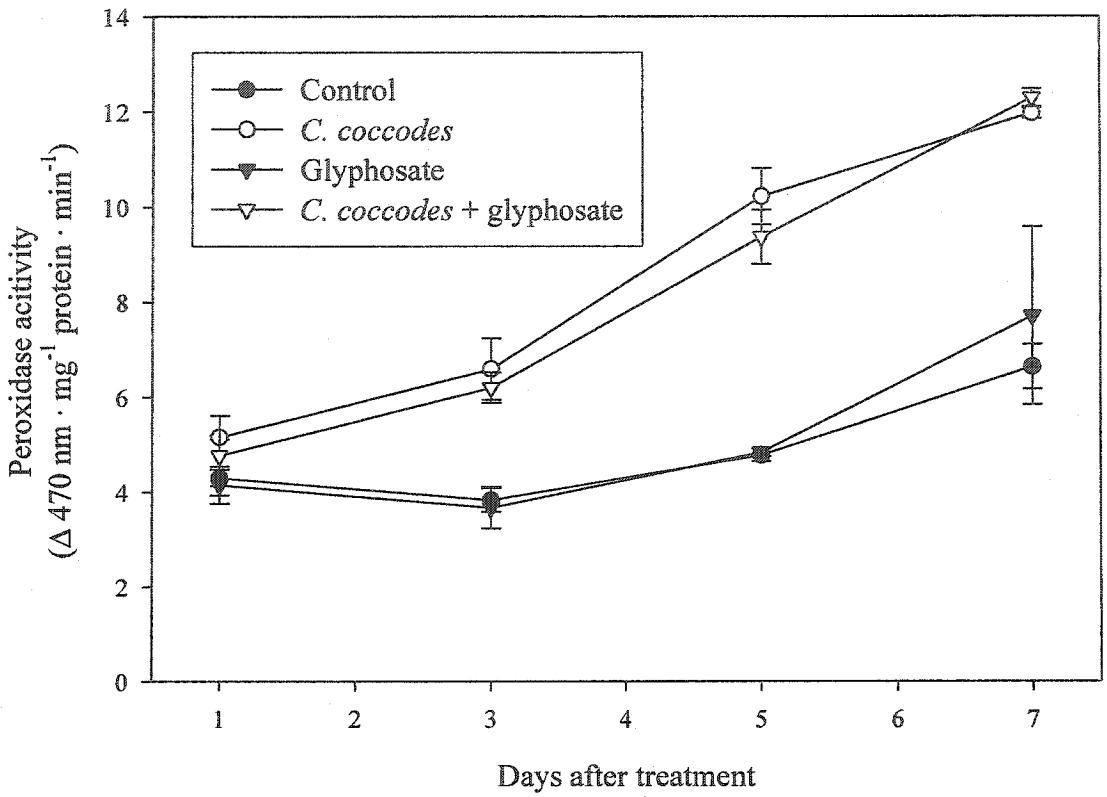

Figure 4.5. Effect of glyphosate and *Colletotrichum coccodes* on peroxidase activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and glyphosate was applied at the rate of 0.2 kg a.i. (active ingredient) ha^{-1}.

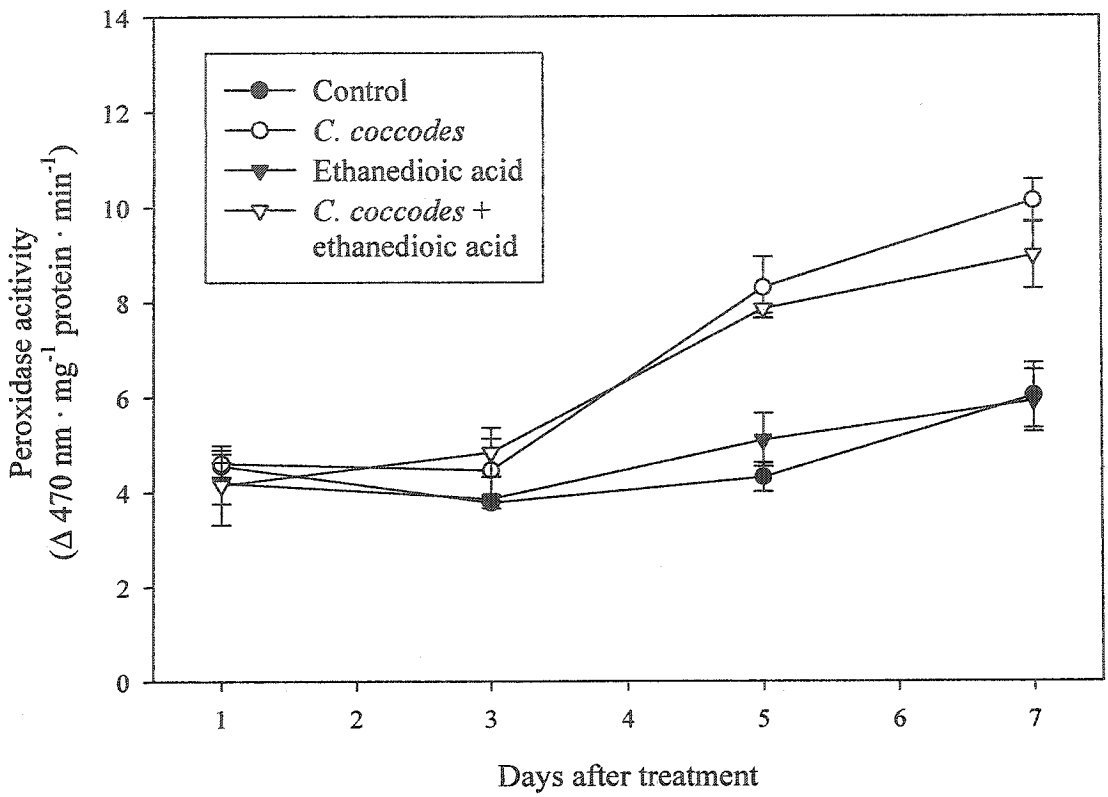

Figure 4.6. Effect of oxalic acid diammonium salt (ethanedioic acid) and *Colletotrichum coccodes* on peroxidase activity. Bars indicate standard errors. *C. coccodes* was applied at the rate of 10^9 conidia m^{-2} and 50 ml of 0.01 % ethanedioic acid solution was applied per m^{-2}.

Chapter 5. General Conclusion

Colletotrichum coccodes has been researched as a bioherbicide for *Abutilon theophrasti*. However, weak virulence has hindered practical use of this pathogen, and enhancement of the virulence has been a major research target. Better understanding of resistance mechanisms of *A. theophrasti* could facilitate attaining enhanced biological weed control activity. Responses of *A. theophrasti* were observed after the various stresses with *C. coccodes*, and effects on efficacy of *C. coccodes* were determined.

Induced resistance of *A. theophrasti* by *C. coccodes*, BTH, acifluorfen, and bentazon has been confirmed. Significant decrease of lesion size in induced plants was observed compared to non-induced plants. Enhanced peroxidase and phenylalanine ammonia lyase (PAL) activities were observed in induced plants after the inducing treatments, providing solid evidences of induced resistance by these agents. Induction of resistance appeared two days after the inducing treatments, indicating that it takes a minimum of two days for a signal molecule to transmit and systemically activate resistance in other plant tissues. BTH strongly induced resistance to *C. coccodes* in *A. theophrasti* and the induction persisted, while resistance induced by herbicides and *C. coccodes* was weak compared to the induction by BTH and disappeared rapidly. However, the harmful effect to a biological control agent can be expected and prevented in an integrated weed management system by arranging the application timing and method since the degree and persistence of induced resistance varied with the type of inducing agent and timing.

A. theophrasti exploits a variety of biochemical defense mechanisms. If the defense mechanism of *A. theophrasti* is clarified and suppressed, virulence of *C. coccodes*

could be enhanced. α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, oxalic acid, and analogues of oxalic acid and mannose were tested for the antifungal effects and enhancement of *C. coccodes* virulence. In general, vegetative growth, conidia production, germination of conidia, and appressoria formation of *C. coccodes* were inhibited by the presence of the chemicals. Despite the inhibitory effects of the chemicals on the growth of the fungus, the virulence of *C. coccodes* was significantly enhanced when *C. coccodes* was applied after mannose and oxalic acid were vacuum-infiltrated through the leaf cuticle. However, lipophilic analogues of the chemicals, the (1-amino-ethyl)-phosphonic acid diisopropyl ester of oxalic acid (ester OA), oxalic acid diammonium salt (ethanedioic acid), and 4,6-di-O-methyl-D-mannose (methyl mannose) did not enhance *C. coccodes* virulence or affect *A. theophrasti* growth. Strong antifungal effects, poor inhibitory effects on plant defense mechanisms, or minor dependence of *A. theophrasti* on the defense mechanisms that the chemicals affected could be reasons of these results.

The efficacy of *C. coccodes* to reduce the dry weight of *A. theophrasti* increased in the presence of 0.25 kg a.i. ha^{-1} bentazon, more than when *C. coccodes* was applied alone, while the effect of glyphosate was minimal. PAL activity in *A. theophrasti* was not affected by infection with *C. coccodes*, but PAL activity was significantly inhibited by the presence of bentazon at five days after treatment and it remained until seven days after treatment. The inhibition was stronger when bentazon alone was applied than when bentazon was applied with *C. coccodes*. The treatment with glyphosate or a mixture of glyphosate and *C. coccodes* did not affect PAL activity. PAL activity was not affected by treatment with ethanedioic acid at any time. Increase of PAL and peroxidase activities

was observed as plants grew. Peroxidase activity was strongly induced by the treatment with *C. coccodes* and its activity increased over time. A significant difference of peroxidase activity in the *C. coccodes* treatment compared to the control appeared three days after the treatment. Peroxidase activity was not induced by 0.25 kg a.i. ha^{-1} bentazon alone. However, when bentazon was applied in combination with *C. coccodes*, it prevented the activation of peroxidase caused by the infection of *C. coccodes*. Treatment with 0.2 kg a.i. ha^{-1} of glyphosate and ethanedioic acid did not affect peroxidase activity. These results suggest that these enzymes are involved in the resistance mechanism of *A. theophrasti,* probably functioning to delay infection of *C. coccodes.* These results also indicate that suppression of weed defense mechanisms can bring enhanced pathogen virulence thus rendering improved weed biocontrol systems.

In general, *A. theophrasti* seems to defend itself from *C. coccodes* by combination of physiological and morphological defense arsenals. Spread of disease from the local infection site to other tissue may be delayed by the structural defense mechanism that requires activation of peroxidase. On the other hand, the plant abscises severely infected leaves, and grows rapidly and recovers from the infection since the apical meristem is protected from infection by leaf primordia and other young leaves. The survived plant tissue, then, achieves increased degree of resistance induced by the infection. The chemical suppression of the defense mechanisms in *A. theophrasti* enhanced *C. coccodes* virulence when the chemical inhibitors were infiltrated into the leaf cuticle. Further research on the delivery of the chemical inhibitors through the leaf cuticle is required. In addition, other defense mechanisms that *A. theophrasti* may exploit to defend itself from *C. coccodes* need to be investigated and clarified.

Contribution to knowledge

The research described in this thesis had focused on clarifying defense responses of *Abutilon theophrasti* to a biocontrol agent, *Colletotrichum coccodes*, thus obtaining clues to facilitate the development of an enhanced biological weed control system. Key contributions to original knowledge arising from the research are considered as follow:

1. This study provides an initial characterization of resistance responses in *A. theophrasti* to *C. coccodes*.
2. Infection of *C. coccodes*, herbicidal stress by acifluorfen and bentazon, and a plant activator, benzothiadiazole, can systemically induce resistance in *A. theophrasti*. This is the first demonstration of induced resistance in *A. theophrasti* to *C. coccodes* caused by these agents.
3. The details of induced resistance in *A. theophrasti* provide vital information that will aid in the development of improved weed biocontrol systems.
4. It was demonstrated that chemical inhibitors of defense mechanisms could enhance the virulence of *C. coccodes* if the chemicals were infiltrated into the leaf cuticle.
5. Bentazon treatment inhibited the elevation of defense related enzymes after *C. coccodes* infection. Thus the mechanism whereby bentazon enhances the efficacy of *C. coccodes* has been determined.

Appendices

Table 1. Effects of α-amino-oxy acetic acid (AOA), 2-deoxy-D-glucose (DDG), mannose, and oxalic acid (OA) on germ tube growth of *Colletotrichum coccodes.*

Treatment	Germ tube length [a] (μm)
Control	207.8 ± 0.9
0.01 % AOA	168.5 ± 9.4
0.1 % AOA	200.5 ± 7.1
0.01 % DDG	53.6 ± 10.4
0.1 % DDG	18.7 ± 2.0
0.01 % Mannose	141.0 ± 11.6
0.1 % Mannose	170.3 ± 11.3
0.01 % OA	113.7 ± 17.1
0.1 % OA	139.5 ± 1.7

[a] Mean germ tube length with standard error.

Germ tube length was measured 16 hours after incubation of conidia on water agar.

Table 2. Effects of oxalic acid and its analogues on the growth of velvetleaf.

Treatment	Plant Height [a] (mm)	Fresh Weight [a] (g)
Control	138.6	4.23
0.001 % OA[b]	148.2	4.38
0.01 % OA	151.2	4.62
0.1 % OA	158.4	4.54
0.001 % Salt-OA[c]	154.6	4.48
0.01 % Salt-OA	135.6	4.19
0.1 % Salt-OA	146.8	4.09
0.001 % Ester-OA[d]	149.0	4.38
0.01 % Ester-OA	137.0	3.94
0.1 % Ester-OA	132.3	4.17

[a] Means are not significantly different at 5 % level.

[b] Oxalic acid.

[c] [Oxalic acid diammonium salt (ethanedioic acid)].

[d] [(1-amino-ethyl)-phosphonic acid diisopropyl ester, compound with oxalic acid].

Table 3. Effects of mannose and methyl mannose on the growth of velvetleaf.

Treatment	Plant Height [a] (mm)	Fresh Weight [a] (g)
Control	90.7	2.71
0.001 % Mannose	94.9	2.72
0.01 % Mannose	85.6	2.51
0.1 % Mannose	83.0	2.71
0.001 % Methyl-mannose[b]	89.4	2.65
0.01 % Methyl-mannose	88.1	2.59
0.1 % Methyl-mannose	84.3	2.58

[a] Means are not significantly different at 5 % level.

[b] 4,6-di-O-methyl-D-mannose.

Table 4. Effects of oxalic acid and its analogues on the efficacy of *Colletotrichum coccodes*.

Treatment	Plant Height [a] (mm)	Fresh Weight [a] (g)
Control	84.7 a	2.97 a
C. coccodes	78.7 a	2.07 cd
C. c[b] + 0.001 % OA[c]	81.8 a	2.12 bcd
C. c + 0.01 % OA	77.5 a	1.91 c
C. c + 0.1 % OA	82.3 a	2.41 bc
C. c + 0.001 % Salt-OA[d]	79.8 a	2.13 bcd
C. c + 0.01 % Salt-OA	78.0 a	1.92 c
C. c + 0.1 % Salt-OA	76.3 a	2.19 bcd
C. c + 0.001 % Ester-OA[e]	85.0 a	2.13 bcd
C. c + 0.01 % Ester-OA	85.9 a	2.20 bcd
C. c + 0.1 % Ester-OA	90.2 a	2.44 b

[a] Means with the same letter within a column are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *Colletotrichum coccodes*.

[c] Oxalic acid.

[d] [Oxalic acid diammonium salt (ethanedioic acid)].

[e] [(1-amino-ethyl)-phosphonic acid.diisopropyl ester, compound with oxalic acid].

Table 5. Effects of mannose and methyl mannose on the efficacy of *Colletotrichum coccodes.*

Treatment	Plant Height [a] (mm)	Fresh Weight [a] (g)
Control	76.3 a	2.41a
C. coccodes	77.7 a	1.63 b
C. c[b]. + 0.001 % Mannose	72.5 a	1.61 b
C. c. + 0.01 % Mannose	72.4 a	1.63 b
C. c. + 0.1 % Mannose	74.8 a	1.68 b
C. c. + 0.001 % Methyl-mannose[c]	75.0 a	1.67 b
C. c. + 0.01 % Methyl-mannose	74.5 a	1.64 b
C. c. + 0.1 % Methyl-mannose	75.9 a	1.79 b

[a] Means with the same letter within a column are not significantly different at 5 % level according to Duncan's Multiple Range Test (DMRT).

[b] *Colletotrichum coccodes.*

[b] 4,6-di-O-methyl-D-mannose.