MYCOFUMIGATION WITH *MUSCODOR ALBUS*: EFFECTS ON VERTICILLIUM WILT AND BLACK DOT ROOT ROT OF POTATO, EFFECTS ON *GLOMUS INTRARADICES* AND ECTOMYCORRHIZAL FUNGI, AND *M. ALBUS* PROLIFERATION IN SOIL

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

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in

Plant Science

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November 2008

DEDICATION

This dissertation is dedicated to my husband Oliver for his never ending love and support, and to my parents, brother, and grandparents for their love and encouragement to follow my dreams.

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ABSTRACT

Muscodor albus Worapong, Strobel & Hess, isolate CZ-620 (MA) is an endophytic fungus that produces volatile organic compounds (VOCs) and non-volatile antimicrobial compounds. The use of these VOCs to inhibit or kill a wide range of microorganisms is termed mycofumigation. This dissertation focuses on parameters of MA mycofumigation including: production and bioactivity of previously un-described water-soluble antimicrobial compounds produced by MA; distribution of antimicrobial compounds from a MA point source in three soil types as measured by effects on *Verticillium dahliae* and *Colletotrichum coccodes*; control of *V. dahliae* and *C. coccodes* on potato; the ability of MA to colonize soil; and the effects of mycofumigation on ectomycorrhizal fungi (EMF) *in vitro* and on the colonization of onion roots by the arbuscular mycorrhizal (AM) fungus *Glomus intraradices*. The bioactivity of water-soluble compounds produced in potato dextrose broth was significantly increased as measured in growth reduction of *C. coccodes, V. dahliae,* and *Rhizoctonia solani*. No reduction was observed for *Aphanomyces cochlioides* and *Pythium ultimum*. Antimicrobial compounds from a MA colonized barley point source reduced *V. dahliae* and *C. coccodes* populations in soils by 60-100% at distances up to 9 cm from the inoculation source depending on soil type. Mortality rate ranging from 70-100% was observed within a 3 cm radius from the inoculation source. In both field and greenhouse trials, MA colonized barley formulation reduced Verticillium wilt and black dot root rot severity and reduced populations of both pathogens in potato tissue as measured by real-time quantitative PCR and serial dilution. Planting directly into mycofumigated soil previously infested with *V. dahliae* or *C. coccodes* resulted in equal control of the pathogens when compared to a one-week mycofumigation period prior to planting. After six weeks of incubation MA did not colonize sterile soil further than 0.5 cm away from a MA inoculation point. *In vitro* experiments showed that most of the tested EMF were inhibited in the presence of MA VOCs, but were able to resume growth when removed from VOCs. Incorporating MA into soil had no negative but supportive effect on onion root colonization by the AM fungus *G. intraradices*.

PROLIFERATION OF *MUSCODOR ALBUS* IN SOIL, DISTRIBUTION AND EFFECTS OF ITS ANTIMICROBIAL COMPOUNDS AS MEASURED BY MORTALITY TO *COLLETOTRICHUM COCCODES* AND *VERTICILLIUM DAHLIAE* IN SOIL

Introduction

Muscodor albus Worapong, Strobel & Hess, isolate CZ-620 is an endophytic fungus isolated from *Cinnamomum zeylanicum* in Honduras (Worapong et al., 2001) that produces volatile organic compounds (VOCs). These VOCs effectively inhibit or kill microorganisms including bacteria, fungi, and nematodes (Strobel, et al., 2001; Stinson et al., 2003; Grimme et al., 2007). The potential of *M. albus* as biological control agent (BCA) for control of soil-borne pathogens has been recognized and the chemical composition of the antimicrobial VOCs and their bioactivity have been described both *in vitro* (Strobel et al., 2001; Ezra and Strobel, 2003) and *in vivo* (Stinson et al., 2003; Jacobsen et al., 2004; Grimme et al., 2007). The terms mycofumigation and biofumigation are both used when efficacies of *M. albus* as BCA are described (Stinson et al., 2003; Zidack et al., 2001 and 2002). However the term biofumigation is mostly used where plant residues are involved (Bhat and Subbarao, 2001; Berbegal et al., 2008; Ploeg, 2008).

In greenhouse experiment, fumigation with the BCA *M. albus* resulted in successful control of soil-borne pathogens such as *Phytophthora capsici* (Camp et al., 2008), *Rhizoctonia solani*, *Pythium ultimum*, *Aphanomyces cochlioides*, *Colletotrichum*

coccodes, and *Verticillium dahliae* (Stinson et al., 2003; Grimme, 2004; Mercier and Manker, 2005; Grimme et al., 2007) and plant pathogenic nematodes like *Meloidogyne incognita* (Grimme et al., 2007), *M. chitwoodi*, *M. hapla*, *Paratrichodorus allius* and *Pratylenchus penetrans* (Riga et al., 2008).

The soil-borne pathogens *C. coccodes* and *V. dahliae* are the primary causal agents of potato early dying (PED) (Rowe and Powelson, 2002; Andrivon et al., 1997, 1998). Potato early dying is difficult to manage and may cause yield losses up to 50%, but are more commonly in the range of 10 to 15% (Rowe and Powelson, 2002; Powelson and Rowe, 2008). Potato early dying is characterized by a reduction in photosynthetic activity, reduced gas exchange of leaves, and in premature senescence of potato plants, affecting yield by shortening the tuber bulking period (Read and Hide, 1995; Lees and Hilton, 2002; Bae et al., 2007). The severity of PED developing in a given crop is a function of populations of *V. dahliae* and associated pathogens such as *Pratylenchus penetrans* and *C. coccodes* in the soil at planting (Tsror (Lahkim) et al., 1999; Tsror (Lahkim) and Hazanovsky, 2001). Management tactics for PED are aimed at reducing the population density of these pathogens in the soil and altering the efficacy of these inocula (Powelson and Rowe, 1993, 2008).

At present the only practical controls for black dot root rot, Verticillium wilt, and PED in heavily infested soils is soil fumigation with metham sodium or metham potassium, growing of resistant cultivars, soil solarization, or incorporation of green manures (Arbogast et al., 1999; Berbegal et al., 2008; Powelson and Rowe, 2008). Tsror (Lahkim) et al. (2005) reported that applications of 600 to 900 L/ha of metham sodium

significantly reduced the disease severity of *V. dahliae* in stems and daughter tubers. Recent research shows promising results in controlling *C. coccodes* using azoxystrobin as in furrow treatment followed by chlorothalonil or mancozeb as foliar application when plants are 15-28 cm tall (Nitzan et al., 2005; Jacobsen *unpublished*).

Recently, the US EPA and California Department of Pesticide Regulation (www.cdpr.ca.gov) proposed restrictions on the use of soil fumigants including metham sodium and metham potassium. These restrictions could make the use of these fumigants impractical for many producers and alternatives such as cultural methods, green manures (Davis et al., 1996) or the application of BCAs such as *Talaromyces flavus* (Spink and Rowe, 1989; Engelkes et al., 1997), *Gliocladium* spp., *Trichoderma* spp. (Wilson et al., 2008), and *M. albus* (Grimme et al., 2007) may be needed for control of soil-borne pathogens in potato production. The maximum effectiveness of a fungal BCA is evaluated by its ability to establish in crop ecosystems and its remaining activity against target pathogens during periods favorable for plant infection (Orr and Knudsen, 2004). By introducing a non-indigenous fungus like *M. albus* into soils it is important to understand, detect, and quantify the hyphal proliferation, and its ability to survive in natural and commercial soils (Orr and Knudsen, 2004). In addition, production of VOCs also generates questions about their effects on plant pathogens, non-target organisms, and their ability to disseminate away from the point of inoculation. Ezra and Strobel (2003) investigated the influence of substrates on the bioactivity of VOCs of *M. albus* on several plant pathogenic fungi. They demonstrated that *M. albus*, grown on high nutrient media,

produced VOCs which were more effective in controlling fungal microorganisms than those produced on a low nutrient media.

Production and distribution of water-soluble components and their effects on microorganisms are also of interest especially when *M. albus* is applied as soil treatment. However, little is known about the antimicrobial bioactivity of these non-volatile compounds. The effects of water-soluble compounds produced by the fungus *M. albus* was first investigated by Jacobsen and Zidack (2004, *unpublished data*) who tested the effects of culture broth, incubation time, and temperature on *M. albus* control of *P. ultimum in vitro*. Broth media amended with different concentrations of broth extracts of *M. albus* cultures were inoculated with *P. ultimum* cultures and percent growth relative to the *Pythium* control was recorded. Potato dextrose broth (EMD Chemicals Inc., Darmstadt, Germany) was found to be the media type that best supported the production of water-soluble antimicrobial compounds.

As described above the need for new alternatives to replace conventional fumigants and the promising *in vitro* results for the control of multiple microorganisms including soil-born fungal pathogens such as *C. coccodes* and *V. dahliae* through mycofumigation with *M. albus* initiated this research. The focus of this research was the evaluation of the potential of *M. albus* as BCA for the control of soil-borne pathogens under greenhouse and field conditions and its reaction to different soils and growth conditions.

The objectives of this research were (1) to evaluate the *in vitro* effects of water-soluble antimicrobial compounds emitted by *M. albus* on growth of *A. cochlioides*, *C.*

coccodes, *P. ultimum*, *R. solani*, and *V. dahliae*; (2) to evaluate the hyphal proliferation of *M. albus* in sterile soil over time; and (3) to understand the distribution of antimicrobial compounds emitted by *M. albus* in soil infested with *C. coccodes* and *V. dahliae*.

Materials and Methods

Fungal Isolates

Preparation of *M. albus* Isolate CZ-620 Inoculum: *Muscodor albus* was grown and maintained in 10 cm Petri plates on potato dextrose agar (PDA; EMD Chemicals Inc., Darmstadt, Germany) at 23 ± 1 °C for three weeks. Inoculum was produced by transferring 15 agar plugs (5 mm diameter) from 3-week-old cultures into 2-liter flasks containing 1 liter autoclaved potato dextrose broth (PDB; EMD Chemicals Inc., Darmstadt, Germany), and placing the flasks on a rotary shaker (Model OS-500, VWR International, West Chester, PA) (110 rpm) for 2 weeks at 23 ± 1 °C. The *M. albus* barley formulation was prepared by adding 100 ml of colonized PDB to mushroom cultivation bags (Polypropylene bags with one filter, Unicorn Imp. & Mfg. Corp., Garland, TX 75042) containing 500 g of sterilized barley (*Hordeum vulgare* L). After growth on the substrate for three weeks at 23 ± 1 °C, the colonized seeds were dried for 48 h in a laminar flow-hood and stored in plastic bags at 4 °C.

Preparation of Plant-Pathogen Inocula: A culture of *Verticillium dahliae*, isolate 502, was obtained from the MSU fungal culture collection, courtesy of Dr. Alan T. Dyer. *Colletotrichum coccodes* and *Rhizoctonia solani* were isolated from infected potato plants

and tubers from Montana. *Aphanomyces cochlioides* and *Pythium ultimum* were originally isolated from infected sugar beet seedlings grown in Montana. All fungal pathogens were stored on PDA slants at 4 ± 1 °C and oomycetes were stored in sterile distilled water at room temperature.

Colletotrichum coccodes and *V. dahliae* were grown and maintained in Petri dishes with PDA at 23 ± 1 ˚C for two weeks. Ten 5 mm agar plugs from PDA cultures of *Verticillium* or *Colletotrichum* were transferred to 1-liter flasks containing 0.5 liter autoclaved PDB, followed by incubation on a rotary shaker (110 rpm) for 2 weeks at 23 ± 1 °C. Sixty milliliters of the resulting liquid cultures were then added to 300 g of sterilized barley contained in Mason jars and incubated for three weeks at 23 ± 1 ˚C. The colonized barley was dried as described above and ground in a Waring blender (Waring Inc., Santa Monica, CA) to produce a homogenous inoculum formulation with a mean particle size of 300 µm.

One gram of the ground inoculum of both pathogens was diluted 10-, 100-, and 1,000 fold in sterile distilled water and 100 µl of each dilution was evenly spread with a sterile glass rod on polygalacturonic acid selective media (NP-10 medium) for *V. dahliae* (Sorensen et al., 1991) and Farley's medium for *C. coccodes* (Farley, 1972) to determine colony forming units (CFUs) of *C. coccodes* and *V. dahliae* per gram of inoculum. All plates were incubated at 22 ± 1 °C for one week. Colonies of *C. coccodes* and *V. dahliae* were counted and CFUs/g inoculum were determined and resulted in 1 x 10^7 CFUs/1g barley inoculum for *V. dahliae* and 2 x 10^4 CFUs/1g barley inoculum for *C. coccodes*.

Effect of Water-Soluble Antimicrobial
Compounds from *M. albus* on Fungal Pathogens

Three different broth media were used to test differences in water-soluble antimicrobial compositions on growth of plant pathogenic fungi following the recommendations of Ezra and Strobel (2003) regarding the carbohydrate and nitrogen sources. Broth media used included: a) minimal media (HS) containing only 20 g of sucrose (EMD Chemicals Inc., Darmstadt, Germany) as carbon source per 1 l distilled water; b) water agar with 20 g sucrose as carbon source and 10 g yeast extract (Difco) per 1 l distilled water (HSY); and c) PDB broth as high nutrient broth with dextrose and potato starch as carbon sources. The media broths were sterilized and inoculated with *M. albus* culture as described above. The cultures were grown for 14 days at 23 ± 1 °C on a rotary shaker. The liquid cultures were aseptically filtered through sterile cheesecloth, followed by fine-filtration through sterile membranes of a Vacuum Filtration System (pore size: 0.45 μm) (VWR International). Different amounts of the sterile culture broth filtrates were separately added under sterile conditions to regular PDA to obtain PDA media plates with filtrate ratios of 10, 20, and 30%. Agar plugs (5-mm diameter) of *A. cochlioides*, *C. coccodes*, *P. ultimum*, *R. solani*, and *V. dahliae* were transferred to the modified PDA plates and incubated at 23 ± 2°C. Fungal growth was monitored for 14 days and compared to control cultures. Data were expressed as reduction of mycelial growth. Mycelial growth reductions were calculated as Area Under the Mycelial Growth Progress Curve (AUMGPC) using the formula: $AUMGPC = \sum[(y_i + y_{i+1})/2 \times (t_{i+1} - t_i)$ where y_i = initial mycelial growth and t_i = initial time of growth reduction.

Proliferation of
M. albus Hyphae in Soil

MSU soil mix (1/3 Bozeman silt loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent) used for the experiments was sieved (2 mm mesh opening) and autoclaved at 121°C and at a pressure of 21 kg/cm^2 for 60 min. Glass Petri dishes (10 cm x 5 cm, VWR International) were filled with 200 g of MSU soil mix around a plastic cylinder (2.5 cm x 4.5 cm) which was placed in the middle as a place holder. *M. albus* grown on barley (12 g) was added into the empty space in the soil and moistened with 12 ml of sterile distilled water. The Petri dishes were covered loosely with Parafilm to reduce water evaporation while allowing some air exchange. Six replicate Petri dishes were prepared and placed in an incubator at 22 ˚C. Soil samples (approximately 0.22 g) were taken in a circle around the *M. albus* point of inoculation with a sterile cork borer (0.2 cm^2) after two, four, and six weeks of incubation. The samples were taken at four different distances (peripheral, 0.5 cm, 1.0 cm, and 1.5 cm) from the point of *M. albus* barley inoculation to monitor mycelial proliferation in the soil. Eight soil samples were taken per distance to evaluate the hyphal soil proliferation of *M. albus* with half of the samples used for DNA extraction and PCR amplification. The other samples were used for determination of *M. albus* CFUs using serial dilution plating.

Molecular Quantification of
M. albus by Polymerase Chain Reaction

M. albus DNA Extraction and Sequencing: *Muscodor albus* was cultured on PDA for three weeks at 22 ˚C ± 1 ˚C until the majority of the Petri plate was covered with mycelium. DNA was extracted using the FastDNA Kit (Qbiogene Inc., Carlsbad, CA) and FastPrep FP120 homogenizer (Qbiogene Inc.) following the instructions provided by the manufacturer (Schroeder, 2006). DNA concentrations were quantified using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) with absorbance measurements at 260 and 280 nm. The internal transcribe spacer (ITS) regions of *M. albus*, as well as the 5.8S genes of the ribosomal DNA (rDNA), were amplified using PCR and ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. PCR reactions consisted of 65 to 120 ng of fungal DNA, 1.5 µl (10 pmol) of each primer and 12.5 µl 1X GoTaq® Colorless Master Mix (Promega, Madison, WI) in a total reaction volume of 25 µl. PCR was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories Ltd, Hemel Hempsted, United Kingdom) with the following program settings: initial denaturation 4 min at 95 ºC, 60 s denaturation at 95 ºC, 45 s annealing at 55 ºC, extension 2 min at 72 ºC for 30 cycles, final extension 5 min at 72 ºC, 4 ºC hold temperature. Amplicons for DNA sequencing were separated from excess nucleotides and salts using electrophoresis on a 1.2 % agarose gel or the QIAquick PCR purification kit (Qiagen Sciences Inc., Germantown, Maryland) (Okubara et al., 2008). Purified DNA samples were prepared

for sequencing according to the specifications provided by the sequencing facility and sent to the University of California at Berkeley DNA sequencing facility (Berkeley, CA).

Primer Design: Based on the internal transcribed spacer regions (ITS regions) of the fungal ribosomal DNA (rDNA), specific primer pairs for *M. albus* were designed for conventional PCR and real-time quantitative PCR (QPCR) using the Invitrogen Oligo perfect software with the following design criteria: (i) minimum oligonucleotide length of 16 bases, (ii) melting temperatures of 50 to 60 ºC, and (iii) primer % GC content of 40 to 55. The following specific primer pairs were designed (Table 1.1; Image 1.1).

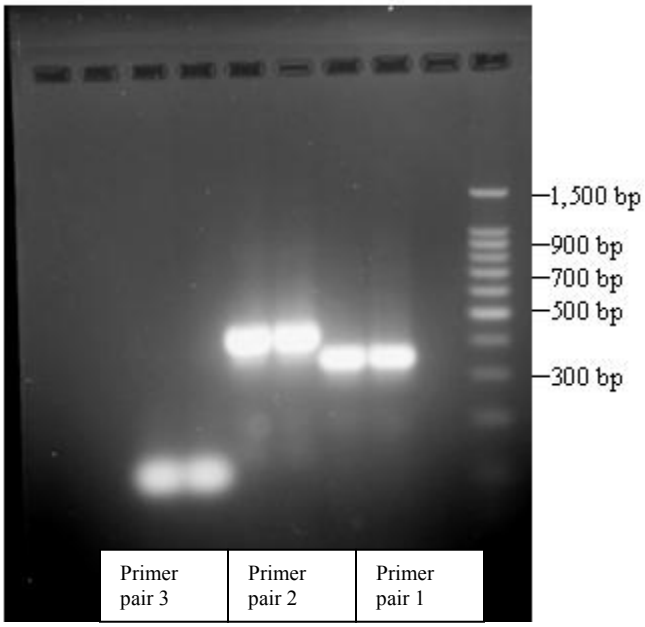


Image 1.1: PCR products after amplification of *Muscodor albus* DNA with different primer pairs on a 2 % agarose gel; 100 bp ladder.

Table 1.1: Specific primer pairs for *Muscodor albus*, isolate CZ-620 were designed using the Invitrogen Oligo perfect software.

	Primer Name	Sequence	Melting Temperature (ºC)
Primer pair 1	MaF1	5'-TCGTGCTCAACGTCTTATCG-3'	54.8
	MaR1	5'-AGAGTGAGAACCAACTCCGCCAAT-3'	60.3
Primer pair 2	Ma F2	5'-AACTTACCTTTGTTGCTTCGGCGG-3'	60.2
	Ma R1	5'-AGAGTGAGAACCAACTCCGCCAAT-3'	60.3
Primer pair 3	MaF3	5'-ATTTCACCACTTAAGCCCTGTT-3'	54.7
	MaR3	5'-CTACGCCTAGAGTGAGAACCAA-3'	55.9

The specificity of each primer pair was confirmed by using the Basic Local Alignment Search Tool (BLAST) of the GenBank database (National Resource for Molecular Biology Information). Primer sequences were verified to have low homodimer and heterodimer potential, as well as a low probability of forming a hairpin loop, using the OligoAnalyzer 3.0 web software (IDT, Coralville, IA). All primers were synthesized by IDT-DNA Technologies.

Detection of *M. albus* in the Soil by PCR: DNA was extracted from soil samples (150 mg) with the FastDNA Kit (Qbiogene Inc., Carlsbad, CA) as described by the manufacturer. Extracted samples were stored at – 20 ºC.

Polymerase chain reactions consisted of 65 to 120 ng of fungal DNA, 1.5 µl (10 pmol) of each primer and 12.5 µl 1X GoTaq® Colorless Master Mix (Promega, Madison, WI) in a total reaction volume of 25 µl. Polymerase chain reactions were performed using an iCycler Thermal Cycler (Bio-Rad Laboratories Ltd, Hemel Hempsted, United

Kingdom) with the following program settings: initial denaturation 4 min at 95 ºC, 60 s denaturation at 95 ºC, 45 s annealing at 52 ºC, extension 2 min at 72 ºC for 30 cycles, final extension 5 min at 72 ºC, 4 ºC hold temperature. Amplicons for DNA sequencing were separated from excess nucleotides and salts using electrophoresis on a 1.2 % agarose gel or the QIAquick PCR purification kit (Qiagen Sciences Inc., Germantown, Maryland).

Quantification of *M. albus*
in Soil Using Serial Dilution Plating

Subcultures of *M. albus* were grown for one week at 22 ºC on PDA, containing 10 ppm chloramphenicol. The soil samples (0.2 g per sample) were diluted 10 to 1000 fold with sterile distilled water. One hundred microliters of each dilution were evenly spread with a sterile glass rod onto PDA containing 10 ppm chloramphenicol. The plates containing the soil samples were inverted above plates containing the one-week-old *M. albus* culture (Image 1.2), sealed with parafilm, and incubated at 22 ºC ± 1 ºC for one week. Colonies of *M. albus* were counted and CFUs/g soil determined.

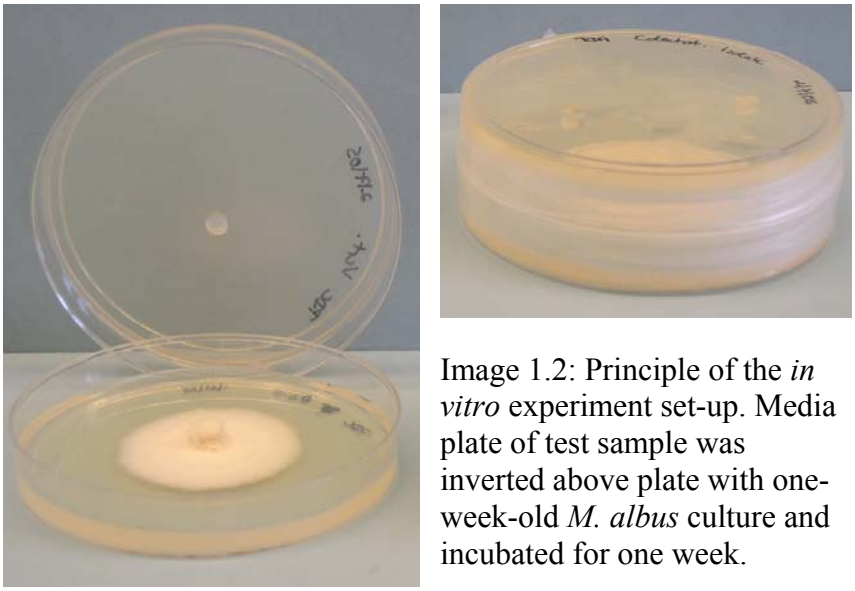


Image 1.2: Principle of the *in vitro* experiment set-up. Media plate of test sample was inverted above plate with one-week-old *M. albus* culture and incubated for one week.

Distribution of Antimicrobial
Compounds Emitted by *M. albus* in Soil

Three different soils (MSU mix, Lihen sandy loam, and Amsterdam silt loam) were used to evaluate the distribution of antimicrobial compounds emitted by a point source of *M. albus*. A soil analysis of all three soils was obtained from A & L Western Agricultural Laboratories, Modesto, CA, (Table 1.2; Appendix A). All growing substrates were sieved through 2 mm mesh and air dried at 22 ˚C ± 1 ˚C.

Table 1.2: Soil analysis obtained from A & L Western Agricultural Laboratories, Modesto, CA of three growing substrates.

Soil	**Soil Texture**	**Fraction (%)**			**pH**
		Sand	**Silt**	**Clay**	
MSU mix	Sandy loam	72	10	17	6.8
Amsterdam silt loam	Silt loam	26	56	17	8.0
Lihen sandy loam	Sandy loam	70	16	13	7.0

Soils were adjusted to a moisture content of 15% and artificially infested with *C. coccodes* or *V. dahliae* barley inoculum by mixing the inocula throughout the soils. Soil chambers approximately 30 x 30 x 2.54 cm in size (2.3 liter volume; Image 1.3) were filled with 2.5 kg of *Colletotrichum*- or *Verticillium*-infested soil. Eleven grams of *M. albus* barley formulation were packaged into a nylon bag, moistened with 6 ml of sterile distilled water, and placed in the center of the soil chamber. Soil chambers with only *Verticillium*- or *Colletotrichum*-infested soil were prepared as pathogen controls. Aluminum foil was placed on top of the chambers to avoid excessive water evaporation. The soil chambers were stored in the dark and incubated at 21 ºC for 7 or 14 days. Two replicate soil chambers were set up per pathogen and incubation time.

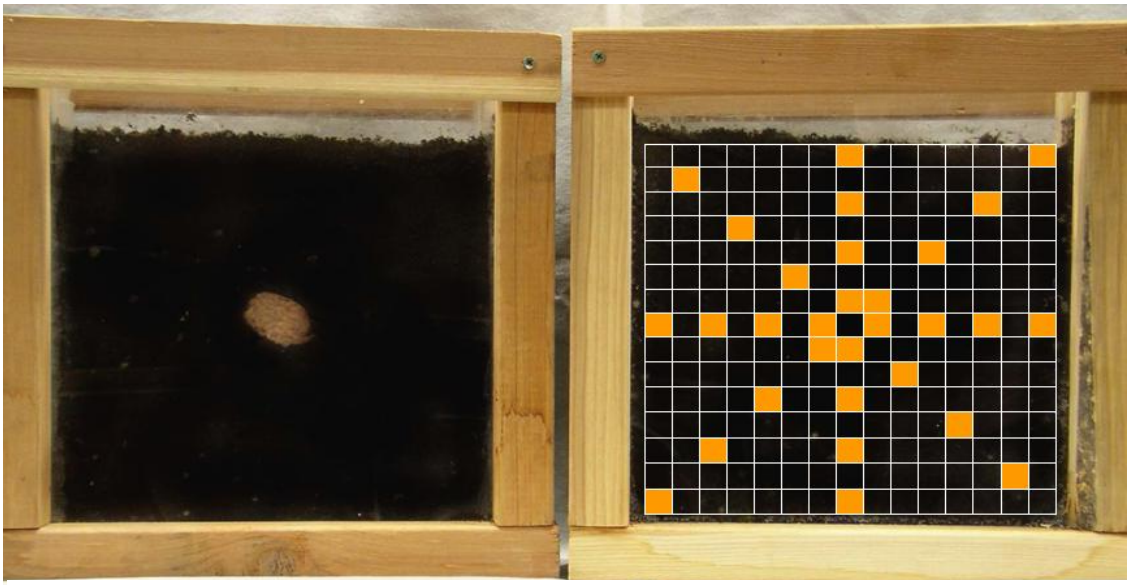


Image1.3: Soil chambers, constructed of wood and plexiglass. Left: soil chamber filled with pathogen-infested soil and *M. albus* barley formulation as point source placed in the center; Right: sample grid with orange field marking the position of samples taken after fumigation period.

After the respective incubation time, a plastic grid (pixel size 1 cm x 1 cm) was laid over the front site of the soil chambers (Image 1.3) and soil samples (approximately 5 g) were taken with a cork borer (1.33 cm^2) in radial distances at 3, 5, 7, and 9 cm from point of *M. albus* inoculation. The exact location of each sample on the grid was recorded in space (x, y coordinates) (Collins et al., 2003). The soil samples were allowed to air-dry under a laminar flow hood at room temperature for 48 h. A 1 g sub-sample of each soil sample was serially diluted (10 to 1,000 fold) in sterile distilled water and 100 µl of each dilution were evenly spread with a sterile glass rod on selective NP-10 medium for *V. dahliae* and Farley's medium for *C. coccodes*. All plates were incubated at 22 ºC for one week. Colonies of *C. coccodes* and *V. dahliae* were identified by colony color and morphology of conidiophores and conidia. Numbers of CFUs per g soil were

determined and marked with the corresponding location in the plastic grid to monitor the distribution of VOC of *M. albus* in soil. Colony forming units of fumigated soil chambers were compared to CFUs of respective control soils and data were expressed as reduction in CFUs compared to the respective pathogen control.

Spatial distribution maps of fungal density distribution in soil after fumigation with *M. albus* were prepared by use of VESPER (Variogram Estimation and Spatial Prediction with ERror, Australian Center for Precision Agriculture, Sydney, AUS) with neighborhood and kriging method analyses. VESPER performs interpolation with punctual kriging using global variogram estimation. Punctual kriging predicts an exact value at each grid point and assigns that value to the grid point. The global variogram calculation uses all the data in the grid to produce one variogram. This global variogram is then used to calculate the interpolated values at all points on the sample grid.

Statistical Analysis

All experiments with were repeated twice. Statistical analysis of the data was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at $P = 0.05$.

Results

Effect of Water-Soluble Antimicrobial Compounds from *M. albus* on Fungal Pathogens

The tested water-soluble compounds of *M. albus* had no inhibitory effects on the mycelial growth of *A. cochlioides* and *P. ultimum* regardless of media type or filtrate concentration. Consequently these data are not presented in the following chapter.

Potato dextrose media supplemented with high nutrient filtrates derived from *M. albus* grown in PDB resulted in the highest reduction of mycelial growth of *V. dahliae*, *C. coccodes*, and *R. solani* regardless of the concentration of the filtrates (Tables 1.3, 1.4, and 1.5) compared to filtrates of HS and HSY. However, PDB filtrates at a concentration of 30% showed the significantly highest reduction ($P \leq 0.0001$) of *V. dahliae, R. solani, and C. coccodes* mycelium growth compared to concentrations of 10 and 20% filtrate. Potato dextrose agar plates supplemented with PDB filtrates showed significantly ($P \leq 0.0001$) higher growth reduction of *V. dahliae*, *R. solani*, and *C. coccodes* when compared to plates with HS and HSY filtrates (Table 1.3, 1.4, and 1.5). Media plates supplemented with HSY filtrate resulted in higher AUMGPC of *C. coccodes* and *R. solani* plates than plates with HS filtrate, but was lower for *V. dahliae*. There was no significant difference in growth reduction of PDA plates supplemented with HS or HSY filtrates. The concentration of these filtrates had no significant influence on the mycelial growth reduction of *V. dahliae C. coccodes* or *R. solani* (Table 1.3 and 1.4).

Table 1.3: Effect of different concentrations of water-soluble compounds of *M. albus*, produced in three different culture broths, on fungal mycelial growth of *Verticillium dahliae* expressed as reduction in Area Under the Mycelial Growth Progress Curve (AUMGPC).

Concentration / **Medium** [x]	**10 %**			**20 %**			**30 %**			***LSD*$_{0.05}$**
PDB	293.56	**a** [y]	*c* [z]	587.05	**a**	*b*	836.56	**a**	*a*	*110.68*
HS	147.50	**b**	*a*	172.22	**b**	*a*	163.69	**b**	*a*	*27.23*
HSY	90.90	**b**	*b*	142.20	**b**	*a*	163.29	**b**	*a*	*44.94*
LSD$_{0.05}$	**64.55**			**50.89**			**73.40**			

[x] PDB = Potato Dextrose Broth, high nutrient broth with dextrose and potato starch as carbon source; HS = minimal media containing only 20 g of sucrose as carbon source per 1 l distilled water; HSY = water agar with 20 g sucrose as carbon source and 10 g yeast extract per 1 l distilled water.

[y] same bold letters within the same column are not significantly different from each other according Fisher's protected LSD=0.05.

[z] same italic letters within the same row are not significant differently from each other according Fisher's protected LSD=0.05.

Table 1.4: Effect of different concentrations of water-soluble compounds of *M. albus*, produced in three different culture broths, on fungal mycelial growth of *Rhizoctonia solani* expressed as reduction in Area Under the Mycelial Growth Progress Curve (AUMGPC).

Concentration / **Medium** [x]	**10 %**	**20 %**	**30 %**	***LSD*$_{0.05}$**
PDB	305.34 **a** [y] *b* [z]	160.46 **a** *c*	633.64 **a** *a*	*48.56*
HS	5.78 **b** *a*	6.72 **b** *a*	10.11 **b** *a*	*5.15*
HSY	28.65 **b** *a*	35.67 **b** *a*	40.25 **b** *a*	*11.85*
LSD$_{0.05}$	**25.12**	**40.71**	**31.81**	

[x] PDB = Potato Dextrose Broth, high nutrient broth with dextrose and potato starch as carbon source; HS = minimal media containing only 20 g of sucrose as carbon source per 1 l distilled water; HSY = water agar with 20 g sucrose as carbon source and 10 g yeast extract per 1 l distilled water.

[y] same bold letters within the same column are not significantly different from each other according Fisher's protected LSD=0.05.

[z] same italic letters within the same row are not significantly different from each other according Fisher's protected LSD=0.05.

Table 1.5: Effect of different concentrations of water-soluble compounds of *M. albus*, produced in three different culture broths, on fungal mycelial growth of *Colletotrichum coccodes* expressed as reduction in Area Under the Mycelial Growth Progress Curve (AUMGPC).

Concentration / **Medium** [x]	**10 %**		**20 %**		**30 %**		***LSD*$_{0.05}$**
PDB	638.24	**a** [y] *c* [z]	728.97	**a** *b*	954.18	**a** *a*	*56.15*
HS	29.06	**b** *a*	58.89	**b** *a*	30.85	**b** *a*	*32.30*
HSY	41.34	**b** *ab*	91.20	**b** *a*	4.08	**c** *b*	*51.91*
LSD$_{0.05}$	**39.51**		**88.74**		**16.88**		

[x] PDB = Potato Dextrose Broth, high nutrient broth with dextrose and potato starch as carbon source; HS = minimal media containing only 20 g of sucrose as carbon source per 1 l distilled water; HSY = water agar with 20 g sucrose as carbon source and 10 g yeast extract per 1 l distilled water.

[y] same bold letters within the same column are not significantly different from each other according Fisher's protected LSD=0.05.

[z] same italic letters within the same row are not significantly different from each other according Fisher's protected LSD=0.05.

Proliferation of
M. albus Hyphae in Soil

Colony forming units were successfully detected in soil after two weeks by serial dilution plating and PCR amplification when samples were taken directly at the periphery of the *M. albus* barley formulation. No CFUs were detected from samples taken at a distance of 0.5 cm (Figure1.1). After four weeks of incubation CFUs were detected with dilution plating and PCR directly at the periphery but not in samples 0.5 cm and 1.0 cm away. After six weeks of incubation, CFUs were detected at the periphery of *M. albus* formulation and at a distance of 0.5 cm. No CFUs were discovered at other distances (Figure 1.1; Image 1.4).

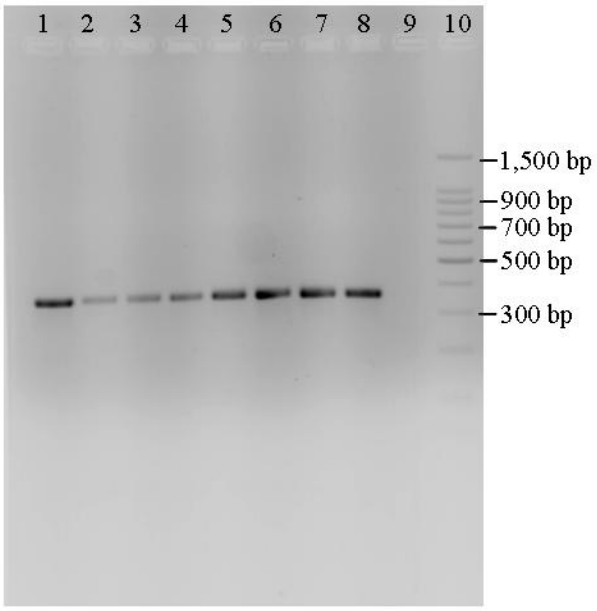


Image 1.4: PCR products after amplification of soil samples with specific primer pairs on a 1.2 % agarose gel; Lane 1: *M. albus* culture plate DNA control; Lanes 2 – 4: samples at distance 2 (0.5 cm away from inoculum); Lanes 5 – 8: samples at distance 1 (direct at periphery of *M. albus* inoculum); Lane 9: negative control; Lane 10: 100 bp ladder

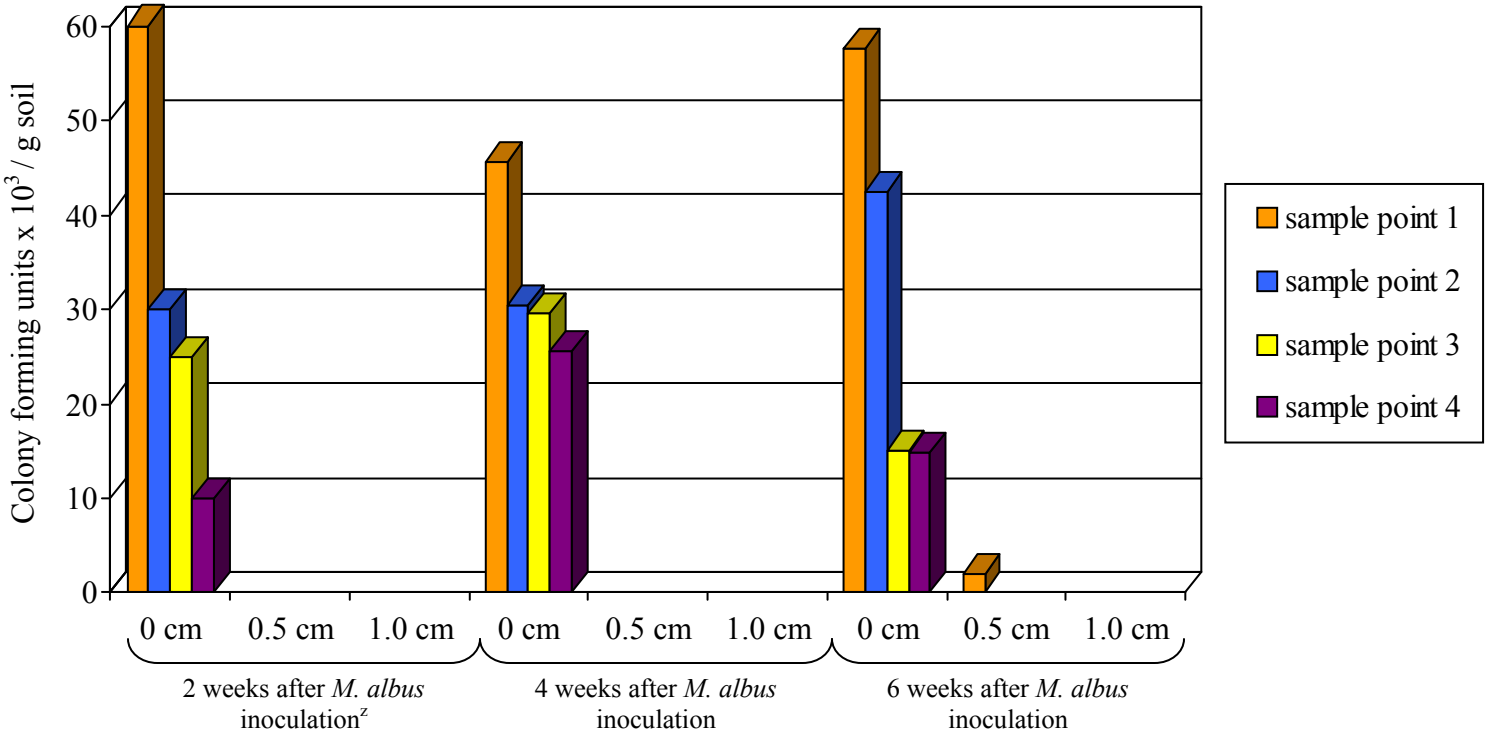


Figure 1.1: Detection of *Muscodor albus* proliferation by serial dilution plating after 2, 4, and 6 weeks of incubation in sterile MSU mix.

[z] *M. albus* was point inoculated in the center of a Petri dish filled with soil. Soil samples were taken after two, four, and six weeks of incubation. The samples were taken at different distances from the point of *M. albus* barley inoculation to monitor mycelial proliferation in the soil; distance 1: direct at the periphery of *M. albus* barley formulation, distance 2: 0.5 cm away from formulation, and distance 3: 1.0 cm away from formulation. CFUs/soil sample at different distances were determined.

Distribution of Antimicrobial Compounds
from a *M. albus* Point Source in Three Different Soils

In all three tested soils the fumigation time of fourteen days was superior in reducing CFUs of *V. dahliae* per gram of soil compared to the fumigation time of seven days (Figure 1.2). Data were expressed as percent reduction of CFUs compared to the *Verticillium* control. In MSU mix *Verticillium* CFUs were reduced up to 90% after seven days at the distances of 1 and 3 cm away from *M. albus* point of inoculation. Colony forming units were significantly higher at the distance of 5 cm, but decreased again at 7 and 9 cm away from the *M. albus* point source. In MSU mix fourteen days of fumigation resulted in 100% reduction of *V. dahliae* CFUs at 0 to 9 cm from the point source.

In Lihen sandy loam soil the seven day fumigation period with *M. albus* resulted in 84% reduction of *Verticillium* CFUs at 1 cm from the point source with significantly increasing CFUs at distances of 3 (65%) and 5 cm (65%) from *M. albus* inoculum (Figure 1.2). At the distance of 7 cm CFUs decreased slightly up to 69%. CFU reduction was less effective at the distance of 9 cm with approximately 46% reduction. In Lihen sandy loam fourteen day fumigation resulted in a reduction of CFUs of 84% at the distance of 1 cm and 69% at 3 cm with decreasing reduction at 5 (66%) to 9 cm (54%) from the *M. albus* point source.

In Amsterdam silt loam soil fumigation with *M. albus* resulted in less *Verticillium* CFUs reduction after seven days when compared to the other test soils. Reduction of CFUs range from 82% at the distance of 1 cm to 78% at a distance of 9 cm from the *M. albus* point source. There were no significant differences in CFU reduction within all distances tested. The fumigation time of fourteen days did not result in significantly

different reductions of *Verticillium* CFUs at all distances. At the distance of 1 cm 62% of CFUs were reduced to a reduction of 48% at a distance of 9 cm (Figure 1.2).

Spatial distribution maps were created using the software VESPER to visualize the number of *Verticillium* CFUs after seven and fourteen days of fumigation with *M. albus*. For MSU mix the spatial distribution map of *V. dahliae* confirmed the results of figure 1.2 and showed that numbers of CFUs were lower around the point source of *M. albus* and that CFUs were increasing with increasing distance from the point source. After a fumigation period of fourteen days 100% of *V. dahliae* was controlled (Figure 1.3).

Spatial distribution maps of *V. dahliae* in Lihen sandy loam after seven and fourteen days of fumigation clearly showed a reduction of CFUs around the point source of *M. albus* with increasing numbers of CFUs with increasing distance (Figure 1.4). For Amsterdam silt loam, spatial distribution of *Verticillium* CFUs after seven and fourteen days of fumigation were declining around and below the point source of *M. albus* (Figure 1.5).

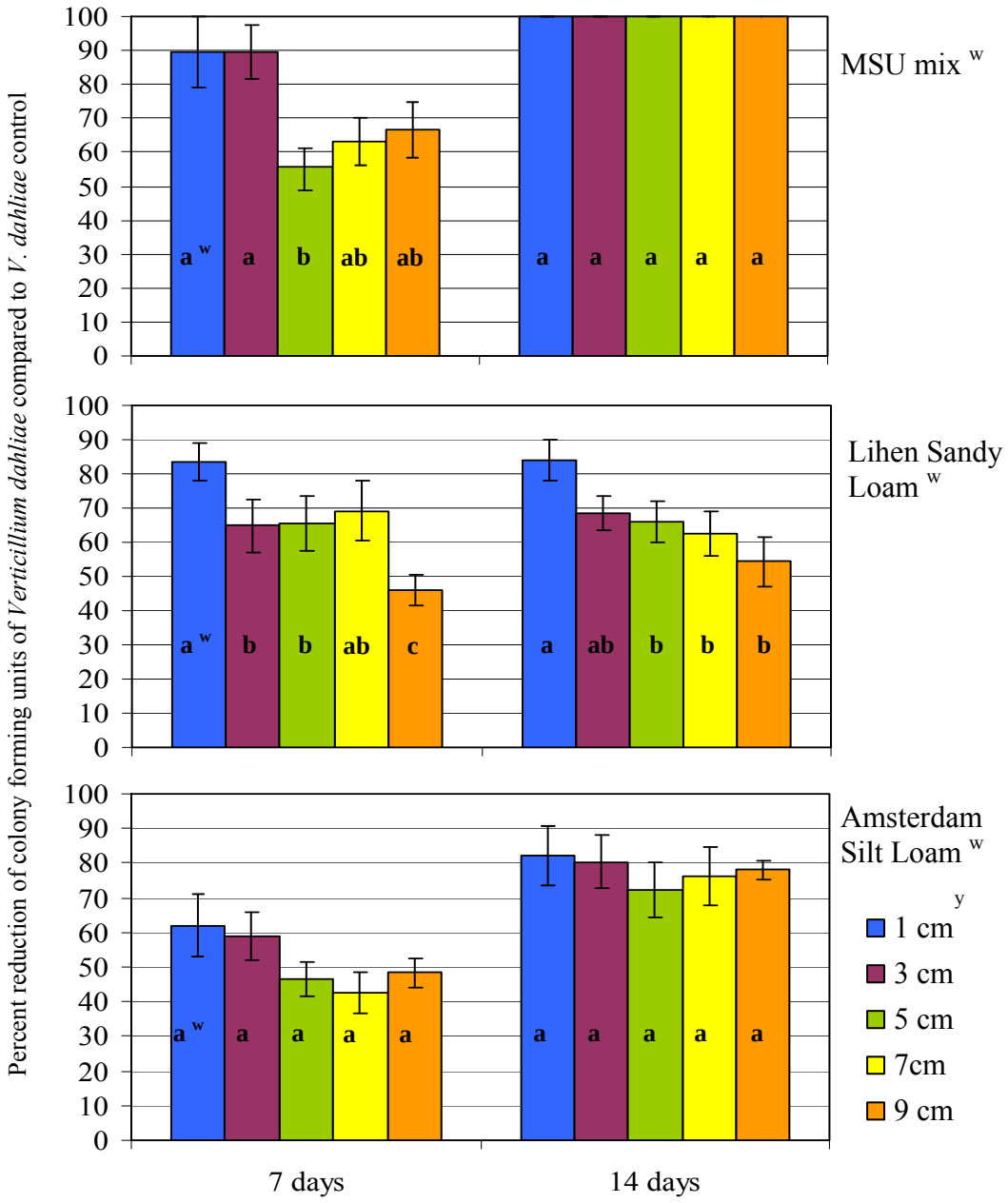


Figure 1.2: Reduction of *Verticillium dahliae* CFUs by antimicrobials emitted by *Muscodor albus* at two fumigation periods and in three different test soils compared to respective *V. dahliae* controls.

[w] different soils were infested with *C. coccodes* grown on sterile barley at a rate of 10 CFUs/g soil.

[x] *M. albus* grown on sterile barley was point inoculated at a rate of 4.5 g/ kg soil

[y] distance from point of inoculation with *M. albus*.

[z] not significantly different according to Fisher's protected $LSD_{0.05}$.

A. **B.**

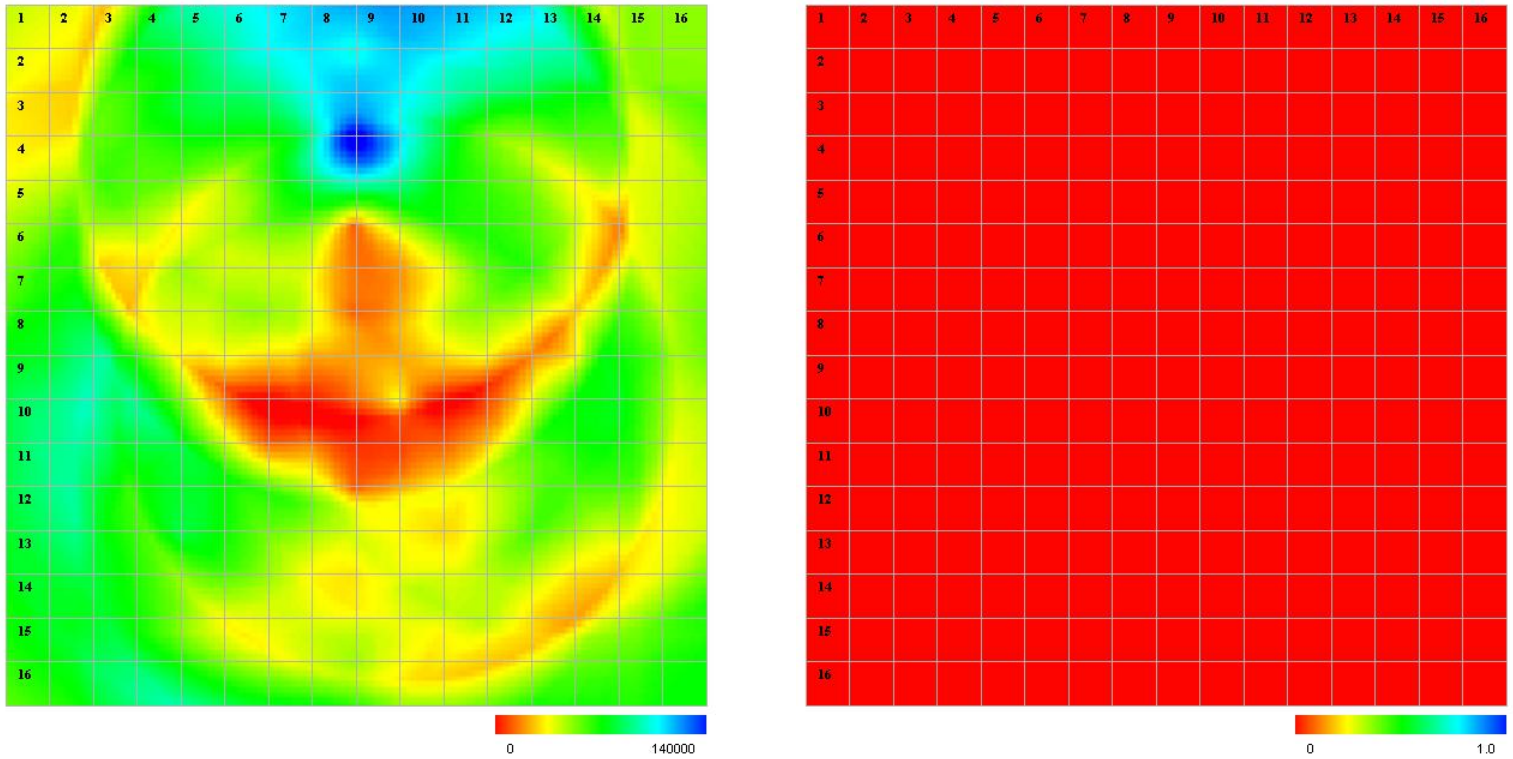


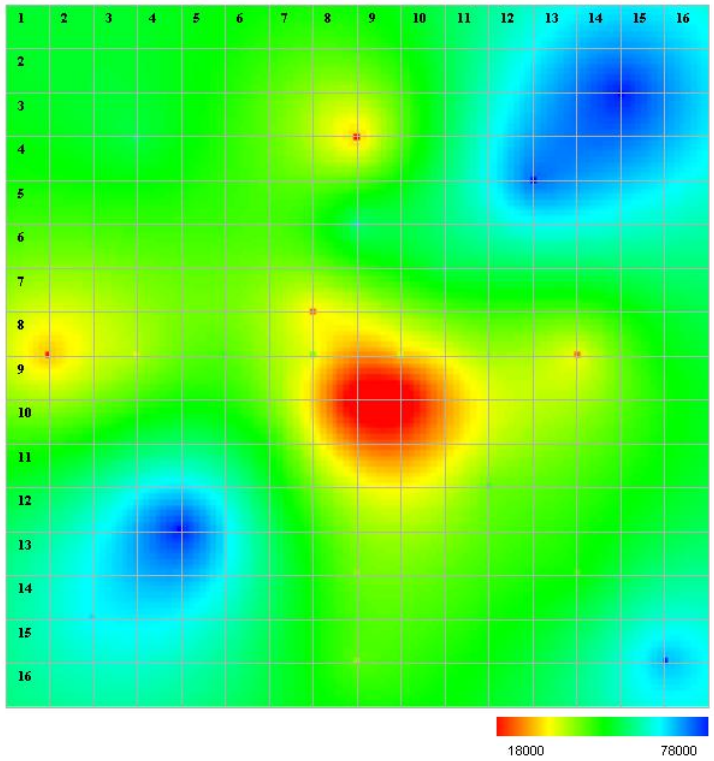
Figure 1.3: Spatial [x] distribution analysis of *Verticillium dahliae* [y] colony forming units (CFUs) in artificially infested soils originating from MSU mix after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).

[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).

[y] Soil was inoculated with *V. dahliae* grown on sterile barley at a rate of 10 CFUs/g soil.

[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

A.



B.

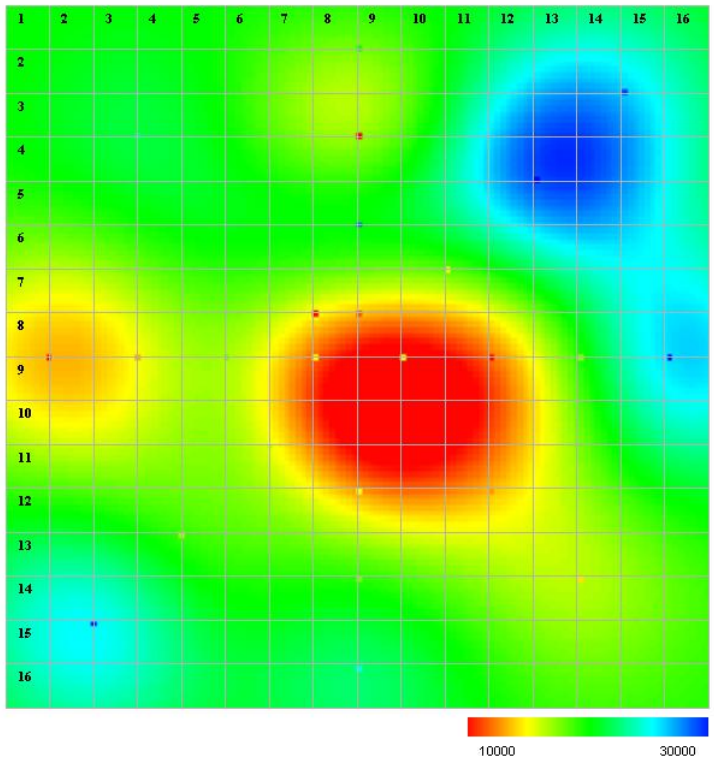


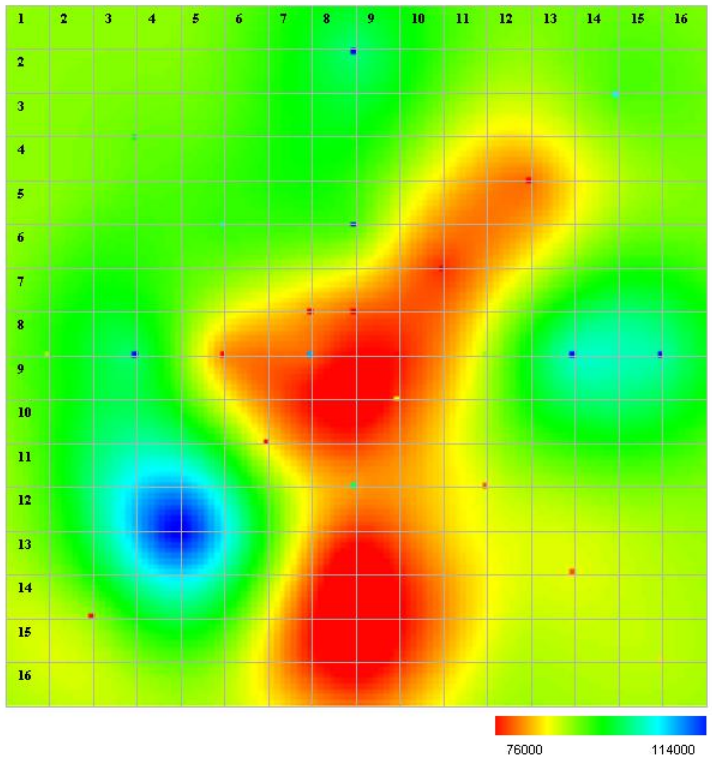
Figure 1.4: Spatial [x] distribution analysis of *Verticillium dahliae* [y] colony forming units (CFUs) in artificially infested Lihen sandy loam after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).

[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).

[y] Soil was inoculated with *V. dahliae* grown on sterile barley at a rate of 10 CFUs/g soil.

[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

A.



B.

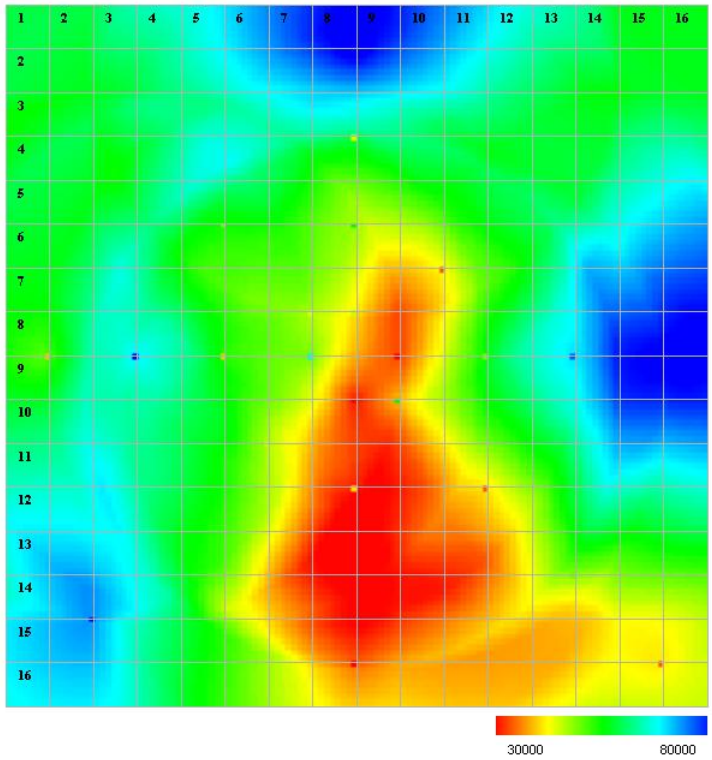


Figure 1.5: Spatial [x] distribution analysis of *Verticillium dahliae* [y] colony forming units (CFUs) in artificially infested Amsterdam silt loam after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).

[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).

[y] Soil was inoculated with *V. dahliae* grown on sterile barley at a rate of 10 CFUs/g soil.

[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

In all three tested soils the fumigation time of fourteen days was superior in reducing CFUs of *C. coccodes* per gram of soil compared to the fumigation time of seven days (Figure 1.6). In MSU mix *Colletotrichum* CFUs were reduced up to 89% after seven days at the distances of 1 cm with slightly decreasing reduction at 3 cm (85%) to 7 cm (86%). At the distance of 9 cm away from *M. albus* point source CFUs were significantly higher than CFUs at the other distances. Fourteen days of fumigation in MSU mix resulted in 100% reduction of *C. coccodes* CFUs at the distance of 1 cm, and 98 % to 96 % at a distance up to 7 cm. At 9 cm distance from the point source of *M. albus*, CFU reduction was 75% (Figure 1.6).

In Lihen sandy loam soil a fumigation period of seven days with *M. albus* resulted in 87% reduction of *Colletotrichum* CFUs at 1 cm distance with significantly increasing CFUs at distances of 3 and 5 cm from *M. albus* point source (Figure 1.6). At the distance of 7 cm CFUs decreased slightly to 71%. Colony forming units reduction was lower at the distance of 9 cm with approximately 59% reduction. In Lihen sandy loam fourteen days of fumigation resulted in a reduction of CFUs of 100% at the distance of 1 cm and 3 cm and in a slightly decreased reduction at 5 cm (95%) to 9 cm (84%) from the *M. albus* point source.

In Amsterdam silt loam the seven day soil fumigation with *M. albus* at 1 cm distance resulted in *Colletotrichum* CFU reduction of 94% and 85% at 3 cm distance. Number of CFUs increased significantly with increasing distance from the point source of *M. albus* inoculation. The fumigation time of fourteen days was more effective in reducing *Colletotrichum* CFUs and no significant difference in CFU reduction within

different distances was detected (Figure 1.6). At the distance of 1 cm 96% of CFUs were reduced with a reduction of 90% at a distance of 9 cm (Figure 1.6).

Spatial distribution maps were created using the software VESPER to visualize the number of *Colletotrichum* CFUs after seven and fourteen days of fumigation with *M. albus*. For MSU mix the spatial distribution map of *C. coccodes* showed that numbers of CFUs were lower at the point source of *M. albus* and reduced in the upper part of the soil chamber. Colony forming units were increasing in the area below the point source. After a fumigation period of fourteen days the spatial distribution map clearly showed a reduction of CFUs around the point source of *M. albus* (Figure 1.7).

Spatial distribution maps of *C. coccodes* in Lihen sandy loam after seven and fourteen days of fumigation clearly showed a reduction of CFUs around the point source of *M. albus* with numbers of CFUs increasing with distance (Figure 1.8). In comparison to Lihen sandy loam and MSU mix, spatial distribution of *Colletotrichum* CFUs in Amsterdam silt loam, after seven and fourteen days of fumigation, was declining around and below the point source of *M. albus* (Figure 1.9).

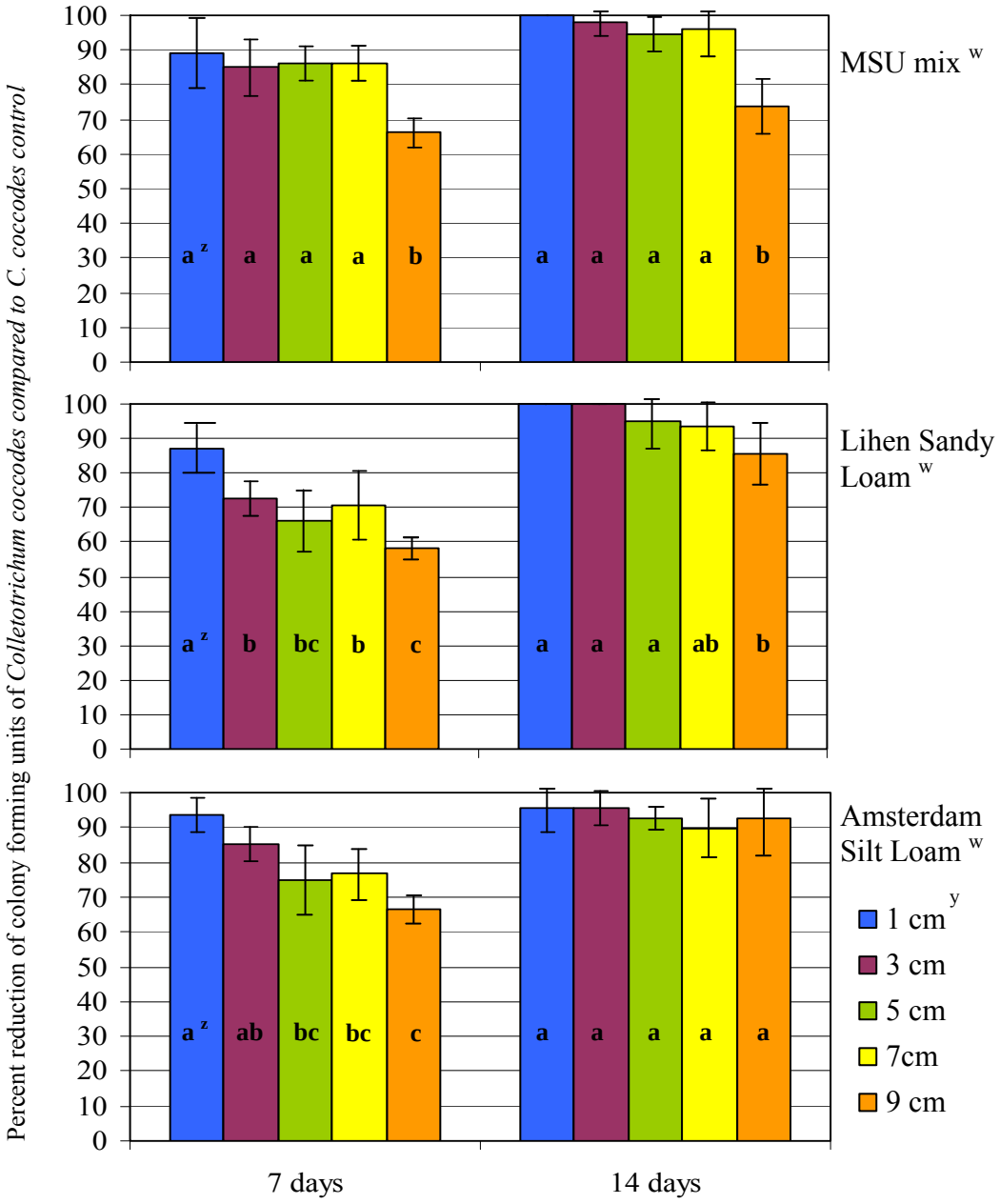


Figure 1.6: Reduction of *Colletotrichum coccodes* CFUs by antimicrobials emitted by *Muscodor albus* at two fumigation periods and in three different test soils compared to respective *C. coccodes* controls.

[w] different soils were infested with *C. coccodes* grown on sterile barley at a rate of 7 CFUs/g soil.

[x] *M. albus* grown on sterile barley was point inoculated at a rate of 4.5 g/ kg soil.

[y] distance from point of inoculation with *M. albus*.

[z] not significant differently according to Fisher's protected $LSD_{0.05}$.

A. **B.**

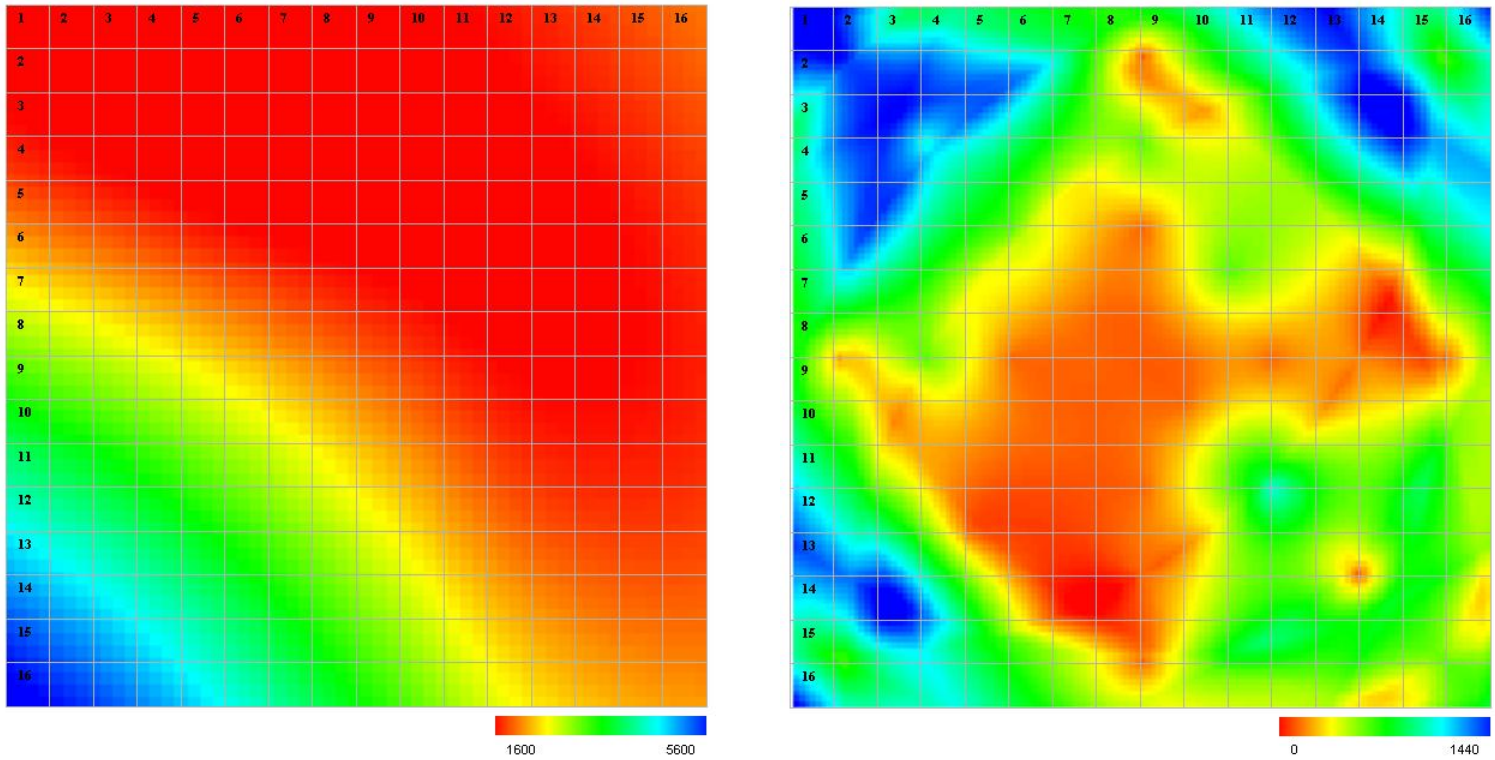


Figure 1.7: Spatial [x] distribution analysis of *Colletotrichum coccodes* [y] colony forming units (CFUs) in artificially infested soils originating from MSU mix after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).
[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).
[y] Soil was inoculated with *C. coccodes* grown on sterile barley at a rate of 7 CFUs/g soil.
[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

A.

B.

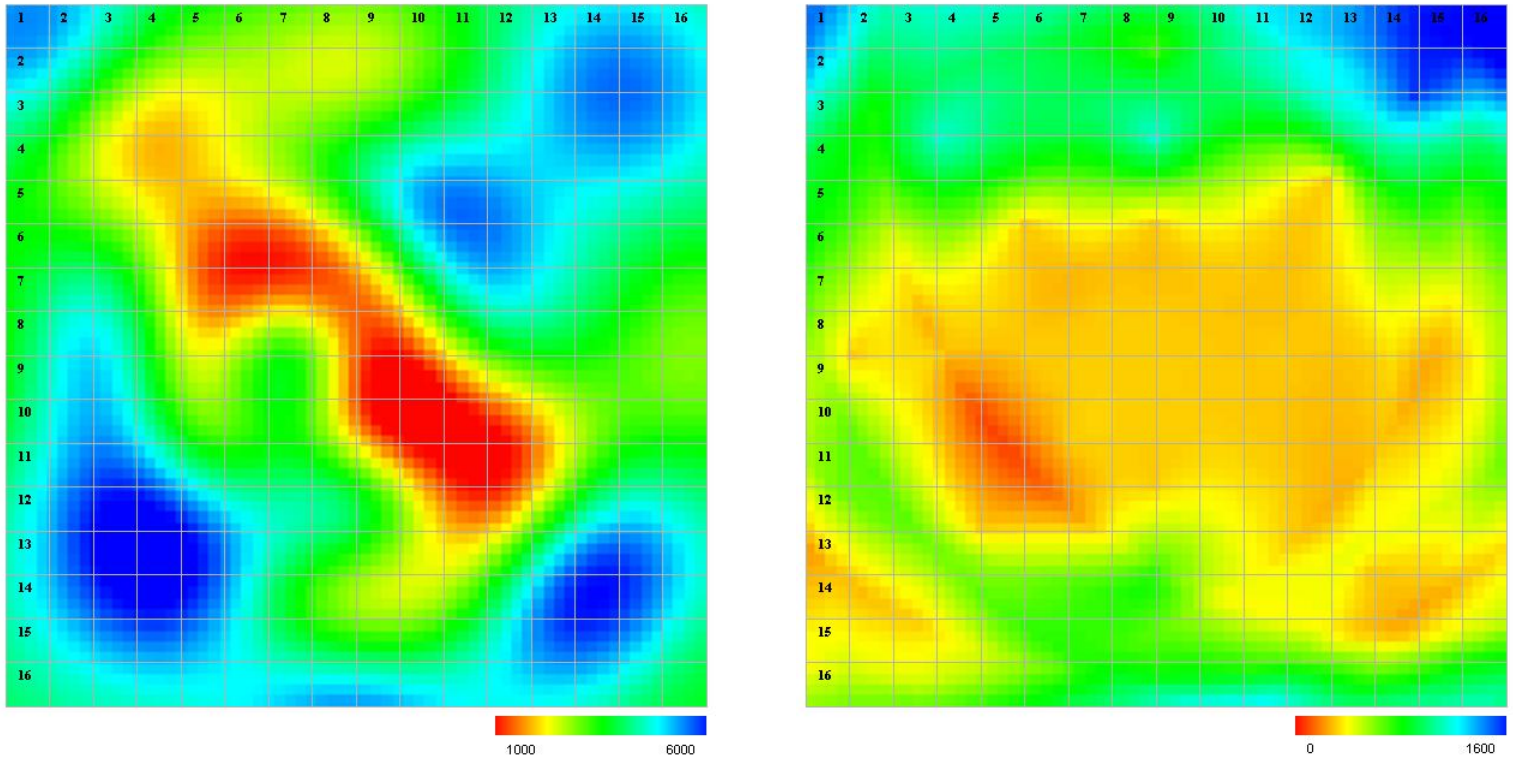


Figure 1.8: Spatial [x] distribution analysis of *Colletotrichum coccodes* [y] colony forming units (CFUs) in artificially infested soils originating from Lihen sandy loam after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).
[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).
[y] Soil was inoculated with *C. coccodes* grown on sterile barley at a rate of 7 CFUs/g soil.
[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

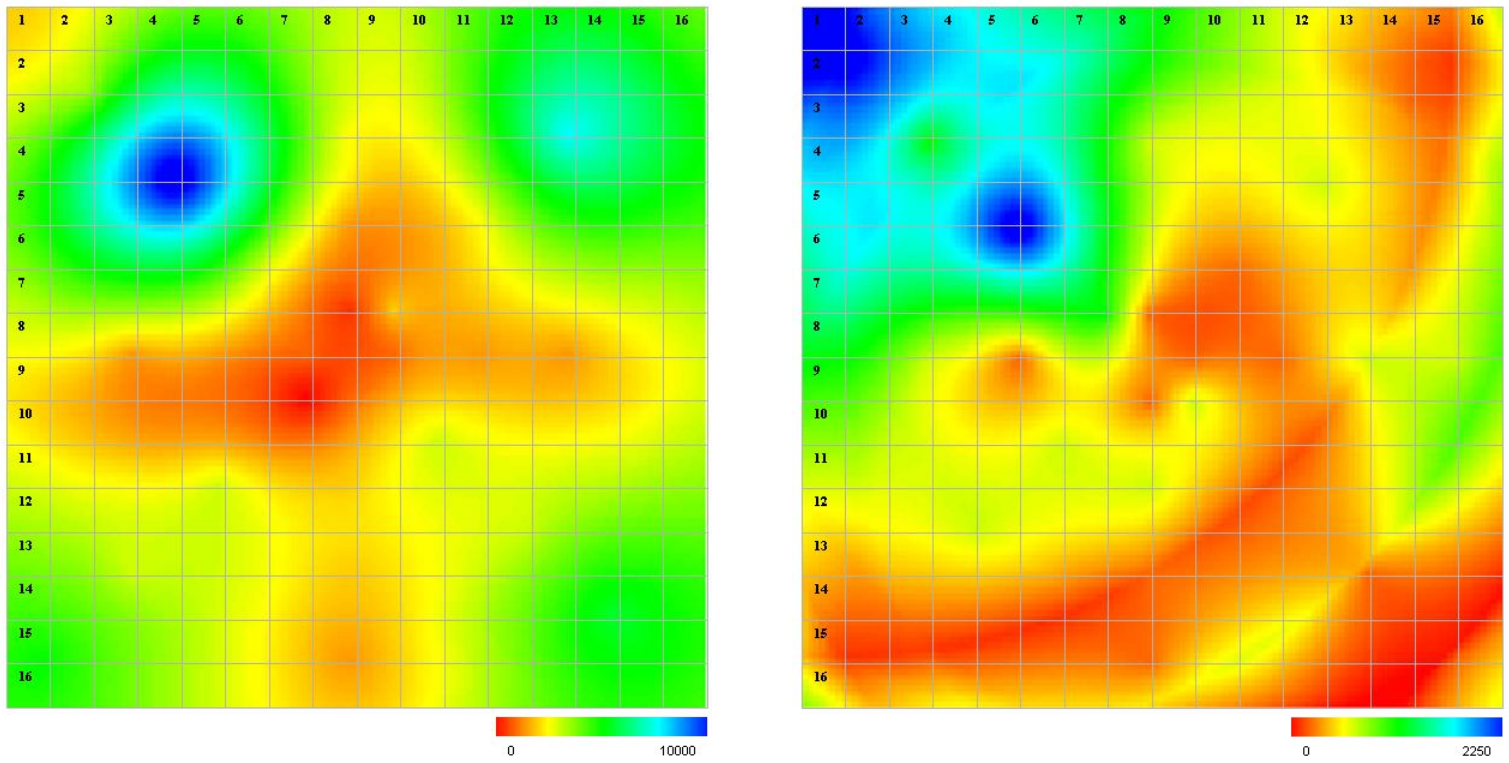


Figure 1.9: Spatial [x] distribution analysis of *Colletotrichum coccodes* [y] colony forming units (CFUs) in artificially infested soils originating from Amsterdam silt loam after a fumigation period with *Muscodor albus* [z] of 7 days (**A.**) and 14 days (**B.**).
[x] Spatial distribution of CFUs estimated by VESPER software (Australian Centre for Precision Agriculture, 2006).
[y] Soil was inoculated with *C. coccodes* grown on sterile barley at a rate of 7 CFUs/g soil.
[z] Eleven grams of *M. albus* grown on sterile barley were positioned in the center of the soil chamber.

The effects of soil type and texture on the efficacy of fumigation with *M. albus* on reduction of *Verticillium* CFUs were investigated. After seven days of fumigation no significant difference was detected at a distance of 1 cm between the MSU mix, Lihen sandy loam, and Amsterdam silt loam (Table 1.6). At distances of 3 and 9 cm from point source of *M. albus*, MSU mix and Amsterdam silt loam resulted in equal reduction of *Verticillium* CFUs and had significantly ($P \leq 0.0001$) higher CFU reductions when compared to CFUs of Lihen silt loam. There were no significant differences in CFU reduction at distances of 5 and 7 cm after seven days of fumigation.

The fumigation time of fourteen days resulted in 100% reduction of *Verticillium* CFUs in MSU mix at all distances. At all distances CFU reduction in MSU mix was significantly higher ($P \leq 0.0001$) than in Lihen sandy loam and Amsterdam silt loam (Table 1.6). No significant difference in CFU reduction was observed between Lihen sandy loam and Amsterdam silt loam.

In *C. coccodes* infested soil, fumigation with *M. albus* for seven days resulted in no significant differences in CFUs between all soils at a distance of 1 cm away from *M. albus* point source (Table 1.7). At distances of 3, 5, and 9 cm, CFU reduction of *Colletotrichum* in MSU mix and Amsterdam silt loam were significantly higher ($P \leq 0.0003$) when compared to CFU reduction in Lihen sandy loam. There was no significant difference in CFU reduction at the mentioned distances between MSU mix and Amsterdam silt loam. At the distance of 7 cm from the point source, CFU reduction in MSU mix was significantly higher than in Lihen sandy loam and Amsterdam silt loam (Table 1.7). *Colletotrichum* CFU reduction in all soils was not significantly different

after fourteen days of fumigation at distances of 1, 3, 5, and 7 cm. At a distance of 9 cm CFU reduction was significantly higher in Lihen sandy loam and Amsterdam silt loam when compared to MSU mix. There was no significant difference in CFU reduction at the distance of 9 cm between Lihen sandy loam and Amsterdam silt loam (Table 1.7).

Table 1.6: Effects of soil type on the percent reduction of colony forming units (CFUs) of *Verticillium dahliae* [w] caused by volatile organic compounds emitted by *Muscodor albus* [x]. Data were expressed as percent reduction of CFUs compared to *V. dahliae* pathogen control.

	Fumigation period									
	7 days					**14 days**				
Soil type [w] / **Distance** [y]	**1 cm**	**3 cm**	**5 cm**	**7 cm**	**9 cm**	**1 cm**	**3 cm**	**5 cm**	**7 cm**	**9 cm**
MSU mix	89.58	89.58	55.56	63.19	66.67	100	100	100	100	100
Lihen Sandy Loam	83.64	64.85	65.46	69.09	45.99	83.94	68.65	65.90	62.54	54.33
Amsterdam Silt Loam	82.28	80.38	72.36	76.37	78.00	62.01	58.91	46.51	42.64	48.29
LSD $_{0.05}$	ns [z]	13.31	ns	ns	14.97	15.84	8.57	20.66	19.87	9.11

[w] different soils were infested with *V. dahliae* grown on sterile barley at a rate of 10 CFUs/1g soil.
[x] *M. albus* grown on sterile barley was point inoculated at a rate of 4.5 g/1 kg soil.
[y] distance from point of inoculation with *M. albus* [x].
[z] not significant different according to Fisher's protected $LSD_{0.05}$.

Table 1.7: Effects of soil type on the percent reduction of colony forming units of *Colletotrichum coccodes* [w] caused by volatile organic compounds emitted by *Muscodor albus* [x]. Data were expressed as percent reduction of CFUs compared to *C. coccodes* pathogen control.

	Fumigation period									
	7 days					**14 days**				
Soil type [w] / **Distance** [y]	**1 cm**	**3 cm**	**5 cm**	**7 cm**	**9 cm**	**1 cm**	**3 cm**	**5 cm**	**7 cm**	**9 cm**
MSU mix	89.25	84.95	86.02	86.02	65.53	100	98.24	94.56	96.14	74.71
Lihen Sandy Loam	87.08	72.79	65.99	70.75	58.80	100	100	95.24	93.65	83.89
Amsterdam Silt Loam	93.56	85.38	74.85	76.61	65.92	95.65	95.65	92.75	89.85	89.82
LSD $_{0.05}$	ns [z]	7.25	17.47	15.15	6.26	ns	ns	ns	ns	12.42

[w] different soils were infested with *C. coccodes* grown on sterile barley at a rate of 7 CFUs/1g soil.
[x] *M. albus* grown on sterile barley was point inoculated at a rate of 4.5 g/1 kg soil.
[y] distance from point of inoculation with *M. albus* [x].
[z] not significant different according to Fisher's protected $LSD_{0.05}$.

Discussion

The endophytic fungus *Muscodor albus* produces both volatile and non-volatile metabolites that adversely affect growth of different fungi. In this study the *in vitro* effects of the water-soluble antimicrobial compounds produced by *M. albus* on growth of fungal plant pathogens were investigated. Ezra and Strobel (2003) reported that the composition of the solid medium used to support the growth of *M. albus* had a great influence on the quality and microbial effectiveness of the VOCs emitted by this fungus. *Muscodor albus* grown on high nutrient substrate like PDA produced a variety of VOCs which were lethal or inhibitory to most of the tested plant pathogens. We were able to confirm these results and extended the observation to water-soluble antimicrobial compounds of *M. albus* produced in culture broth. The composition of the culture broth had a significant influence on the effectiveness of antimicrobial compounds of *M. albus* in inhibiting plant pathogenic fungi *in vitro*. These results are similar to the observations made by Jacobsen and Zidack (2004, *unpublished data*) where the antimicrobial fractions of *M. albus* grown in PDB were more effective in controlling growth of *P. ultimum in vitro* than other culture broth media. In our presented research, high nutrient culture broth (PDB) resulted in more effective water-soluble compounds regarding the inhibitory effects of plant pathogenic fungi than the tested low nutrient broths (HS and HSY). The critical components for the production of highly effective water-soluble compounds might be potato starch and dextrose as carbon sources as it is provided by the PDB broth. Amendments of sucrose or the combination of sucrose and yeast were less effective in producing inhibitory water-soluble compounds with antimicrobial properties. In contrast

to the work of Jacobsen and Zidack (2004, *unpublished data*) the tested water-soluble compounds of *M. albus* in our research had no inhibitory effects on the mycelial growth of *A. cochlioides* and *P. ultimum* regardless of media type or filtrate concentration. This may be due to the fact that culture broth filtrates of *M. albus* were added to PDA and poured as regular media plates while Jacobsen and Zidack (2004, *unpublished data*) directly inoculated the culture broth with media plugs of *P. ultimum*.

When introducing a non-indigenous fungus like *M. albus* into soils it is important to be able to detect and quantify the hyphal proliferation, and understand the organisms ability to survive in natural and commercial soils (Orr and Knudsen, 2004). Culture-independent molecular and culture-dependent CFU assays successfully detected *M. albus* in soil. Detection of fungi in plant tissue or soil via CFUs is commonly used for pathogenic and beneficial fungi (Paulitz and Linderman, 1991; Li et al., 2008) and has been used in the assessment of plant diseases like Verticillium wilt and black dot of potato (Harris et al., 1993; Barkdoll and Davis, 1992). Culture-dependent methods rely on the assessment of viable cells which are counted on media plates. Li et al., 2008, cautions that the number of CFUs is estimated by visual identification which may be less accurate due to different microorganisms present in samples. The use of selective or semi-selective media for dilution plating is therefore essential for an accurate assessment of CFUs (Farley, 1976; Sorenson et al., 1991). Detection of *M. albus* using PCR was successful and supported the CFUs of serial dilution plating data in regard to the distance of hyphal proliferation of *M. albus* in soil. In our experiment *M. albus* colonized the sterile barley kernels and was detected after 2 and 4 weeks of inoculation directly at the

periphery of the inoculum. After 6 weeks of inoculation, *M. albus* was also detected 0.5 cm away from the point source. No *M. albus* proliferation was observed at greater distances away from the point source. This observation supports the findings of Grimme (2004) who showed that *M. albus* does not colonize the soil and will not survive after its food source is exhausted. This observation differs from research investigation of the proliferation of *Trichoderma* spp. in soil. *Trichoderma* sp. have the ability to proliferate and establish themselves in the soil (Lewis and Papavizas, 1984; Howell, 2003). Orr and Knudsen (2004) stated that the maximum effectiveness of a fungal BCA is evaluated by its ability to establish in crop ecosystems and remain active against target pathogens during periods favorable for plant infection. *Muscodor albus* does not establish itself in the soil or plant tissue. Nevertheless, the water-soluble and volatile compounds produced by this fungus have antimicrobial properties that kill a wide range of plant pathogenic microorganisms (Strobel at al., 2001; Grimme, 2004) and remain active up to fourteen days tested. Even without the ability to proliferate in the soil, *M. albus* is an effective BCA that can be applied for the control of soil-borne pathogens without implications of detrimental effects on beneficial microorganisms or plants (Zidack et al., 2002; Stinson et al., 2003; Grimme et al., 2007).

The production of VOC and water-soluble compounds by *M. albus* generated questions about their effects on plant pathogens, non-target organisms, and their ability to disseminate away from the point of inoculation. *Muscodor albus* was effective in all three soils tested and a fumigation period of fourteen days is more effective in reducing CFUs of both pathogens than a fumigation time of seven days. These results may be due

to the fact that the fungus was applied in dehydrated barley formulation and required some time to reactivate mycelial growth and VOC production. The reduction of CFUs of both pathogens via VOCs was effective at the point source up to 9 cm away. In MSU mix and Amsterdam silt loam *M. albus* VOCs significantly reduced pathogen CFUs when compared to Lihen sandy loam. Organic matter or pH value of the soils had no apparent effect on these results since all soils had a similar composition in respect to these. A possible factor for the lower effectiveness of *M. albus* in the Lihen sandy loam might be the higher salinity and zinc levels in this substrate which were twice and three times, respectively, higher than in the MSU mix and Amsterdam silt loam (Appendix A). Colpaert and Van Assche (1992) showed that high zinc concentrations inhibit the growth of fungi in soil. The zinc level in Lihen sandy loam may have had little or no impact on the mycelial growth of *M. albus*, but may have no or just a reduced impact on resting structures of *V. dahliae* and *C. coccodes*.



Image 1.5: *Trichoderma* sp. colonizing *M. albus* barley formulation and soil around point source.

Recently new state and federal regulations proposed by EPA and the California Department of Pesticide Regulation addressed the reduction of pesticide VOC emissions and to ensure worker and bystander safety. These proposed regulations might limit and

restrict the use of chemical soil fumigants like methyl bromide and metam sodium and include the use of tarping and water seals to reduce emission which will result in higher costs for the growers.

If applied correctly, soil fumigation is highly effective in reducing populations of nematodes and other soil-borne fungal pathogens, in controlling weeds and insects, and thereby improving the quality of harvested tubers (Hamm et al., 2003). Soil fumigants such as Vapam or K-Pam are highly efficient, fast working soil fumigants used for the control of Verticillium wilt and black dot of potato. However, due to toxicity concerns, plant-back intervals need to be considered before potatoes can be planted. Other problematic issues of chemical fumigants are the potential for contaminating ground water, drift of sprinkler-applied products, destruction of beneficial soil microorganisms, and incompatibility with some biocontrol practices (Powelson and Rowe, 1993, 2008). Ibekwe et al., (2001) is emphasizing the importance of the microbial community composition for sustaining the health of natural and agricultural soils. Changes in microbial communities due to the phytotoxic nature of soil fumigants may have a negative effect on the overall soil health (Ibekwe, 2004) and the chemicals are also extremely toxic to workers. In comparison U.S. EPA (2005) classified the biological control agent *M. albus* and its VOCs as not harmful for honeybees, birds, other non-target insects, and plants, and is considered non-toxic to humans and therefore only requires minimal worker protection equipment.

In multiple studies, *M. albus* showed a distinct selectivity for soil-borne pathogens *in vitro* (Strobel et al., 2001; Grimme, 2004), and *in vivo* (Stinson et al., 2003;

Grimme, 2004, 2007) under greenhouse and field conditions (Grimme et al., 2007, 2008) without the detrimental effects on beneficial microorganisms like *Trichoderma* spp. (Image 1.5) and *Gliocladium* sp., or plants (Zidack et al., 2002; Stinson et al., 2003; Grimme et al., 2007). Due to the fact that there is no phytotoxic reaction, plant-back intervals to secure plant safety are not necessary. *M. albus* might be a powerful tool in potato production for the control of multiple fungal pathogens including *R. solani* (Jacobsen, *personal communication*), *V. dahliae*, and *C. coccodes*, bacterial pathogens like *Streptomyces scabies* (Jacobsen, *personal communication*) as well as nematodes like *P. penetrans* (Grimme et al. 2007), *Meloidogyne chitwoodi*, *M. hapla*, *Paratrichodorus allius* (Riga et al., 2008) at the same time, without interfering with other cultural practices.

Economic thresholds for *V. dahliae* CFUs/g soil have been established at 2-30 CFUs/g depending on the presence of the root lesion nematode (*P. penetrans*) (Powelson and Rowe, 1993). The results of the soil chamber fumigation experiments demonstrate that in the MSU mix and Amsterdam silt loam soils populations of *V. dahliae* were achieved below threshold at up to 9 cm from the point source of *M. albus* barley formulation. However this level was only achieved at 3 cm from point source of *M. albus* barley formulation in the Lihen sandy loam soil. Therefore, the barley formulation in the Lihen sandy loam soil would have to be uniformly distributed to achieve effective mycofumigation for Verticillium wilt control.

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CHAPTER 2

CONTROL OF *COLLETOTRICHUM COCCODES* AND *VERTICILLIUM DAHLIAE* ON POTATO WITH VOLATILE ORGANIC COMPOUNDS OF *MUSCODOR ALBUS*, EVALUATED BY SERIAL DILUTION PLATING AND REAL-TIME QUANTITATIVE PCR

Introduction

Verticillium wilt is a major disease of potato (*Solanum tuberosum* L.) and found throughout most of the major potato producing regions worldwide. It is caused by the fungus *Verticillium dahliae* Kleb., vegetative compatibility groups (VCG) Group 4A and 4B (Rowe and Powelson, 2002). Symptoms include uneven chlorosis, premature wilting of leaves and vascular browning in stems and tubers. Yellowing of leaves and death proceeds upward from the base of infected stems, which often remain erect (Arbogast et al., 1999; Powelson and Rowe, 2008). Early dying of potato plants results in yield reductions of up to 30% (Powelson and Rowe, 1993; Read and Hide, 1995; Ibekwe et al., 1999). The pathogen survives in soil as microsclerotia and in infected plant residues and it can also be spread by seed potatoes (Andrivon et al., 1998; Denner et al., 1998; Nitzan et al., 2008). Economic thresholds based on microsclerotia counts have been established and work well for table stock and processing potatoes. However, production of certified disease-free seed potatoes can be difficult particularly under low inoculum levels where symptoms are not easily seen.

Black dot of potato caused by the fungus *Colletotrichum coccodes* (Wallr.) Hughes, occurs world-wide with yield losses ranging from 12 – 32 % (Johnson et al., 1997; Tsror (Lahkim) et al., 1999; Lees and Hilton, 2002; Nitzan et al., 2005, 2006). Disease symptoms include yellowing and wilting of the foliage, below-ground rot of roots, stems and stolons, and premature death of plants (Read and Hide, 1995; Tsror (Lahkim), 2004). In senescent and dead plant tissue, *C. coccodes* produces small, black sclerotia internally and externally on stems, stolons, and tubers (Glais-Varlet et al., 2004; Tsror (Lahkim), 2004). On potato tubers the pathogen produces a brownish-gray blemish that reduces the value of the fresh market crop (Andrivon et al., 1998). This blemish is usually superficial, but extensive infection may cause the tubers to shrivel or to develop deep sunken lesions which also reduce the value for the fresh market (Tsror (Lahkim), 2004). Research has shown that this disease is both soil- and seed-borne, and is also disseminated by airborne inoculum (Nitzan et al., 2006 and 2008; Aqeel et al., 2008).

Verticillium dahliae and *C. coccodes* are both primary causal agents of potato early dying (PED) (Andrivon et al., 1997; Rowe and Powelson, 2002). This syndrome is characterized by a reduction in photosynthetic activity, reduced gas exchange of leaves, and in premature senescence of potato plants, affecting yield by shortening the tuber bulking period. Yield losses associated with PED may reach 50%, but are more commonly in the range of 10 to 15% (Rowe and Powelson, 2002). The severity of PED developing in a given crop is dependent on populations of *V. dahliae* and is also associated with other pathogens such as *Pratylenchus penetrans* and *C. coccodes* in the soil at planting (Tsror (Lahkim) and Hazanovsky, 2001; Rowe and Powelson, 2002).

Cultivar susceptibility and the favorability of environmental conditions for disease development also play important roles in determining both disease severity and yield losses. Management strategies for this disease complex are aimed at reducing the population density of these associated pathogens in the soil, altering the efficacy of these inocula, and changing the susceptibility of the host (Powelson and Rowe, 1993, 2008).

Potato early dying cannot be completely controlled by any single practice. Management strategies must emphasize integrated crop management systems, including, but not limited to, soil fumigation, cultural methods (crop rotation, green manures, fertilization, irrigation), host resistance and biological control (Powelson and Rowe, 1993, 2008).

At present the only practical control of Verticillium wilt in heavily infested soils is soil fumigation with metham sodium or metham potassium, growing of resistant cultivars, soil solarization, and incorporation of green manures (Arbogast et al., 1999; Berbegal et al., 2008; Powelson and Rowe, 2008). Available Verticillium resistant cultivars may not suit the market needs of all growers. Tsror (Lahkim) et al. (2005) showed that applications of 600 to 900 L/ha of metham sodium significantly reduced the disease severity of *V. dahliae* in stems and daughter tubers and is a widely used practice in the Columbia Basin of Oregon (Hamm et al., 2003).

Effective chemical control methods for *C. coccodes* are soil fumigation with metham sodium and metham potassium (Powelson and Rowe, 2008). Recent research shows promising results in controlling *C. coccodes* using azoxystrobin as in furrow treatment followed by chlorothalonil or mancozeb as foliar application when plants are

15-28 cm tall (Nitzan et al., 2005, Jacobsen *unpublished*). As a consequence of increased incidence and recognition of this disease, it has become a major focus of our research.

Recently, the US EPA and California Department of Pesticide Regulation (www.cdpr.ca.gov) proposed restrictions on the use of soil fumigants including metham sodium and metham potassium. These restrictions may make use of these fumigants impractical for many producers. Due to their biocidal nature, chemical soil fumigants, as well as soil sterilization methods including solarization, may have negative effects on the soil microbial diversity (Ibekwe et al., 1999, 2001; Ibekwe 2004). The need for more environmentally friendly alternatives to the use of chemical soil fumigants on potatoes makes soil fumigation using volatile organic compounds (VOC), emitted by the fungus *Muscodor albus* Worapong, Strobel & Hess, isolate CZ-620 a logical alternative (Stinson et al., 2003; Jacobsen et al., 2004; Grimme et al., 2008).

Anti-microbial VOCs can be emitted by decaying plant residues, mainly from plants belonging to the Brassica family, but also from microorganisms including fungi (Bjurman and Kristensson, 1992). Many fungal species are known to produce different concentrations of diverse VOCs (Bjurman and Kristensson, 1992). Dennis and Webster (1971) reported that species of *Trichoderma* emit VOCs that inhibit growth of other fungi. Most recently, *M. albus* has been described as an endophytic fungus isolated from *Cinnamomum zeylanicum* in Honduras (Worapong et al., 2001) that produces VOCs, which effectively inhibit or kill microorganisms including soil-borne plant pathogens like *V. dahliae* and *C. coccodes* (Strobel, et al., 2001; Stinson et al., 2003; Jacobsen et al., 2004; Grimme et al., 2008). The antimicrobial use of *Muscodor* species and their volatile

compounds is termed “mycofumigation” (Stinson et al., 2003; Grimme, 2004). The chemical composition of these VOCs and the beneficial biological properties of *Muscodor* sp. have been described previously *in vitro* (Strobel et al., 2001; Ezra and Strobel, 2003) and *in vivo* (Stinson et al., 2003, Grimme et al., 2007 and 2008). The efficacy of these VOCs to control soil-borne pathogens prompted this study.

Real-time quantitative polymerase chain reaction (QPCR) is an effective method for the rapid identification and quantification of a wide range of plant-pathogenic microorganisms including fungi, bacteria, and viruses from infected host tissue (Sayler et al., 2007; Schaad and Frederick, 2002; Schena et al., 2004). Real-time quantitative PCR is faster than conventional PCR and is considered to be more sensitive and specific than PCR. These factors have prompted its use by researchers (Cullen et al., 2002; Dauch et al., 2006) for quantification of plant pathogens in plant tissues and soils through DNA quantification (Vandermark and Barker, 2003; Alkan et al., 2004; Schena et al., 2004).

The objectives of this research were: (1) to evaluate the effects of VOC, emitted by the fungus *M. albus* isolate CZ-620, on Verticillium wilt (*V. dahliae*) and black dot root rot (*C. coccodes*) on potato under greenhouse and field conditions; (2) Development of real-time quantitative polymerase chain reaction (QPCR) assays to rapidly detect and quantify the amount of *V. dahliae* and *C. coccodes* genomic DNA in infected potato stems in order to measure the effects of *M. albus* VOCs on the amount of fungal pathogens in host tissue. The correlation between colony forming units (CFUs) assays and culture-independent QPCR (Li et al., 2008) was also analyzed.

Materials and Methods

Greenhouse Bioassays for Testing the Efficacy of *M. albus* for Control of *C. coccodes* and *V. dahliae* on Potato

Preparation of *M. albus* Isolate CZ-620 Inoculum: *Muscodor albus*, isolate CZ-620, was grown and maintained in 10 cm Petri plates on potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) at 23°C ± 1°C for three weeks. Inoculum was produced by transferring 15 agar plugs (5 mm diameter) from 3-week-old cultures into 2-liter flasks containing 1 liter autoclaved potato dextrose broth (PDB, EMD Chemicals Inc., Darmstadt, Germany), and placing the flasks on a rotary shaker (Model OS-500, VWR International, West Chester, PA) (110 rpm) for 2 weeks at 23 °C ± 1 °C. The *M. albus* barley formulation was prepared by adding 100 ml of colonized PDB to mushroom cultivation bags (Polypropylene bags with one filter, Unicorn Imp. & Mfg. Corp., Garland, TX 75042) containing 500 g of sterilized barley (*Hordeum vulgare* L). After growth on the substrate for three weeks at 23°C ± 1°C, the colonized seeds were dried for 48 h in a laminar flow-hood and then stored in plastic bags at 4 °C.

Preparation of Plant-Pathogen Inocula: A culture of *V. dahliae*, isolate 502, was obtained from the MSU fungal culture collection, courtesy of Dr. Alan T. Dyer. *C. coccodes* was isolated from infected potato plants, grown in Gallatin County, Montana.

Colletotrichum coccodes and *V. dahliae* were grown and maintained in Petri dishes with PDA at 23 ˚C ± 1 ˚C for two weeks. Ten 5 mm agar plugs of the *Verticillium* or *Colletotrichum* cultures were transferred to 1-liter flasks containing 0.5 liter

autoclaved PDB, and placing the flasks on a rotary shaker (110 rpm) for 2 weeks at 23 °C ± 1 °C. Sixty milliliters of the liquid cultures were added into Mason jars containing 300 g of sterilized barley for inoculum production and incubated for three weeks at 23 ˚C ± 1 ˚C. The colonized barley was dried as described above and ground in a Waring blender (Waring Inc., Santa Monica, CA) to produce a homogenous inoculum formulation. Inoculum concentrations were determined using serial dilution plating and resulted in 1 x 10^7 CFUs/g for *V. dahliae* and 2 x 10^4 CFUs/g for *C. coccodes*.

Plant Materials and Experimental Design: The potato (*S. tuberosum*) cultivars used in this study were 'Alturas' and 'Russet Norkotah'. 'Alturas' is susceptible to *C. coccodes* (Meyer, 2007) and 'Russet Norkotah' is susceptible to *V. dahliae* (Bae et al., 2007). Disease-free, certified potato plantlets were obtained from the MSU Potato Laboratory, Bozeman, Montana.

Greenhouse bioassays were designed to measure the efficacy of the VOC produced by *M. albus* in barley formulation for the control of *C. coccodes* and *V. dahliae* on potato. All experiments were carried out in a greenhouse with 24 ± 2 °C day and 18 ± 2 °C night temperatures and with a 16 h photoperiod. Supplemental lighting was provided by SON AGRO 430 WATT HPS lights (Philips Lighting Company, Somerset, NJ). The growing substrate (1/3 Bozeman silt loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent) used for all experiments was sieved (2 mm mesh opening) and steam sterilized for 2 h. Soil was artificially infested with either *V. dahliae* or *C. coccodes* at concentrations of 10 CFUs and 7 CFUs per gram soil respectively. *Muscodor albus* barley formulation

was mixed at 2 different rates (rate 1: 6.7 g/kg soil; rate 2: 13.4 g/kg soil) with the infested soil, and 375 g of soil was filled into round 15 cm plastic pots. In previous experiments sterile barley alone had no pathogen controlling effect (Grimme et al., 2007) and is therefore not included as a control treatment in the present study. The *M. albus* fumigation treatments and the control treatments were incubated in the dark for one week, either non-covered or covered with plastic mulch. After the fumigation period, tissue culture derived 14-day-old potato plantlets of the cultivar 'Russet Norkotah' or 'Alturas' were planted either into *V. dahliae* or *C. coccodes* infested soil, which was treated and fumigated as described above. Plantlets were also planted into non-infested control soil and into soil infested with either *C. coccodes* or *V. dahliae* as pathogen controls. The fumigated treatments were compared to directly prepared soil treatments with both rates of *M. albus* barley formulation and potato plantlets were transplanted into treated soil without the fumigation period.

The greenhouse experiments were arranged in randomized complete designs with 10 replications per treatment. Experiments with both pathogens were repeated two times.

Evaluation of Disease Symptoms and Growth Reduction: Disease severity was monitored during the growing period and foliar disease symptoms were assessed 30 days and 12 weeks after planting. Percentages of chlorosis and necrosis were determined and the area under the disease progress curve (AUDPC) was calculated with the following formula: $AUDPC = \sum[(y_i + y_{i+1})/2 \times (t_{i+1} - t_i)$ where y_i = initial severity of symptoms and t_i = initial time of symptom appearance (Bjarko and Line, 1988; Nitzan et al., 2008). Fresh and dry weights were taken 12 weeks after planting.

Quantification of Fungal Pathogens in
Potato Plants Using Real-Time Quantitative PCR

DNA Extraction and Sequencing: *C. coccodes* and *V. dahliae* were cultured on PDA for three weeks at room temperature until the majority of the Petri plate was covered with mycelium. DNA was extracted using the FastDNA Kit (Qbiogene Inc., Carlsbad, CA) and FastPrep FP120 homogenizer (Qbiogene Inc.) following the instructions provided by the manufacturer (Schroeder et al., 2006). DNA concentrations were quantified using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) with absorbance measurements at 260 and 280 nm. The internal transcribe spacer (ITS) regions of both fungi, as well as their 5.8S genes of the ribosomal DNA (rDNA), were amplified using PCR and ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. PCR reactions consisted of 65 to 120 ng of fungal DNA, 1.5 µl (10 pmol) of each primer and 12.5 µl 1X GoTaq® Colorless Master Mix (Promega, Madison, WI) in a total reaction volume of 25 µl. PCR was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories Ltd, Hemel Hempsted, United Kingdom) with the following program settings: initial denaturation 4 min at 95 ºC, 60 s denaturation at 95 ºC, 45 s annealing at 52 ºC, extension 2 min at 72 ºC for 30 cycles, final extension 5 min at 72 ºC, 4 ºC hold temperature. Amplicons for DNA sequencing were separated from excess nucleotides and salts using electrophoresis on a 1.2 % agarose gel or the QIAquick PCR purification kit (Qiagen Sciences Inc., Germantown, Maryland) (Okubara et al., 2008). Purified DNA samples were prepared for sequencing according to the specifications provided by the

sequencing facility and sent to the University of California at Berkeley DNA sequencing facility (Berkeley, CA).

QPCR Primer Design: Based on the internal transcribed spacer regions (ITS regions) of the fungal ribosomal DNA (rDNA), specific primers for each pathogen were designed for real-time quantitative PCR (QPCR) using the Invitrogen Oligo perfect software with the following design criteria: (i) minimum oligonucleotide length of 16 bases, (ii) melting temperatures of 58 to 60 ºC, (iii) primer % GC content of 40 to 55, and (iv) product size of 75 to 150 bp. The following specific primer pairs were designed: *C. coccodes* forward primer CcF1 5'-CGC AGC GAA ATG CGA TAA GTA ATG TG-3' and reverse primer CcR1 5'-GAG CTT GAG GGT TGA AAT GAC GCT-3'; *V. dahliae* forward primer VdF1 5'-CAG CGA AAC GCG ATA TGT AGT GTG AA-3' and reverse primer VdR1 5'-TTGAAACGACGCTCGGACAGG-3'. The specificity of each primer pair was confirmed by using the Basic Local Alignment Search Tool (BLAST) of the GenBank database (National Resource for Molecular Biology Information). Primer sequences were verified to have low homodimer and heterodimer potential as well as a low probability of forming a hairpin loop, using the OligoAnalyzer 3.0 web software (IDT, Coralville, IA). All primers were synthesized by IDT-DNA Technologies. The specific primer pairs amplify a fragment of 128 bp for *C. coccodes* and a fragment of 130 bp for *V. dahliae*.

Primer specificity was tested using culture plate DNA of each fungal pathogen. QPCR amplifications were conducted in triplicate using the Rotor Gene 3000 (Corbett Life Science) and SYBR Green I (Qiagen) as the fluorescent reporter dye. QPCR

reactions consisted of 1 µl (100 ng) of total sample DNA, 1.5 µl (5 µM) of each primer, 12.5 µl 2X QuantiTect SYBR Green PCR Master Mix (Qiagen), and 8.5 µl molecular grade H_2O in a total reaction volume of 25 µl. The following amplification protocol was used: initial denaturation for 10 min at 95 ºC followed by 40 cycles of 15 s at 95 ºC, 20 s at 58 ºC and 20 s at 72 ºC. Melt curve analysis was used to distinguish potential non-specific amplification products (Atallah et al., 2007).

Extraction of Fungal Pathogen DNA from Potato Plants: Stems of 12-weeks-old potato plants grown under conditions described above were rinsed in sterile-distilled water and dried for four days at 65 ºC. The lower 2 cm of the stems were ground with a Waring blender and stored at 4 ºC. DNA was extracted from 150 mg dried stem material with the FastDNA Kit (Qbiogene Inc., Carlsbad, CA) as described by the manufacturer. Extracted samples contained DNA of fungal pathogen and genomic plant DNA and were stored at – 20 ºC.

Real-Time Quantitative PCR Amplification: QPCR amplifications were carried out in a total volume of 25 µl using the Rotor Gene 3000 and SYBR Green I (Qiagen) as the fluorescent reporter dye. QPCR reactions consisted of 1 µl (100 ng) of total sample DNA, 1.5 µl (5 µM) of each primer, 12.5 µl 2X QuantiTect SYBR Green PCR Master Mix (Qiagen), and 8.5 µl molecular grade H_2O in a total reaction volume of 25 µl. Control reactions consisted of the same mixture of reagents and 1 µl of molecular grade H_2O as replacement for the DNA template. Thermal cycling conditions consisted of 10 min at 95 ºC followed by 40 cycles of 15 s at 95 ºC, 20 s at 58 ºC and 20 s at 72 ºC.

Every DNA sample was analyzed with duplicate QPCR reactions and each QPCR run was repeated twice on different days (two machine replicates). Absolute QPCR was employed to determine the quantities of *C. coccodes* and *V. dahliae* in stem tissues of potato plants. A 10-fold dilution series of DNA of both pathogens from pure cultures were analyzed in each absolute QPCR assay in triplicates to generate a standard curve (Filion et al., 2003). The dilution series of *V. dahliae* was set up with the following DNA concentrations in ng μl^{-1}: 75 x 10^{0}, 75 x 10^{-1}, 75 x 10^{-2}, 75 x 10^{-3}, 75 x 10^{-4}, 75 x 10^{-5}, 75 x 10^{-6}, 75 x 10^{-7}, and 75 x 10^{-8}. The concentrations for the *C. coccodes* dilutions in ng μl^{-1}: were: 25 x 10^{0}, 25 x 10^{-1}, 25 x 10^{-2}, 25 x 10^{-3}, 25 x 10^{-4}, 25 x 10^{-5}, 25 x 10^{-6}, 25 x 10^{-7}, and 25 x 10^{-8}. Threshold cycle (C_t) values of the samples and DNA of dilution series for both pathogens were calculated using the Rotor Gene 6000 software. Absolute quantification of fungal DNA was achieved by comparison of the unknown samples containing either *V. dahliae* or *C. coccodes* to the respective standard curve in with the threshold cycle (C_t) values were plotted against starting fungal DNA quantities. An analysis of the amplicon dissociation temperature was generated to verify the *C. coccodes* and *V. dahliae* primer specificity.

Quantification of Fungal Pathogens in
Potato Plants using Serial Dilution Plating

Stems of 12-weeks-old potato plants grown under conditions described above were rinsed in sterile distilled water and dried for four days at 65 ºC. The lower 2 cm of the stems were ground with a Waring blender and stored at 4 ºC. One gram per replicate of the ground material was diluted 10, 100, and 1,000 fold. Colony forming units (CFUs)

of *C. coccodes* and *V. dahliae* per gram of plant material were determined by spreading, 100 µl of each dilution evenly with a sterile glass rod on polygalacturonic acid selective media (NP-10 medium) described by Sorensen et al. (1991) for *V. dahliae* and Farley's medium described by Farley (1976) for *C. coccodes*. All plates were incubated at 22 ºC for one week. Colonies of *C. coccodes* and *V. dahliae* were counted and CFUs/g stem tissue determined. Effects of *M. albus* treatments on the amount of fungal DNA in potato plants determined with serial dilution plating were compared to the data of the real-time quantitative PCR analysis.

Field Experiments for Testing the Efficacy of *M. albus* for Control of *C. coccodes* and *V. dahliae* on Potato

Plant Materials, Fungal Inocula, and Experimental Design: Field experiments were performed at the Horticulture Farm, Montana State University, Bozeman, where high populations of the pathogens *V. dahliae* and *C. coccodes* occur naturally. *Muscodor albus* in dried barley formulation was prepared as described above. *Muscodor albus* barley formulation was applied as seed treatment and/or in furrow application at the time of planting of seed potatoes. Sterile ground barley was also applied as seed and/or in furrow as control treatment. Seed pieces of the potato cultivar 'Red Lasoda' were pre-moistened with sterile distilled water in plastic bags. *M. albus* barley formulation or the sterile barley treatment were added as a seed treatment at a rate of 15 kg/t to the potatoes in the bags and mixed throughout with the seed pieces. The in furrow treatments consisted of 224 kg/ha or 448 kg/ha of *M. albus* formulation or sterile barley and were added to the planting rows prior to planting of the potato seeds. Twenty seed pieces were

planted 0.3 m apart with a two-row planter into 6 m long plots with a 1.5 m alley in between plots. In October 2004 and 2006, the final stand was evaluated and potatoes were harvested using a one-row harvester (Grimme Landmaschinenfabrik GmbH & Co. KG, Damme, Germany).

Stem colonization of potatoes by *V. dahliae* and *C. coccodes* was evaluated after harvest. Ten potato stems per replication for all treatments were surface sterilized with a 20% Clorox solution for 15 s, rinsed with sterile distilled water, cut with a sterile scalpel into 1-2 mm thick discs, placed onto water agar (EMD) and incubated at 22 ˚C for 5-6 days. Presence of both pathogens was determined by observing the fruiting structures of *V. dahliae* and *C. coccodes* using a dissecting microscope. Percentage of potato stem colonized by *V. dahliae* or *C. coccodes* was calculated.

Statistical Analysis

The greenhouse experiments were arranged in randomized complete designs with 10 replications per treatment. Experiments with both pathogens were repeated two times. Data were analyzed for homogeneity over repeated experiments using Levene's test. Statistical analysis of the data was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at $\alpha = 0.05$. The PROC CORR analysis of SAS was used to compute Pearson's correlation coefficients between the means of treatments from QPCR and CFU values.

The field experiments were arranged in randomized complete designs with 10 replications per treatment. Experiments were repeated in 2004 and 2006. Data were analyzed for homogeneity over years using Levene's test. Statistical analysis of the data was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary NC). The treatment means were separated using Fisher's protected least significant difference test at $\alpha = 0.05$.

Results

Effects of *M. albus* Treatments on Disease Severity and Plant Growth Reduction Caused by *V. dahliae* Infection under Greenhouse Conditions

Levene's test of homogeneity determined that there were no significant differences between the two replicate greenhouse experiments; therefore the pooled data of the two greenhouse assays are presented.

Muscodor albus treatments significantly reduced the area under the disease progress curve (AUDPC) of Verticillium wilt ($P = 0.0001$) on potato. *Muscodor albus* barley formulation at the higher rate of 13.4 g/kg soil provided a significantly more effective disease control as measured by AUDPC than the 6.7 g/kg treatments (Table 2.1). Plants grown in soil with 13.4 g/kg soil, non-covered fumigation, resulted in AUDPC equal to the non-infested control. There was no significant effect of the fumigation method within the treatments of the lower (6.7 g/kg soil) or the higher rate (13.4 g/kg soil) of *M. albus* barley formulation at (Table 2.1).

Potato plants treated with *M. albus* formulation resulted in higher fresh weights than plants in the pathogen control. Treatments with *M. albus* at 6.4 g/kg soil, directly planted, and at the rate of 13.4 g/kg soil, covered fumigation, resulted in significantly (P = 0.0001) higher fresh weights relative to the Verticillium control (Table 2.1).

Table 2.1: Effects of *Muscodor albus* barley formulation on the AUDPC, fresh and dry weights of the susceptible potato cultivar 'Russet Norkotah' grown for 12 weeks in *Verticillium dahliae* [x] infested soil.

Treatment		AUDPC [y]	Fresh weight (g)	Dry weight (g)
Non-infested control		7.70 f [z]	26.46 a	3.58 a
V. dahliae control		73.50 a	7.95 c	2.15 bc
6.7g *M. albus*/ kg soil	direct planting	33.37 bcd	19.85 ab	2.85 ab
	covered fumigation	38.47 b	10.21 c	2.22 bc
	non-covered fumigation	37.60 bc	8.86 c	1.71 c
13.4g *M. albus*/ kg soil	direct planting	25.12 de	12.85 bc	2.23 bc
	covered fumigation	26.17 cde	18.38 b	2.52 bc
	non-covered fumigation	17.95 ef	12.82 bc	1.75 c

[x] Soil was infested with *Verticillium dahliae* at 10 CFUs/g soil.
[y] Area under the disease progress curve (AUDPC) was calculated for two ratings.
[z] Means followed by the same letter are not significantly different based on Fisher's protected LSD at P = 0.05.

The fumigation method influenced the fresh weight of potato plants in the treatments with *M. albus* at 6.7 g/kg soil. Direct planting in pathogen-infested and *M. albus* treated soil resulted in significantly higher fresh weights relative to plants in the covered and

non-covered fumigation treatments. There was no significant effect of fumigation method within the treatments of the higher rate of *M. albus* barley formulation at 13.4 g/kg soil (Table 2.1). Dry weights of plants fumigated with *M. albus* barley formulation were similar to weights of the pathogen control.

Effects of *M. albus* Treatments on Disease Severity and Plant Growth Reduction Caused by *C. coccodes* Infection under Greenhouse Conditions

Colletotrichum coccodes inoculum grown on barley kernels proved to be a very effective method for inoculating potato plants. The inoculated potato plants of the pathogen control showed severe stunting, chlorosis, and necrosis (Image 2.1).

Levene's test of homogeneity determined that there were no significant differences between the sets of greenhouse bioassays; therefore combined data of two greenhouse assays are presented.

All *M. albus* treatments significantly reduced the disease severity of black dot on potato as measured by AUDPC ($P = 0.0001$) (Table 2.2). *Muscodor albus* applied at the higher rate of 13.4 g/kg soil provided

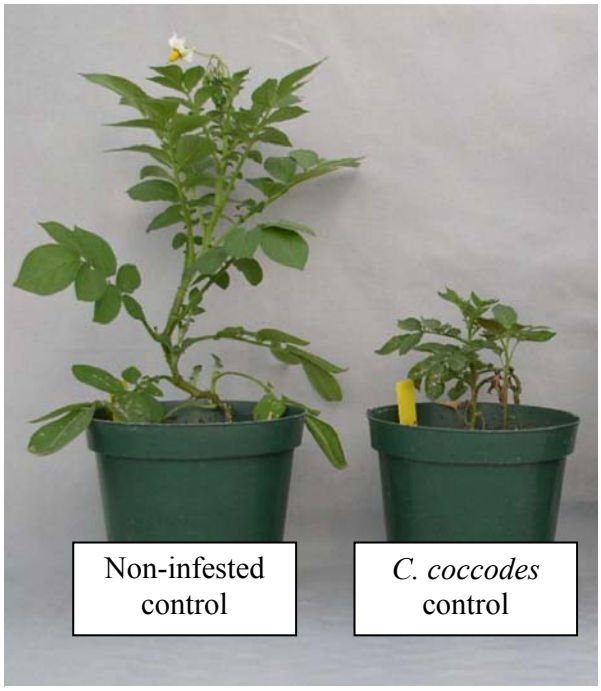


Image 2.1: Effect of *Colletotrichum coccodes* infection on potato plants of the cultivar 'Alturas' under controlled greenhouse conditions.

a significantly lower AUDPC than the 6.7g/kg treatment. (Table 2.2; Image 2.2). Only *M. albus*, 6.7 g/kg soil, non-covered fumigation, resulted in AUDPC equal to the covered fumigation treatment with 13.4 g/kg soil. There was no significant effect of the fumigation method on the AUDPC within the treatments of the lower (6.7 g/kg soil) or the higher rate (13.4 g/kg soil) of *M. albus* barley formulation (Table 2.2).

Potato plants treated with *M. albus* formulation resulted in higher fresh weights than plants in the pathogen control (Table 2.2). All plants of the *M. albus* treatments, except for the treatment 6.7 g/kg soil, direct planting, resulted in significantly higher fresh weights relative to the *C. coccodes* control ($P = 0.0001$). Treatments with *M. albus* at the rate 13.4 g/kg soil resulted in fresh weights equal to the non-infested control ($P = 0.0001$). The plants treated with 13.4 g/kg of *M. albus* barley formulation yielded significantly higher fresh weights ($P = 0.0001$) relative to the lower *M. albus* rate of 6.7 g/kg soil in the direct planting and covered fumigation treatments. The treatment of *M. albus* at 6.7 g/kg soil was statistically equal to the treatments at the 13.4 g/kg. The fumigation method influenced the fresh weight of potato plants in the treatments with *M. albus* at 6.7 g/kg soil. Direct planting in pathogen-infested and with *M. albus* treated soil in these treatments resulted in significantly lower fresh weights relative to plants in the non-covered fumigation treatments. There was no significant effect of the fumigation method within the treatments of the higher rate of *M. albus* barley formulation at 13.4 g/kg soil (Table 2.2).

Plants treated with *M. albus* resulted in significantly ($P = 0.0001$) higher dry weights than plants of the pathogen control. Dry weights were significantly higher in

treatments at the *M. albus* rate of 13.4 g/kg soil when compared to the 6.7g/kg rate of *M. albus*. Non-covered fumigation at 6.7 g/kg soil resulted in significantly higher dry weights than direct planted plantlets. There was no significant difference of the fumigation method between 6.7 g/kg soil, covered and non-covered fumigation and within *M. albus* treatments at the higher rate of 13.4 g/kg soil (Table 2.2).

Table 2.2: Effects of *Muscodor albus* barley formulation on the AUDPC, fresh and dry weights of the susceptible potato cultivar 'Alturas' grown for 12 weeks in *Colletotrichum coccodes* [x] infested soil.

Treatment		**AUDPC** [y]	**Fresh weight (g)**	**Dry weight (g)**
Non-infested control		1.71 e [z]	31.22 ab [z]	4.24 ab
C. coccodes control		30.00 a	9.20 d	1.34 d
6.7g *M. albus*/ kg soil	direct planting	20.57 b	17.80 cd	2.77 c
	covered fumigation	18.00 b	24.40 bc	3.51 bc
	non-covered fumigation	16.28 bc	34.59 ab	4.67 ab
13.4g *M. albus*/ kg soil	direct planting	10.29 d	30.14 ab	4.28 ab
	covered fumigation	12.86 cd	37.45 a	5.20 a
	non-covered fumigation	11.14 d	35.18 a	4.51 ab

[x] Soil was infested with *Colletotrichum coccodes* at 7 CFUs/g soil.
[y] Area under the disease progress curve (AUDPC) was calculated for two ratings.
[z] Means followed by the same letter are not significantly different based on Fisher's protected LSD at $P = 0.05$.

Quantification of *V. dahliae* and
C. coccodes in Potato Plants by Serial
Dilution Plating and Real-Time Quantitative PCR

Quantification of *V. dahliae* by Serial Dilution Plating: Levene's test of homogeneity determined that there were no significant differences between the sets of greenhouse bioassays and combined data are presented. Effect of *M. albus* treatments on the amount of *V. dahliae* in potato stems was determined by serial dilution plating. All *M. albus* treatments with the higher rate of barley formulation at 13.4 g/kg soil showed significantly lower CFUs/g potato stem tissue ($P = 0.0001$) relative to the Verticillium control and were equal to the non-infested control (Table 2.3). The *M. albus* treatment with 6.7 g/kg soil, direct planted, resulted in CFUs equal to the non-infested control, whereas the treatments at the same rate but covered and non-covered fumigation showed CFUs similar to the pathogen control. There was no significant difference in CFUs within the treatments at 6.7g /kg soil and 13.4 g/kg soil (Table 2.3).

Table 2.3: Effect of soil fumigation with *Muscodor albus* barley formulation and quantification of *Verticillium dahliae* [y] in potato stems 12 weeks after inoculation of the susceptible cultivar 'Russet Norkotah' by colony forming units (CFUs) and real-time quantitative PCR reaction from 100 ng of total DNA extracted from potato stems.

Treatment	**CFUs/g stem tissue**	**ng *V. dahliae*/100 ng total DNA**
Non-infested control	0.0 c [z]	0 c
Pathogen control	28.6 x 10^3 a	71.84 a
6.7 g *M. albus*, direct planting	8.8 x 10^3 bc	1.83 b
6.7 g *M. albus*, covered fumigation	17.1 x 10^3 ab	20.88 b
6.7 g *M. albus*, non-covered fumigation	20.9 x 10^3 ab	17.02 b
13.4 g *M. albus*, direct planting	12.4 x 10^3 bc	0.67 b
13.4 g *M. albus*, covered fumigation	10.9 x 10^3 bc	0.3 b
13.4 g *M. albus*, non-covered fumigation	6.7 x 10^3 bc	16.64 b

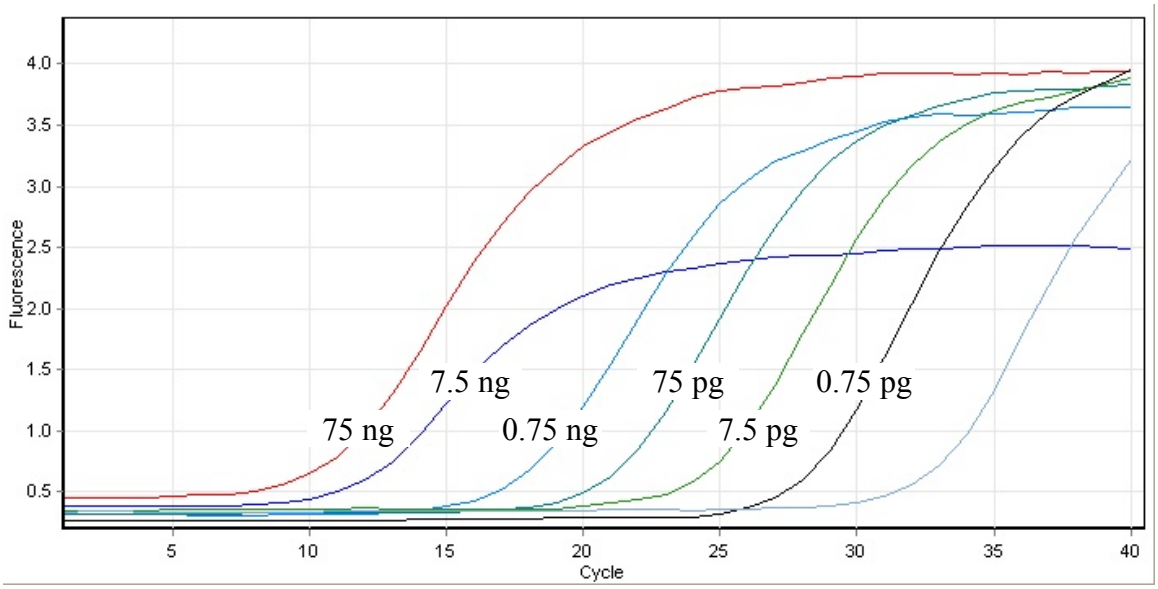
[y] Soil was artificially infested with *Verticillium dahliae* at 10 CFUs/g soil.
[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

Quantification of *V. dahliae* with Real-Time Quantitative PCR: The sensitivity of the real-time quantitative PCR assay (QPCR) was determined by 10-fold dilutions from 75 ng/µl to 0.75 pg/µl of *V. dahliae* DNA. The analysis of known concentrations of DNA showed that amounts as low as 0.75 pg/µl could be detected by the developed QPCR assay (Figure 2.1 a). Quantification showed a negative linear relationship between the threshold cycle (C_t) value and the diluted pathogen DNA ($R^2 = 0.9828$) (Figure 2.1 b).

Real-time quantitative PCR showed that all treatments with *M. albus* barley formulation significantly reduced the amount of *V. dahliae* DNA in potato stems of the cultivar 'Russet Norkotah' ($P = 0.0001$) relative to the pathogen control (Table 2.3). Treatments with the higher rate at 13.4 g/kg soil of *M. albus* barley formulation resulted in reduced pathogen DNA concentrations than the same treatments with the lower rate of 6.7 g/kg soil. Only the treatment with 6.7 g/kg soil, direct planting, resulted in lower DNA concentrations than the treatments with the same rate but covered and non-covered fumigation method. No significant differences in pathogen DNA concentration were calculated within the *M. albus* treatments (Table 2.3). No DNA was amplified in the non-template controls.

Correlation analysis between treatment means of QPCR and CFU data showed that QPCR results are significantly ($P = 0.0094$) correlated with the CFU values with a correlation coefficient of 0.838.

A. *Verticillium dahliae* standard curve



B.

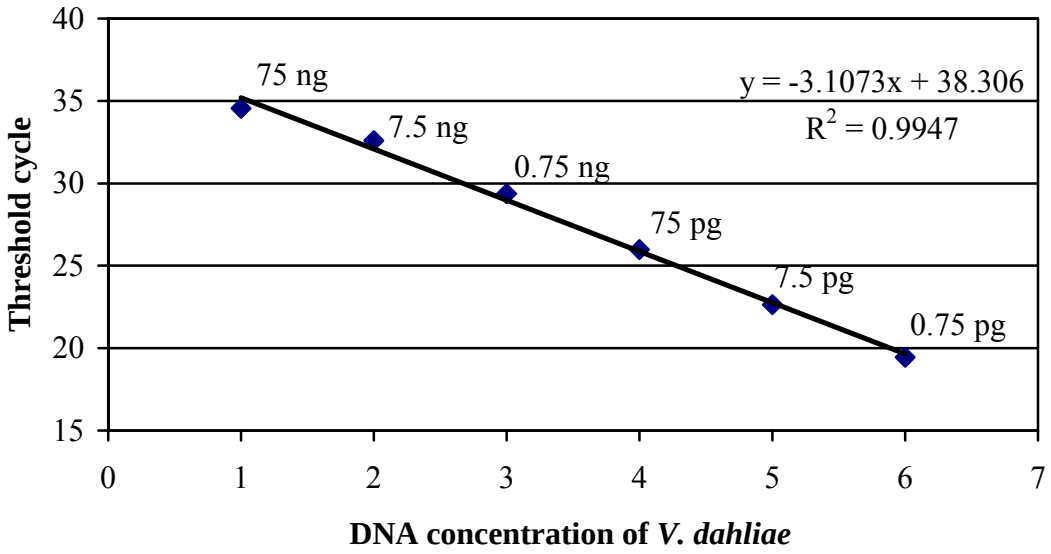


Figure 2.1: *Verticillium dahliae* DNA dilutions measured by real-time quantitative PCR cycle threshold (C_t) values. **A.** Representative real-time PCR fluorescence curves for *V. dahliae* DNA concentrations from left to right are 75 ng, 7.5 ng, 0.75 ng, 75 pg, 7.5 pg, and 0.75 pg. **B.** Correlation of C_t value and DNA concentrations of *V. dahliae*.

Quantification of *C. coccodes* by Serial Dilution Plating: Levene's test of homogeneity determined that there were no significant differences between the sets of greenhouse experiments, therefore combined data of the two experiments are presented.

All *M. albus* treatments significantly lowered the CFUs/g potato stem tissue (P = 0.0001) relative to the *Colletotrichum* control (Table 2.4). *M. albus* treatments with 6.7 g/kg soil, direct planted, and treatments with 13.4 g/kg, direct planted and covered fumigation, resulted in CFUs equal to the non-infested control (P = 0.0001). There was no significant difference in CFUs within the treatments at 6.7g /kg soil and 13.4 g/kg soil (Table 2.4).

Table 2.4: : Effect of soil fumigation with *Muscodor albus* barley formulation and quantification of *Colletotrichum coccodes* [y] in potato stems 12 weeks after inoculation of the susceptible cultivar 'Alturas' by colony forming units (CFUs) and real-time quantitative PCR reaction from 100 ng of total DNA extracted from potato stems.

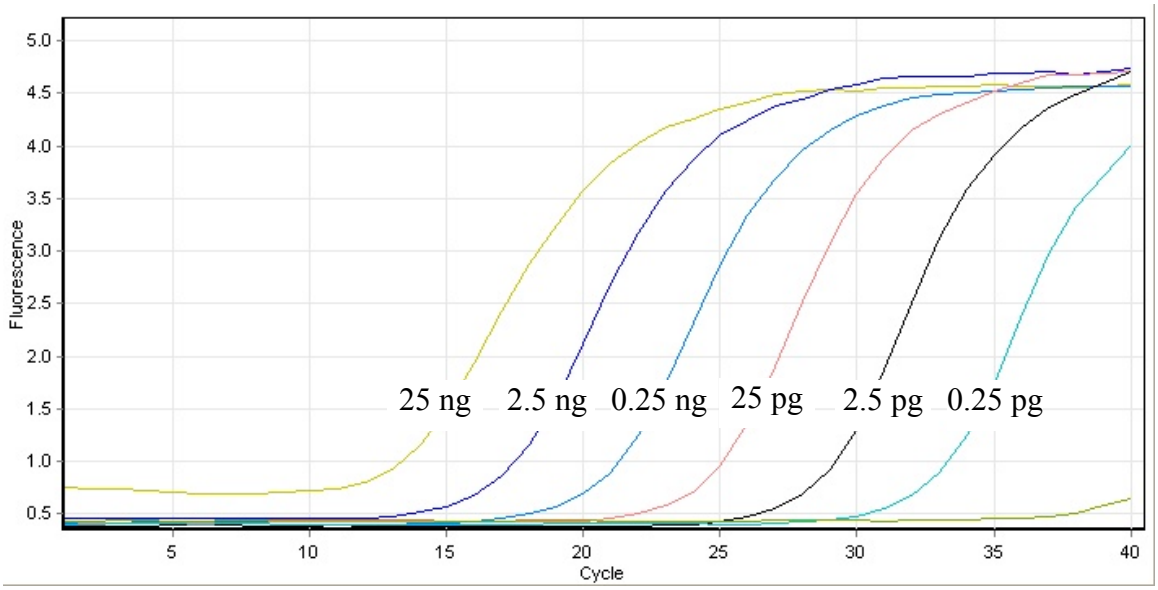
Treatment	**CFUs/g stem tissue**	**ng *C. coccodes*/100 ng total DNA**
Non-infested control	0.0 c [z]	0 c
C. coccodes control	480.0 a	0.0606 a
6.7 g *M. albus*, direct planting	120.0 bc	0.0035 b
6.7 g *M. albus*, covered fumigation	100.0 b	0.0119 b
6.7 g *M. albus*, non-covered fumigation	140.0 b	0.0119 b
13.4 g *M. albus*, direct planting	120.0 bc	0.0027 b
13.4 g *M. albus*, covered fumigation	80.0 bc	0.0033 b
13.4 g *M. albus*, non-covered fumigation	120.0 b	0.0127 b

[y] Soil was artificially infested with *Colletotrichum coccodes* at 7 CFUs/g soil.
[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

Quantification of *C. coccodes* with Real-Time Quantitative PCR: A series of 10-fold dilutions from 25 ng/µl to 0.25 pg/µl of *C. coccodes* was prepared to determine the sensitivity of the real-time quantitative PCR assay (QPCR). The analysis of known concentrations of DNA showed that amounts as low as 0.25 pg/µl could be detected by the developed QPCR assay (Figure 2.2 a). Quantification showed a negative linear relationship between the threshold cycle (C_t) value and the diluted *C. coccodes* DNA (R^2 = 0.9979) (Figure 2.2 b).

Real-time quantitative PCR showed that all treatments with *M. albus* barley formulation significantly reduced the amount of *C. coccodes* DNA in potato stems of the cultivar 'Alturas' (P = 0.0001) relative to the pathogen control (Table 2.4). Treatments with *M. albus* barley formulation at a rate of 13.4 g/kg soil, direct planting and covered fumigation, resulted in the lowest DNA concentrations. Direct planting at a rate of 6.7 g/kg soil resulted in the lowest DNA concentration of the low-rate treatments and was equal to the best treatments at the higher rate. There were no significant differences in pathogen DNA concentration within the *M. albus* treatments (Table 2.4).
Correlation analysis between treatment means of QPCR and CFU results showed that QPCR results are significantly (P = 0.0025) correlated with the CFU values with a correlation coefficient of 0.897.

A. *Colletotrichum coccodes* standard curve



B.

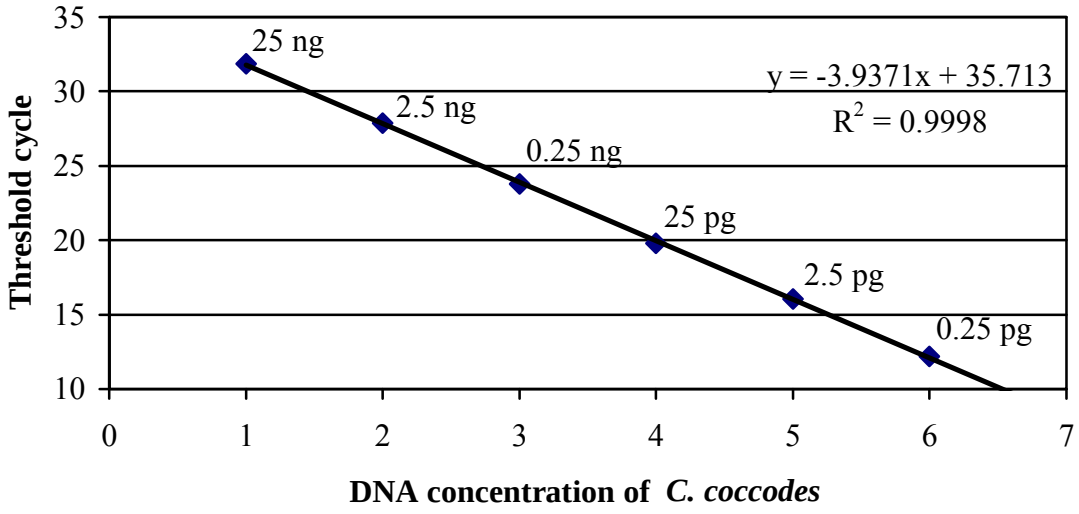


Figure 2.2: *Colletotrichum coccodes* DNA dilutions measured by real-time quantitative PCR cycle threshold (C_t) value. **A.** Representative real-time PCR fluorescence curves for *C. coccodes* DNA concentrations from left to right are 25 ng, 2.5 ng, 0.25 ng, 25 pg, 2.5 pg, 0.25 pg, and a non-template control (NTC). **B.** Correlation of C_t value and DNA concentrations of *C. coccodes*.

Field Experiments for Testing
the Efficacy of *M. albus* for Control
of *C. coccodes* and *V. dahliae* on Potato

Soil fumigation and potato seed treatments with *M. albus* barley formulation were evaluated for the control of *V. dahliae* and *C. coccodes* under natural conditions. Colonization of potato stems by both fungal pathogens was used as an indicator for the effectiveness of the *M. albus* treatments. The *M. albus* seed treatment at a rate of 15 kg/t potatoes was the most effective treatment of controlling *V. dahliae* ($P = 0.0121$) and *C. coccodes* ($P = 0.0001$) and resulted in significantly lower percentage of stem colonization relative to the pathogen controls (Table 2.5). In furrow applications of *M. albus* at a rate of 448 kg/ha also resulted in significantly lower stem colonization of both fungal pathogens and equaled the *M. albus* seed treatment. The lower in furrow application of *M. albus* with 224 kg/ha significantly reduced the stem colonization by *C. coccodes*, whereas colonization by *V. dahliae* was not significantly reduced when compared to the untreated control (Table 2.5).

Only *M. albus* applied as a seed treatment was able to increase the final stand significantly (P=0.05) when compared to the untreated control. *Muscodor albus* applied as in furrow treatment at a rate of 224 kg/ha was not significantly different than the seed treatment, nor the untreated control. There were no significant differences between the in furrow application with *M. albus* at a rate of 448 kg/ha and the untreated control.

Both in furrow treatments with *M. albus* significantly increased the yield ($P = 0.0328$) when compared to the untreated control (Table 2.6). There was no significant difference between the two in furrow treatments. The *M. albus* seed treatment also

increased the potato yield but yields were not significantly different to the untreated control (Table 2.6).

Table 2.5: Effect of potato seed treatments and in furrow applications with *Muscodor albus* barley formulation on the potato stem colonization of *Verticillium dahliae* and *Colletotrichum coccodes* under field conditions.

Treatment	**Application method**	**Stems colonized by *V. dahliae* (%)**	**Stems colonized by *C. coccodes* (%)**
Pathogen control	untreated control	35.8 a [z]	31.0 a
M. albus 15 kg/t	seed treatment	21.0 b	7.6 b
M. albus 224 kg/ha	in furrow	30.2 ab	8.3 b
M. albus 448 kg/ha	in furrow	24.4 b	7.7 b

[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

Table 2.6: Effect of potato seed treatments and in furrow applications with *Muscodor albus* barley formulation on the final stand and yield of potatoes grown in natural soils infested with *Verticillium dahliae* and *Colletotrichum coccodes*.

Treatment	**Application method**	**Percent healthy plants**	**Yield t/ha**
Pathogen control	untreated control	55.5 a [z]	6.3 b
M. albus 15 kg/t	seed treatment	73.8 b	7.7 ab
M. albus 224 kg/ha	in furrow	64.7 ab	9.5 a
M. albus 448 kg/ha	in furrow	61.3 a	10.1 a

[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

Discussion

The incidences of *V. dahliae* and *C. coccodes* on potato were significantly reduced in greenhouse bioassays and under field conditions by treatments of *M. albus* barley formulation. These results indicate that VOC emitted by *M. albus* are effective in reducing pathogen concentration in the plant tissue and disease severity caused by both pathogens.

In this study, culture-independent molecular and culture-dependent CFU assays successfully detected *V. dahliae* and *C. coccodes* in potato plant tissues. Quantification of pathogens in plant tissue via CFUs is commonly used for pathogenic fungi (Li et al., 2008) and has been used in the assessment of Verticillium wilt and black dot of potato (Harris et al., 1993; Barkdoll and Davis, 1992). Culture-dependent methods rely on the assessment of viable cells which are counted on media plates. Li et al. (2008) cautions that the number of CFUs is estimated by visual identification which may be less accurate due to different microorganisms present in samples. The use of selective or semi-selective media for dilution plating is therefore essential for an accurate assessment of CFUs (Farley, 1976; Sorenson et al., 1991). The number of CFUs can be underestimated due to high organism densities. Counted colonies may also contain more than one viable cell (Li et al., 2008). Plating of serial dilutions on selective media and the comparison of CFUs at different dilution concentrations can reduce this problem. Quantification of *V. dahliae* and *C. coccodes* in potato tissue by QPCR can provide researchers with an unbiased method for comparing differences in fungal colonization following chemical and biological treatments. QPCR using SYBR green as reporter dye was sufficient for

the objective of our study in detecting and quantifying *V. dahliae* and *C. coccodes* in potato stem tissues and showed results similar to serial dilution plating. TaqMan probes may offer an additional level of specificity over SYBR green alone but are also more expensive than the SYBR Green dye (Sayler et al., 2007).

The importance of inoculum source of pathogens was demonstrated by Denner et al. (1998) and Nitzan et al. (2008), who showed that the contribution of soil-borne inoculum of *C. coccodes* to the incidence of black dot on progeny tubers is at least twice that of seed-borne inoculum. Soil fumigation is therefore the essential tool in disease control in potato production.

Soil fumigants such as Vapam or K-Pam are highly efficient, fast working soil fumigants used for the control of Verticillium wilt and black dot of potato. In addition, if applied correctly, soil fumigation is highly effective in reducing populations of nematodes and other soil-borne fungal pathogens, in controlling weeds and insects, and thereby improving the quality of harvested tubers. Due to toxicity concerns, plant-back intervals need to be considered before potatoes can be planted. Other problematic issues of chemical fumigants are the potential for contaminating ground water, drift of sprinkler-applied products, destruction of beneficial soil microorganisms, and incompatibility with some biocontrol practices (Powelson and Rowe, 1993, 2008). Recently proposed new state and federal regulations proposed by EPA and the California Department of Pesticide Regulation to reduce pesticide VOC emissions and to ensure worker and bystander safety might limit and restrict the use of chemical soil fumigants like methyl bromide and metam sodium. These proposed regulations include the use of

tarping and water seals to reduce emission, which will result in higher costs for the growers. Other methods for the control of soil-borne pathogens could include, but are not limited to soil solarization, organic amendments such as green manure crops and composts, crop rotation (Powelson and Rowe, 2008), and the use of BCAs.

Ibekwe et al., (2001) is emphasizing the importance of the microbial community composition for sustaining the health of natural and agricultural soils. Changes in microbial communities due to the phytotoxic nature of soil fumigants may have a negative effect on the overall soil health and the chemicals are also extremely toxic to workers. Fumigants also require plant-back intervals after application. In comparison U.S. EPA (2005) classified the biological control agent *M. albus* and its VOCs as not harmful for honeybees, birds, other non-target insects, and plants, and it is considered non-toxic to humans and therefore only requires minimal worker protection equipment.

In previous studies *M. albus* showed a distinct selectivity for soil-borne pathogens *in vitro* like *Pythium ultimum*, *Rhizoctonia solani*, *V. dahliae* (Strobel et al., 2001; Grimme, 2004), and *C. coccodes* (Grimme et al., 2008). *Muscodor albus* has also been shown to be effective in controlling *V. dahliae* (Stinson et al., 2003; Grimme, 2004) and *C. coccodes* under greenhouse and field conditions (Grimme et al., 2008) without the detrimental effects on beneficial microorganisms or plants (Zidack et al., 2002; Stinson et al., 2003; Grimme et al., 2007). Since there is no phytotoxic reaction, plant-back intervals are not necessary. The members of eight different plant families tested for *M. albus* phytotoxicity included representatives from the plant families Rosaceae,

Asteraceae, Graminae, Cruciferae, Cucurbitaceae, Leguminaceae, Chenopodiaceae, and Solanaceae (Strobel et al., 2001).

The fungus requires some time to reactivate mycelial growth and VOC production since *M. albus* is generally applied in dried form, like barley formulation. Therefore these volatiles may not be as fast as Vapam in reaching resting structures of the pathogen such as microsclerotia. *Muscodor albus* barley formulation may need to be applied at lower action threshold levels than standard chemical fumigants. Although there is some variability in published action threshold values, they fall in the range of 5 to 30 CFUs/g dry soil for *V. dahliae* alone. If *P. penetrans* is also present at 10 to 20 vermiforms/100g soil, the threshold for *V. dahliae* drops to 2 to13 CFUs/g dry soil (Powelson and Rowe, 1993). Besides this disadvantage, *M. albus* provides multiple advantages when compared to chemical fumigants. For example, *M. albus* barley formulation can be applied as seed treatments or in-furrow applications at time of planting. In greenhouse studies, no significant (P = 0.05) difference were observed between direct planting or a fumigation period of seven days before planting in regard to disease severity and CFUs/g potato stem tissue. *Muscodor albus* mixed into the soil at a rate of 6.7 g and 13.4 g/kg soil at the time of planting (direct planting) was able to significantly reduce the AUDPC for *V. dahliae* by 55% and 66%, and the CFUs/g stem tissue by 69% and 71%, respectively, when compared to the pathogen controls. In soils infested with *C. coccodes*, *M. albus* application at 6.7 g and 13.4 g/kg soil followed by direct planting significantly reduced the AUDPC by 31% and 66%, respectively, and the CFUs/g stem tissue by 75% for both rates. *In vitro* experiments with *M. albus* conducted to elucidate the distribution of its

VOCs (chapter 1) also showed that VOCs produced by *M. albus* reached as far as 9 cm from point of inoculation and provided up to 100 % and 95 % reduction of CFUs of *V. dahliae* and *C. coccodes* respectively. If these data were related to a seed or in furrow treatment, *M. albus* would be able to sufficiently protect the germinating potato tuber. However, the accomplished control depends on the time and the soil type, where porosity, organic matter, salinity levels, and pH might influence the performance of *M. albus*. In contrast to the above described pathogens, oomycetes such as *Aphanomyces cochlioides* and *P. ultimum* require a fumigation period to be effectively controlled by *M. albus* (Grimme, 2004).

M. albus had no negative effects on fresh and dry weights of potato cultivated under controlled environmental conditions and on potato yield in the field. Another advantage of *M. albus* is its selectivity in killing/inhibiting certain pathogens and not affecting beneficial microorganisms like *Trichoderma* spp. or *Gliocladium* sp. (Strobel et al., 2001; Grimme, 2004). Especially for potato production, *M. albus* is a powerful tool since it is able to control multiple fungal pathogens including *R. solani* (Jacobsen, *personal communication*), *V. dahliae*, and *C. coccodes*, bacterial pathogens like *Streptomyces scabies* (Jacobsen, *personal communication*) as well as nematodes like *P. penetrans* (Grimme et al. 2007), *Meloidogyne chitwoodi*, *M. hapla*, *Paratrichodorus allius* (Riga et al., 2008) at the same time, without interfering with other cultural practices. Finally, *M. albus* might be more acceptable by the consumers since it is considered a biological control organism which could also be used in organic production.

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CHAPTER 3

EFFECTS OF VOLATILE ORGANIC COMPOUNDS EMITTED BY *MUSCODOR ALBUS* ON ECTOMYCORRHIZAL FUNGI *IN VITRO* AND ON COLONIZATION OF ONION ROOTS BY THE AM FUNGUS *GLOMUS INTRARADICES*

Introduction

The control of soil-borne plant diseases with biological control agents (BCAs) is an effective management tool in agricultural and horticultural commodities (Whipps and Lumsden, 2001). The use of BCAs is encouraged due to increased concerns about chemicals/pesticides and their impact on soil and ground water quality (Paulitz and Linderman, 1991) as well as on human health. The use of BCAs is in general considered to be a safe and environmentally friendly alternative for disease control compared to conventional pesticides (Kiss, 2004; Whipps and Lumsden, 2001). However, little is known of the effects of BCAs on beneficial mycorrhizal fungi. Mycorrhizae, a mutualistic symbiosis between a fungus and roots of a plant, are the normal state of over 85% of plants in nature (Cripps, 2001). In particular AM fungi form symbioses with most herbaceous plants, providing several benefits to their host plant (Brimmer and Boland, 2003), including an increase in phosphorus availability (McAllister et al., 1994; Perez-Moreno and Read, 2000), drought tolerance (Ruiz-Lozano et al., 1995), and increased resistance to diseases (Morin et al., 1999).

The genera *Gliocladium* and *Trichoderma* are some of the most studied BCAs used for the control of soil-borne pathogens (Paulitz and Linderman, 1991; Papavizas,

1985; Rousseau et al., 1996). Depending on the species, the modes of action of the BCAs can include mycoparasitism, production of antibiotics, competition for nutrients and space, mobilization of nutrients in the soil for uptake by plants, and increased disease resistance in plants (Rousseau et al., 1996; Brimmer and Boland, 2003). Dennis and Webster (1971) showed that certain species of *Trichoderma* also emit volatile organic compounds (VOCs) with inhibiting effects on other fungi. These different modes of action can also affect non-target organisms like beneficial bacteria (*Bacillus* sp. and *Pseudomonas* sp.) and fungi, including those that form ectomycorrhizae (ECM) and arbuscular mycorrhizae (AM).

BCAs like *Gliocladium* and *Trichoderma* may have positive as well as negative effects on the plant root colonization by AM fungi. *Trichoderma* species vary in their effects towards other microorganisms (Zadworny et al., 2004; Hadar et al., 1979). *Trichoderma harzianum* Rifai had a stimulatory effect on root colonization of corn (*Zea mays* L.) by *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe or *Glomus deserticola* (Vázquez et al., 2000). *Trichoderma koningii* decreased AM colonization of *G. mosseae* in maize and lettuce when inoculated into the rhizosphere before or at the same time as *G. mosseae* (McAllister, 1994). *Trichoderma viride* and *Trichoderma polysporum* were shown to be very antagonistic to the ectomycorrhizal fungus *Laccaria bicolor* (Maire) Ort (Summerbell, 1987). The timing of the inoculation with mycorrhizal fungi and biocontrol agents and the host plant species seems to be the essential criterium that determines the success or failure of AM root colonization (McAllister et al., 1994).

Many of the biocontrol agents in the genus *Gliocladium* produce antibiotics (Howell, 1991), spore germination inhibiting endochitinase, and the toxin gliotoxin (Brimmer and Boland, 2003; Di Pietro et al., 1993). Despite these metabolic compounds, *Gliocladium virens* Miller, Giddens & Foster had no negative impact on root colonization of cucumber (*Cucumis sativus* L.) by the AM fungus *Glomus etunicatum* Becker & Gerdemann (Paulitz and Linderman, 1991). *Glomus virens* also produces the compound viridol, which reduces the germination and plant growth of several species including lettuce (*Lactuca sativa* L.) and mustards (*Brassica* sp.) (Brimmer and Boland, 2003).

Muscodor albus Worapong, Strobel & Hess, isolate CZ-620, is an endophytic fungus isolated from *Cinnamomum zeylanicum* in Honduras (Worapong et al., 2001). *Muscodor albus* produces volatile organic compounds (VOCs), which effectively inhibit or kill microorganisms including soil-borne plant pathogens (Strobel, et al., 2001; Stinson et al., 2003; Grimme et al., 2007b). The chemical composition of the VOCs and beneficial biological properties of *Muscodor* sp. were described previously *in vitro* (Strobel et al., 2001; Ezra and Strobel, 2003) and *in vivo* (Stinson et al., 2003, Grimme et al., 2007a). The terms mycofumigation and biofumigation are both introduced when efficacies of *M. albus* as biocontrol agent are described (Stinson et al., 2003; Zidack et al., 2001, 2002; Jacobsen et al., 2004). *Muscodor albus* was approved in 2005 by the EPA (Environmental Protection Agency) for all soil and post-harvest applications (U.S. EPA, 2005).

Fumigation with the biocontrol agent *M. albus* resulted in successful control of soil-borne pathogens such as nematodes *Meloidogyne chitwoodi*, *M. hapla*,

Paratrichodorus allius and *Pratylenchus penetrans* (Riga et al., 2008; Grimme et al., 2007a) and soil-borne fungi like *Phytophthora capsici* Leonian (Camp et al., 2008), *Rhizoctonia solani*, *Pythium ultimum*, *Aphanomyces cochlioides*, *Colletotrichum coccodes*, and *Verticillium dahliae* (Stinson et al., 2003; Grimme, 2004; Mercier and Manker, 2005; Grimme et al., 2007a, 2007b). The effects of *M. albus* and its VOCs on non-target organisms is poorly understood and it is critical to investigate the effects on plant pathogenic as well as beneficial organisms.

The objectives of this study were (1) to investigated the *in vitro* effects of the VOCs produced by *M. albus* on selected ectomycorrhizal fungi in the genera *Hebeloma*, *Laccaria*., *Rhizopogon*, and *Suillus*; (2) to evaluate the effects of antimicrobial compounds emitted by the fungus *M. albus* isolate CZ-620, on colonization of onion (*Allium cepa* L.) roots by the AM fungus *Glomus intraradices* Schenck & Smith.

Materials and Methods

Fungal Cultures

Ectomycorrhizal Fungi: The fruiting bodies of *Hebeloma laterinum*, *Laccaria* sp., *Rhizopogon salebrosus*, *Suillus brevipes*, and *Suillus* sp. were collected in conifer forests in Montana and identified using David Arora's "Mushrooms demystified – A comprehensive guide to the fleshy fungi"(1986) as a reference guide. Tissue samples of each ectomycorrhizal fungus (EMF) were removed using sterile techniques and placed on modified Melin Norkrans medium (MMN) and incubated at 22 ± 1 ºC for three weeks. Cultures were included into the Montana State University culture collection.

Glomus intraradices: A commercial product (AM 120 Symbios) of *G. intraradices* was purchased from Reforestation Technologies International (Salinas, CA). The inoculum of *G. intraradices* consisted of colonized roots, hyphae, and spores. The inoculum was stored at 4 °C.

M. albus isolate CZ-620 Inoculum Production: *Muscodor albus*, isolate CZ-620, was grown and maintained in 10 cm Petri plates on potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) at 22 ± 1°C. Inoculum was produced by transferring 15 agar plugs (5 mm diameter) from 3-week-old Petri plate cultures into 2-liter flasks containing 1 liter autoclaved potato dextrose broth (PDB, EMD Chemicals Inc., Darmstadt, Germany), and placing the flasks on a rotary shaker (Model OS-500, VWR International, West Chester, PA) (110 rpm) for 2 weeks at 22 ± 1 °C. The *M. albus* barley formulation was prepared by adding 100 ml of colonized PDB to mushroom cultivation bags (Polypropylene bags with one filter, Unicorn Imp. & Mfg. Corp., Garland, TX 75042) containing 500 g of sterilized barley (*Hordeum vulgare* L). After growth on the substrate for three weeks at 22 ± 1°C, the colonized seeds were dried for 48 h in a laminar flow-hood and then stored in plastic bags at 4 °C.

Ectomycorrhizal Plate Assays

Subcultures of the test fungi were taken and two plugs (0.5 mm in diameter) of each fungus were placed on MMN and incubated at 22 ± 1 ºC for two weeks. After the incubation time subcultures in the form of two culture plugs of the EMF were placed on the MMN agar and grown for one week at 22 ± 1 ºC. Subcultures of *M. albus* were grown for one week at 22 ± 1 °C on PDA, containing 10 ppm chloramphenicol. The plates of EMF were then inverted onto the plates of the one-week-old *M. albus* culture, parafilmed, and incubated at 22 ºC ± 1 ºC for two or five days (Image 3.1). Mycelial growth of EMF was determined after the respective fumigation period. Ectomycorrhizal subcultures were then transferred to fresh plates without *M. albus* exposure and observed for subsequent mycelial growth after 7 days.

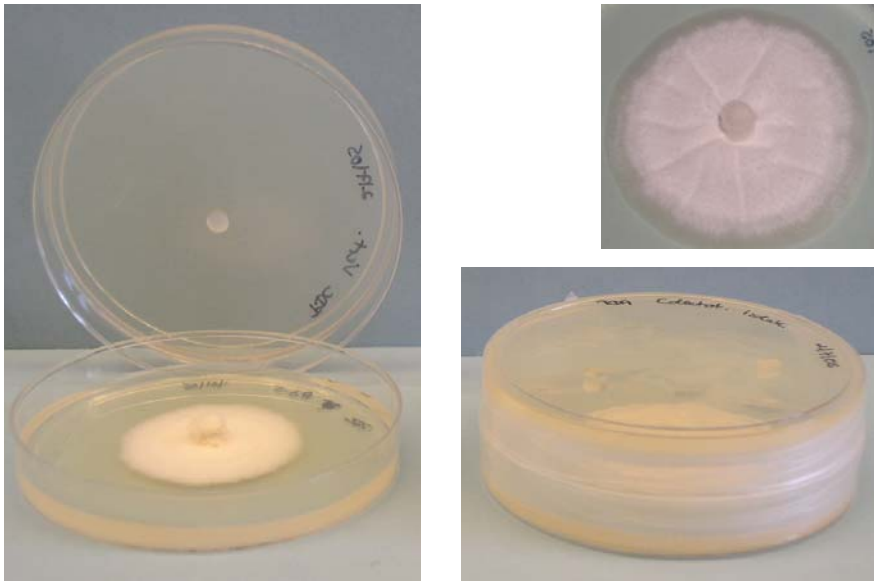


Image 3.1: Principle of the *in vitro* experimental set-up. Subcultures of the test organisms (EMF)were transferred to media plates and parafilmed onto media plates with the one-week-old *M. albus* culture (left). The ectomycorrhizal fungi were exposed to the VOC of *M. albus* for one week (right, bottom). One-week-old *M. albus* culture (right, top).

Greenhouse Assay for
G. intraradices and *M. albus* Interactions

Greenhouse bioassays were designed to measure the effects of the antimicrobial compounds produced by the fungus *M. albus* in barley formulation on the colonization of onion roots by the arbuscular mycorrhizal (AM) fungus *G. intraradices*. The greenhouse bioassay was repeated twice. All experiments were carried out in a greenhouse with 24 ± 2 °C day and 18 ± 2 °C night temperatures and with a 16 h photoperiod. Supplemental lighting was provided by SON AGRO 430 WATT HPS lights (Philips Lighting Company, Somerset, NJ). The growing substrate (1/3 Bozeman silt loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent) used for all experiments was sieved (2 mm mesh opening) and steam sterilized.

Onion (*A. cepa*) seeds of the variety 'Walla Walla' were purchased from Johnny's Selected Seeds (Winslow, ME) and stored at 4 °C. The seeds were surface sterilized in a 5% Clorox solution for one minute, rinsed with sterile distilled water and pre-germinated on moist filter paper for 24 h before planting.

Effects of AM inoculation on growth of onion plants, as well as the effects of AM and *M. albus* co-inoculation were investigated. The *M. albus* treatment was also applied without AM inoculation to determine any effects of the VOC producing fungus on onion plants. *Muscodor albus* barley formulation was applied at a rate of 6 g/kg soil to the respective treatments (Table 3.1) and mixed throughout the soil. *Glomus intraradices* inoculum was applied directly into the planting hole (10 g/planting hole) or throughout mixed into the soil (*G. intraradices*/soil, 1:20, v/v) (Miyasaka et al., 2003). Round

plastic pots with a diameter of 15 cm were prepared with inoculated soils or the non-inoculated control soil. Three pre-germinated onion seeds were planted into each pot and placed into a greenhouse. Plants were grown under controlled conditions for three months. Water was applied as needed once a day.

Table 3.1: Application rates and methods and combinations of the AM fungus *Glomus intraradices* and the biocontrol agent *Muscodor albus*.

Glomus intraradices	**Application method of *G. intraradices***	***Muscodor albus***
non-inoculated control	N/A	N/A
10 g inoculum	direct into planting hole	none
10 g inoculum	direct into planting hole	6 g/kg soil
1:20, v/v soil	mixed through the soil	none
1:20, v/v soil	mixed through the soil	6 g/kg soil
none	none	6 g/kg soil

In both experiments fresh and dry weights of shoots and roots were measured after drying at 65 °C for two days. Fresh root samples (100 mg) were taken, cleared in 10% KOH, and stained with black ink (Schaeffer) following the protocol of Vierheilig et al. (1998). The percentage of root length colonized by hyphae and colonization of arbuscules and vesicles of *G. intraradices* was determined using the magnified intersection method (McGonigle et al., 1990).

Statistical Analysis

The bioassays were arranged in randomized complete designs with 6 replications per treatment. Experiments were repeated two times. Data were analyzed for homogeneity over repeated experiments using Levene's test. Statistical analysis of the pooled data was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary NC). The treatment means were separated using Fisher's protected least significant difference test at $P = 0.05$.

Results

Ectomycorrhizal Fungi

In the presence of the VOCs emitted by *M. albus*, mycelial growth of all tested EMF was inhibited. Regardless of the fumigation time, growth of the mycelium could again be observed after ten days when the two-week-old subcultures of *Laccaria* sp., *Rhizopogon salebrosus*, *Suillus brevipes*, and *Suillus* sp. were transferred to new media plates without the presence of *M. albus*. Fungal cultures of *Hebeloma laterinum* showed no mycelial growth in the presence of *M. albus* even after transference of the two-week-old cultures onto new media plates without exposure to VOCs emitted by *M. albus* (Table 3.2).

Table 3.2: Influence of the volatile organic compounds of *Muscodor albus* on growth of selected ectomycorrhizal fungi [y] *in vitro*.

Ectomycorrhizal Fungi	Growth in presence of *M. albus* Fumigation in days		Growth when transferred to a new plate after fumigation Fumigation in days	
	2	5	2	5
Hebeloma laterinum	N	N	N	N
Laccaria sp.	N	N	Y	Y
Rhizopogon salebrosus	N	N	Y	Y
Suillus brevipes	N	N	Y	Y
Suillus sp.	N	N	Y	Y

[y] Ectomycorrhizal fungi were grown on MMN agar and exposed to *M. albus* VOC for 2 or 5 days. After the respective exposure time, cultures were transferred to new media without *M. albus*. Mycelial growth was monitored while fungi were exposed to the VOC and after being transferred.
[z] N = no mycelial growth, Y = mycelial growth after cultures were transferred to new media without *M. albus*.

Arbuscular Mycorrhizae Assay

Fresh and dry weights of shoots of onion plants grown in soil amended with *G. intraradices* were not significantly different (Fresh weight: $P \leq 0.9337$; Dry weight: $P \leq 0.4123$) from weights of plants grown in soil amended with the AM fungus and the biological control agent *M. albus* (Table 3.3). The *M. albus* control treatment resulted in fresh and dry shoot weights equal to the *G. intraradices* treatments.

Fresh and dry weights of roots for treatments with *G. intraradices* inoculation and/or of treatments amended with *M. albus* were higher than in the non-inoculated control treatment (Table 3.3; Image 3.2). Both *G. intraradices* treatments without *M. albus* inoculation resulted in significantly higher root weights ($P \leq 0.0182$) when compared with the non-inoculated control. *Glomus intraradices* applied at 1:20, v/v,

resulted in significantly higher root weights than the *M. albus* control treatment. There were no significant differences in root weights between the *G. intraradices* amended and the respective *G. intraradices* plus *M. albus* amended treatments (Table 3.3).



Image 3.2: Effect of inoculation with *Glomus intraradices* with and without *Muscodor albus* amendment on development of onion plants. From left to right: **A.** non-inoculated control; **B.** *G. intraradices* at 10 g per planting hole; **C.** *G. intraradices* at 10 g per planting hole plus *M. albus* amendment; **D.** *G. intraradices* mixed throughout soil at 1:20 v/v; **E.** *G. intraradices* mixed throughout soil at 1:20 v/v plus *M. albus* amendment; **F.** *M. albus* control.

Onion roots were successfully colonized by the AM fungus *G. intraradices* (Image 3.3). *Glomus intraradices* in combination with *M. albus* mixed throughout the soil resulted in the highest colonization by hyphae, arbuscules, and vesicles (Table 3.4).

Table 3.3: Fresh and dry weights of shoots and roots of onion plants grown for 12 weeks in *Glomus intraradices* inoculated and with or without *Muscodor albus* barley formulation treated soil [y].

Treatment [y]	Fresh weight (g)		Dry weight (g)	
	Shoot	Root	Shoot	Root
Non-infested control	25.29 a [z]	10.26 c	5.19 a	1.61 c
10 g *G. intraradices*	25.76 a	12.67 ab	5.57 a	1.86 ab
10 g *G. intraradices* & *M. albus* treatment	25.68 a	11.93 abc	5.19 a	1.72 abc
1:20 *G. intraradices* : soil (v/v),	28.63 a	13.70 a	6.29 a	1.89 a
1:20 *G. intraradices* : soil (v/v), & *M. albus* treatment	25.61 a	11.76 abc	4.91 a	1.72 abc
M. albus control	24.02 a	11.33 bc	4.79 a	1.69 bc

[y] Soil was inoculated with *M. albus* at 6 g/kg soil. *G. intraradices* was applied at a rate of 10 g directly into planting hole or throughout mixed into the soil at a rate of 1:20 soil (v/v).

[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

In contrast, inoculation by only *G. intraradices*, mixed throughout the soil, resulted in the lowest AM colonization. Hyphal colonization in treatments where the AM fungus was applied directly into the planting hole, with and without *M. albus* inoculation, and in the treatment of AM fungus and *M. albus* amended soil, was significantly higher ($P \leq 0.0002$) than in the single *G. intraradices* 1:20 (v/v) treatment (Table 3.4).

There was no significant difference in hyphal colonization between treatments with *G. intraradices* applied directly into the planting hole with or without the *M. albus* amendment. In treatments with the AM fungus applied directly into the planting hole, without *M. albus* inoculation, and in the treatment with AM fungus and *M. albus* amended soil, arbuscular colonization was significantly higher ($P \leq 0.0311$) than in the single *G. intraradices* 1:20 (v/v) treatment. *Muscodor albus* amendment with inoculation of the AM fungus into the planting hole was similar in arbuscular colonization to the treatment of AM inoculation alone. Vesicle colonization was highest in the treatment with *G. intraradices* 1:20 (v/v) and *M. albus* amendment and was significantly higher ($P \leq 0.0002$) in vesicle colonization compared to the *G. intraradices* 1:20 (v/v) treatment alone. There was no significant difference in vesicle colonization between treatments with *G. intraradices* applied direct into the planting hole with or without *M. albus* amendment (Table 3.4).

Table 3.4: Effect of *Glomus intraradices* and *Muscodor albus* inoculation treatments on percent arbuscular mycorrhizal fungus root colonization, percent arbuscules and vesicle colonization.

	Colonization of onion roots (%)		
Treatment [y]	**Hyphae**	**Arbuscules**	**Vesicles**
10 g *G. intraradices*	89.89 a [z]	27.00 a	36.11 ab
10 g *G. intraradices* & *M. albus* treatment	88.67 a	20.00 ab	29.11 bc
1:20 *G. intraradices* : soil (v/v)	64.56 b	14.44 b	15.78 c
1:20 *G. intraradices* : soil (v/v) & *M. albus* treatment	93.44 a	29.67 a	44.22 a
M. albus	N/A	N/A	N/A

[y] Soil was inoculated with *M. albus* at 6 g/kg soil. *G. intraradices* was applied at a rate of 10 g directly into planting hole or throughout mixed into the soil at a rate of 1/20 soil (v/v).

[z] Means followed by the same letter are not significantly different based on Fisher's LSD at $P = 0.05$.

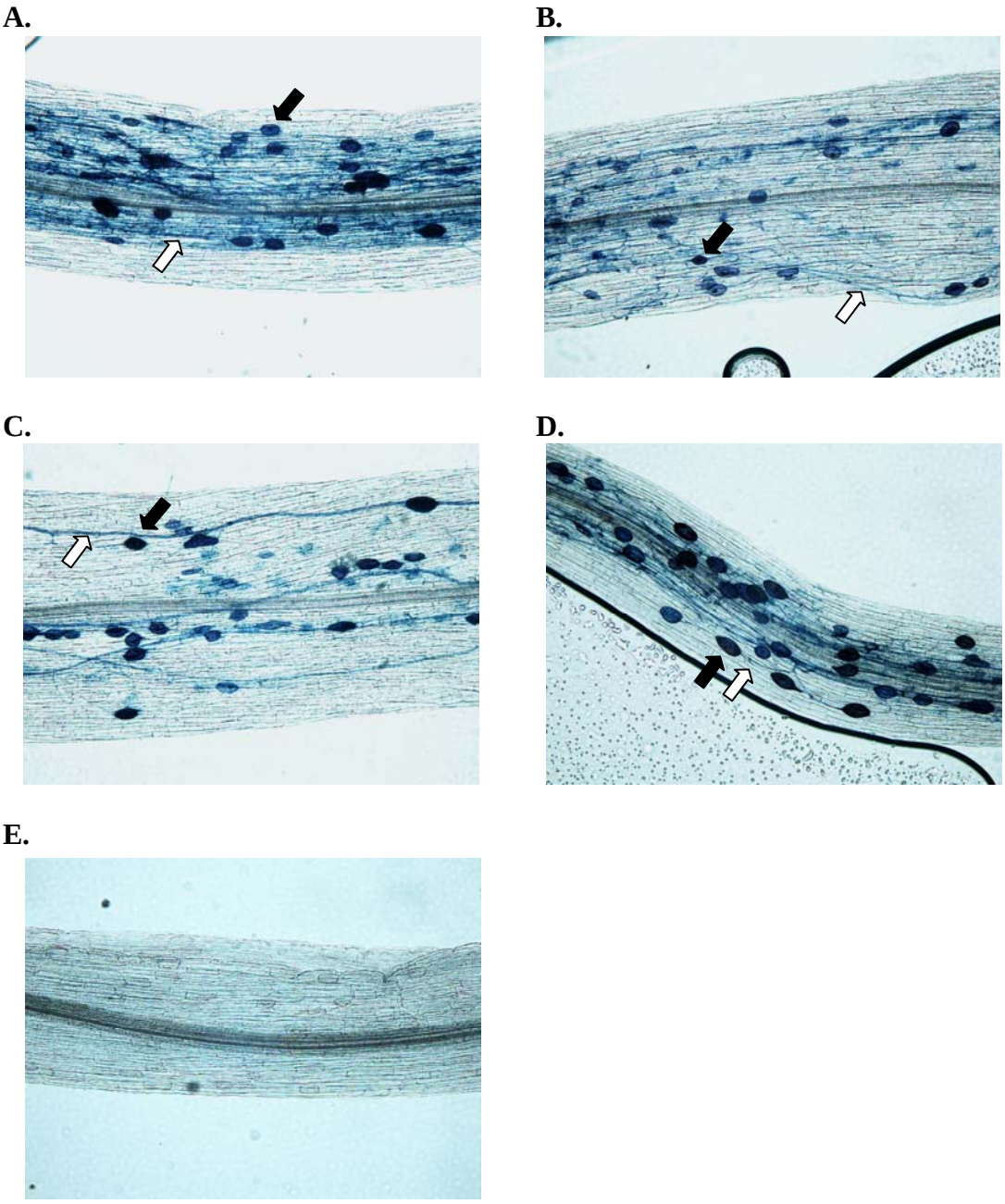


Image 3.3: Microscopic images of onion roots colonized by *Glomus intraradices*: **A.** *G. intraradices* at 10 g per planting hole; **B.** *G. intraradices* at 10 g per planting hole plus *Muscodor albus* amendment; **C.** *G. intraradices* mixed throughout soil at 1:20 v/v; **D.** *G. intraradices* mixed throughout soil at 1:20 v/v plus *M. albus* amendment; **E.** *M. albus* control (⇨ = fungal hyphae, ➡ = fungal vesicles).

Discussion

This is the first study on the effects of antimicrobial compounds produced by *Muscodor albus* on AM and ectomycorrhizal fungi. This research suggests that the biocontrol agent *M. albus* has no negative effect on colonization of onion roots by the AM fungus *Glomus intraradices*.

The *in vitro* exposure of the ectomycorrhizal fungi *Hebeloma laterinum*, *Laccaria* sp., *Suillus brevipes*, *Rhizopogon salebrosus*, and *Suillus* sp. to VOCs emitted by *M. albus* resulted in the inhibition of the mycelial growth of all tested fungi in the presence of *M. albus*. The two-weeks-old cultures of *Laccaria* sp., *R. salebrosus*, *S. brevipes*, and *Suillus* sp. were able to recover and grow again after the removal of *M. albus* from their environment. This observation indicates that the mycelial growth of these fungi was only inhibited when in close proximity to *M. albus* VOCs. The cultures of *H. laterinum* were completely inhibited by the VOCs of *M. albus*. These cultures were not able to initiate mycelial growth again. Since the test organisms were exposed to an extreme dosage of *M. albus* over five days, observations of mycelial growth should also be tested with shorter exposure times. The tested fungi represented only a small fraction of the naturally occurring ectomycorrhizal fungi and the effects of *M. albus* should be tested on a wider range of fungal species as well. While the *in vitro* tests suggest how *M. albus* might affect ectomycorrhizal fungi, *in vivo* experiments will be required to simulate more realistic environmental conditions and to complete the analysis.

The effects of antimicrobial compounds produced by *M. albus* were also tested under *in vivo* conditions by exposing the AM fungus *G. intraradices* to *M. albus* grown

on barley and measuring its effects on fresh and dry weights and on mycorrhizal colonization of onion roots. Application of *G. intraradices*, *M. albus* or a combination of both fungi had no detrimental effect on fresh and dry weights of onion shoots. Root fresh and dry weights were significantly increased when *G. intraradices* was inoculated into the soil with no effect of the application method. The addition of *M. albus* to AM inoculated soil resulted in lower root weights that were not significantly different than those for the respective *G. intraradices* treatments. The inoculation of *M. albus* had no detrimental effect on shoot or root weights.

The hyphal, arbuscular, and vesicle colonization was significantly increased when *M. albus* and *G. intraradices* were inoculated at the same time and mixed throughout the soil. A 45% increase of hyphal colonization could be observed for plants treated with the combination of *M. albus* and *G. intraradices* mixed into the soil when compared to *G. intraradices* alone. No increase in colonization could be observed for treatments where *G. intraradices* was added to the plant hole and *M. albus* was incorporated into the soil. No root colonization by the biocontrol agent *M. albus* was observed when using the staining method of Vierheilig et al. (1998). In general, for treatments without *M. albus*, mycorrhizal colonization was significantly less effective in plants when *G. intraradices* was mixed it into the soil instead of adding it to the plant hole. However, the addition of *M. albus* to *G. intraradices* mixed throughout the soil significantly increased the mycorrhizal colonization for this treatment. Because of the lack of negative effects of antimicrobial compounds produced by *M. albus* on *G. intraradices* and the apparent positive effects manifested in increased root colonization, it may be more effective and

practical to apply *M. albus* and *G. intraradices* together into a soil mix rather than as two separate treatments. These results differ from observations made by McAllister et al. (1994), who found that the biocontrol agent *Trichoderma koningii* decreased AM colonization of *Glomus mosseae* in maize and lettuce when inoculated into the rhizosphere at the same time as *G. mosseae*. Negative effects of BCAs were also described by Summerbell (1987) who demonstrated that *T. viride* and *T. polysporum* were very antagonistic to the ectomycorrhizal fungus *Laccaria bicolor*. Brimmer and Boland (2003) mentioned that the BCA *Gliocladium virens* reduced the germination and plant growth of lettuce and mustards. The modes of action of the BCA such as mycoparasitism, production of antibiotics, competition for nutrients and space (Rousseau et al., 1996; Brimmer and Boland, 2003) also affect non-target organisms like ectomycorrhizal and arbuscular mycorrhizal fungi. It is already known that *M. albus* does not affecting beneficial organisms like *T. harzianum* (Grimme, *unpublished data*) and *Bacillus subtilis* (Strobel et al., 2001), seed germination (Ezra and Strobel, 2003) and plant growth (Zidack et al., 2002; Stinson et al., 2003; Grimme et al., 2007a).

The use of soil fumigants for disease control is a standard practice in agriculture (Ibekwe, 2004). Fumigants like methyl bromide, chloropicrin, and metam sodium have a broad biocidal activity and affect many non-target organisms (Ibekwe et al., 2001) including AM and ECM fungi. The effects on non-target organisms are largely unknown. Ibekwe et al. (2001) emphasized the importance of the microbial community composition for sustaining the health of natural and agricultural soils. Changes is microbial communities may have a negative effect on the overall soil health. Fumigants

also require plant-back intervals after application due to their phytotoxic nature. *Muscodor albus* showed a distinct selectivity for soil-borne pathogens *in vitro* like *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium dahliae* (Strobel et al., 2001; Grimme, 2004), and *Colletotrichum coccodes* (Grimme et al., 2007b) without the detrimental effects on beneficial microorganisms or plants (Zidack et al., 2002; Stinson et al., 2003; Grimme et al., 2007a).

Muscodor albus does not colonize the host plants or the soil itself; therefore it is not competing for nutrients or space with the beneficial soil organisms like mycorrhizal fungi. Additional studies are required to determine the effects of *M. albus* on different soil communities and how these changes in soil microorganism composition may benefit plant health, resulting in lower pesticide applications and in more environmental friendly disease control.

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APPENDICES

APPENDIX A

SOIL ANALYSIS FOR MSU MIX, LIHEN SANDY LOAM, AND AMSTERDAM SILT LOAM

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MONTANA STATE UNIVERSITY
BOZEMAN, MT 59717

GROWER:

DATE OF REPORT: 09/19/08

SOIL PHYSICAL CHARACTERISTICS

Sample ID	Lab Number	% Sand	% Silt	% Clay	Soil Texture	Moisture @ 1/3 Bar	Moisture @ 15 Bar	Available Water %
Lihen	53279	70	16	13	SANDY LOAM			
Amsterdam	53280	26	56	17	SILT LOAM			
MSU mix	53281	72	10	17	SANDY LOAM			

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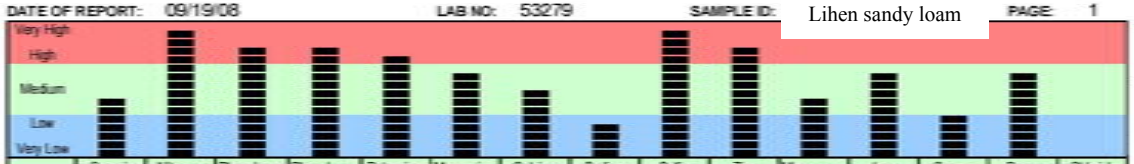
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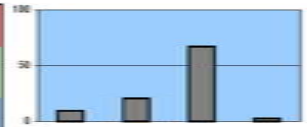
Graphical Soil Analysis Report

DATE OF REPORT: 09/19/08 LAB NO: 53279 SAMPLE ID: Lihen sandy loam PAGE: 1



Analyte	Organic Matter %	Nitrogen NO_3-N ppm	Phosphorus Weak Bray ppm	Phosphorus $NaHCO_3$-P ppm	Potassium K ppm	Magnesium Mg ppm	Calcium Ca ppm	Sodium Na ppm	Sulfur SO_4-S ppm	Zinc Zn ppm	Manganese Mn ppm	Iron Fe ppm	Copper Cu ppm	Boron B ppm	Chloride Cl ppm
Results:	2.9	102	39	41	707	481	2557	118	76	7.4	6	19	0.8	1.2	
			LOW	AVERAGE	HIGH										

Percent Cation Saturation (computed)



Potassium K %	Magnesium Mg %	Calcium Ca %	Sodium Na %
9.5	20.8	67.0	2.7
		ACIDIC	BASIC

ECe	CEC	Ex. Lime	pH
1.9	19.0	L	7.0
dS/m	meq/100g		
INCREASING SALINITY →			← INCREASING NEED FOR LIME

Buffer pH:

Soil Fertility Guidelines

CROP: RATE: NOTES:

Dolomite (70 score)	Lime (70 score)	Gypsum	Elemental Sulfur	Nitrogen N	Phosphate P_2O_5	Potash K_2O	Magnesium Mg	Sulfur SO_4-S	Zinc Zn	Manganese Mn	Iron Fe	Copper Cu	Boron B	

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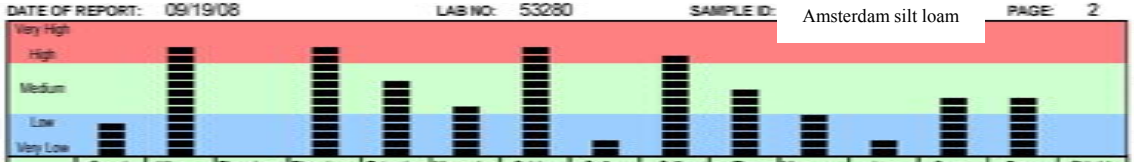
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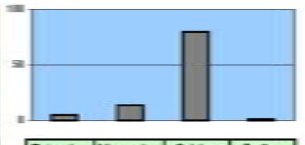
GROWER:

SUBMITTED BY: EVA GRIMME

Graphical Soil Analysis Report

DATE OF REPORT: 09/19/08 LAB NO: 53280 SAMPLE ID: Amsterdam silt loam PAGE: 2





Analyte	Organic Matter %	Nitrogen NO_3-N ppm	Phosphorus Weak Bray ppm	Phosphorus $NaHCO_3$-P ppm	Potassium K ppm	Magnesium Mg ppm	Calcium Ca ppm	Sodium Na ppm	Sulfur SO_4-S ppm	Zinc Zn ppm	Manganese Mn ppm	Iron Fe ppm	Copper Cu ppm	Boron B ppm	Chloride Cl ppm
Results:	2.2	53	6	44	383	337	3187	55	32	2.5	3	4	1.0	0.9	
		LOW	AVERAGE	HIGH											

Potassium K %	Magnesium Mg %	Calcium Ca %	Sodium Na %
4.9	13.9	79.9	1.2
ACIDIC		BASIC	

ECe	CEC	Ex. Lime	pH	Buffer pH
0.8	19.9	H	8.0	
INCREASING SALINITY →	meq/100g		INCREASING NEED FOR LIME ←	

Weak Bray P unreliable at M or H excess lime or pH > 7.5

Soil Fertility Guidelines

CROP: RATE: NOTES:

Dolomite (70 score)	Lime (70 score)	Gypsum	Elemental Sulfur	Nitrogen N	Phosphate P_2O_5	Potash K_2O	Magnesium Mg	Sulfur SO_4-S	Zinc Zn	Manganese Mn	Iron Fe	Copper Cu	Boron B	

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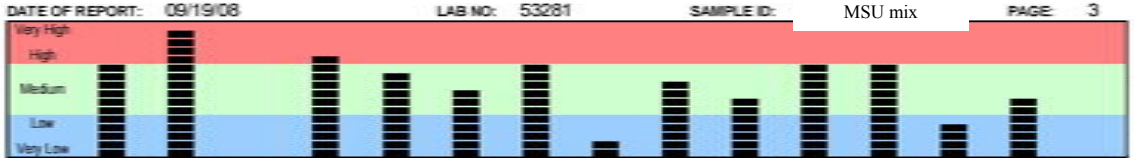
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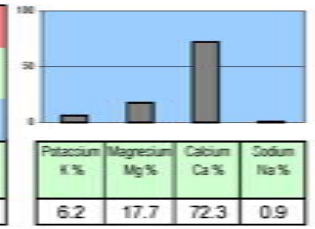
SUBMITTED BY: EVA GRIMME

Graphical Soil Analysis Report

DATE OF REPORT: 09/19/08 LAB NO: 53281 SAMPLE ID: MSU mix PAGE: 3



Analyte	Organic Matter %	Nitrogen NO_3-N ppm	Phosphorus Weak Bray ppm	Phosphorus $NaHCO_3$-P ppm	Potassium K ppm	Magnesium Mg ppm	Calcium Ca ppm	Sodium Na ppm	Sulfur SO_4-S ppm	Zinc Zn ppm	Manganese Mn ppm	Iron Fe ppm	Copper Cu ppm	Boron B ppm	Chloride Cl ppm
Results:	4.3	74	30	21	427	383	2568	35	23	2.1	19	20	0.7	0.8	
		LOW	AVERAGE	HIGH											



Potassium K %	Magnesium Mg %	Calcium Ca %	Sodium Na %
6.2	17.7	72.3	0.9
ACIDIC		BASIC	



Weak Bray P unreliable at M or H excess lime or pH > 7.5

Soil Fertility Guidelines

CROP: RATE: NOTES:

Dolomite (70 score)	Lime (70 score)	Gypsum	Elemental Sulfur	Nitrogen N	Phosphate P_2O_5	Potash K_2O	Magnesium Mg	Sulfur SO_4-S	Zinc Zn	Manganese Mn	Iron Fe	Copper Cu	Boron B	

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APPENDIX B

Muscodor albus DNA SEQUENCE

DEFINITION *Muscodor albus* CZ 620 internal transcribed spacer region sequence

ORGANISM *Muscodor albus* isolate CZ-620

ORIGIN

```
     1 AGNCTCGTGG TGACAGCGGA GGGATCATTA CAGAGTTTTC CAAACTCCCA ACCCTATGTG
    61 AACTTACCTT TGTTGCTTCG GCGGCGGAGG CTACCCTATA GGGGATACCA CATAGTGGTT
   121 ACCCTGTAGT CCCAGGTGCT AGATCGTGCT CAACGTCTTA TCGTCTACGA CTAGCTACCC
   181 GGTGGCCCTC CCCGCCGGCG GCCAACTAAA CTCTGTTTTT ATGGCATTCT GAATTATAAA
   241 CTTAATAAGT TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA
   301 GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC
   361 ACATTGCGCC CATTAGCATT CTAGTGGGCA TGCCTGTTCG AGCGTCATTT CACCACTTAA
   421 GCCCTGTTGC TTAGCGTTGG GAGCCTACGG CACTGCCCGT AGCTCCCTAA AGTGATTGGC
   481 GGAGTTGGTT CTCACTCTAG GCGTAGTAAA TCTATCTCGC CTCTGTAGTG GTTCCGGCCC
   541 CTGCCGTAAA ACCCCCTATA TCAAAGGTTG ACCTCGGATC AGGTAGGAAT ACCCGCTGAA
   601 CTTAAGCATA TCAAAA
```

ANALYSIS

gb|AY927993.1| *Muscodor albus* internal transcribed spacer 1, partial sequence;
5.8S ribosomal RNA gene, complete sequence; and internal
transcribed spacer 2, partial sequence Length = 639

Score = 1107 bits (599), Expect = 0.0
Identities = 599/599 (100%), Gaps = 0/599 (0%)
Strand=Plus/Plus

```
Query  16   GCGGAGGGATCATTACAGAGTTTTCCAAACTCCCAACCCTATGTGAACTTACCTTTGTTG  75
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  26   GCGGAGGGATCATTACAGAGTTTTCCAAACTCCCAACCCTATGTGAACTTACCTTTGTTG  85
```

```
Query  76   CTTCGGCGGCGGAGGCTACCCTATAGGGGATACCACATAGTGGTTACCCTGTAGTCCCAG  135
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  86   CTTCGGCGGCGGAGGCTACCCTATAGGGGATACCACATAGTGGTTACCCTGTAGTCCCAG  145

Query  136  GTGCTAGATCGTGCTCAACGTCTTATCGTCTACGACTAGCTACCCGGTGGCCCTCCCCGC  195
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  146  GTGCTAGATCGTGCTCAACGTCTTATCGTCTACGACTAGCTACCCGGTGGCCCTCCCCGC  205

Query  196  CGGCGGCCAACTAAACTCTGTTTTTATGGCATTCTGAATTATAAACTTAATAAGTTAAAA  255
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  206  CGGCGGCCAACTAAACTCTGTTTTTATGGCATTCTGAATTATAAACTTAATAAGTTAAAA  265

Query  256  CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG  315
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  266  CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG  325

Query  316  TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTA  375
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  326  TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTA  385

Query  376  GCATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCACCACTTAAGCCCTGTTGCTTAGC  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  386  GCATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCACCACTTAAGCCCTGTTGCTTAGC  445

Query  436  GTTGGGAGCCTACGGCACTGCCCGTAGCTCCCTAAAGTGATTGGCGGAGTTGGTTCTCAC  495
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  446  GTTGGGAGCCTACGGCACTGCCCGTAGCTCCCTAAAGTGATTGGCGGAGTTGGTTCTCAC  505

Query  496  TCTAGGCGTAGTAAATCTATCTCGCCTCTGTAGTGGTTCCGGCCCCTGCCGTAAAACCCC  555
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506  TCTAGGCGTAGTAAATCTATCTCGCCTCTGTAGTGGTTCCGGCCCCTGCCGTAAAACCCC  565

Query  556  CTATATCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA  614
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566  CTATATCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA  624
```

APPENDIX C

Colletotrichum coccodes DNA SEQUENCE

DEFINITION *Colletotrichum coccodes* internal transcribed spacer region sequence

ORGANISM *Colletotrichum coccodes*

ORIGIN

```
      1 AGGGCTCGCT GGTGACCAGC TTACTGAGGT TACCGCTCTA TAACCCTTTG CTGAACATAC
     61 CTAACTGTTG CTTCGGCGGG CAGGGGGTGC CGNCTGCGNG ACCCCCCTCC CGGNCCTGCC
    121 CATCNCGGGC GGAGCGCCCG CCGGAGGATA CCAAACTCTA TTTTAACGAC GTTTCTTCTG
    181 AGTGGCACAA GCAAATAATT AAAACTTTCA ACAACGGATC TNTTGGTTCT GGCATCGATG
    241 AANAACGCAA CGAAATGCGA TAAGTAATGT GAATTGCANA ATTCAGTGAA TCATCGAATC
    301 TTTGAACGCA CATTGCGCCC GCCAGCATTC TGGCGGGNAT GCCTGTTCGA GCGTNATTTC
    361 AACCCTCAAG CTCTGCTTGG TGTTGGGGCC CTACGGNTGA CGTACGCCCT TAAAGGNAGT
    421 GGCGGACCCT CTCGGAGCCT CCTTTGCGTA GTAACTAACG TCTCGCACTG GGATTCGGAG
    481 GGACTCTTGC CNTAAAACCC CCAAATTTTT AAAGGTTGAC CTCGGATCAG GTAGGAATAC
    541 CCGCTGAACT TAANCATATC AAAAGGCGGA GGAA
```


ANALYSIS

dbj|AB233340.1| *Colletotrichum coccodes* genes for 5.8S rRNA, ITS1, 18S rRNA,
ITS2, 28S rRNA, partial and complete sequence, isolate:MAFF 306629, Length=576

Score = 955 bits (517), Expect = 0.0
Identities = 553/575 (96%), Gaps = 7/575 (1%)
Strand=Plus/Plus

```
Query  11   GGTG-ACCAGCGGAGGGATCATTACTGAGGTTACCGCTCTATAACCCTTTGCTGAACATA  69
            |||| ||| |||||||||||||||||| |||||||||||||||||||||| ||||||||
Sbjct  7    GGTGAACCTGCGGAGGGATCATTACTGA-GTTACCGCTCTATAACCCTTTG-TGAACATA  64
```

```
Query  70   CCTAACTGTTGCTTCGGCGGGCAGGGGGTGCCGNCTGCGNGACCCCCCTCCCGGNCCTGC  129
            ||||||||||||||||||||||||||||||| ||||| ||||||||||||| |||||
Sbjct  65   CCTAACTGTTGCTTCGGCGGGCAGGGGGTGCCGCCTGCG-GACCCCCCTCCCGGCCCTGC  123

Query  130  CCATCNCGGGCGGAGCGCCCGCCGGAGGATACCAAACTCTATTTTAACGACGTTTCTTCT  189
            || || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  CC-TCACGGGCGGAGCGCCCGCCGGAGGATACCAAACTCTATTTTAACGACGTTTCTTCT  182

Query  190  GAGTGGCACAAGCAAATAATTAAAACTTTCAACAACGGATCTNTTGGTTCTGGCATCGAT  249
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  183  GAGTGGCACAAGCAAATAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  242

Query  250  GAANAACGCAACGAAATGCGATAAGTAATGTGAATTGCANAATTCAGTGAATCATCGAAT  309
            ||| |||||| ||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  243  GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT  302

Query  310  CTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGNATGCCTGTTCGAGCGTNATTT  369
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||| ||||
Sbjct  303  CTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT  362

Query  370  CAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACGGNTGACGTACGCCCTTAAAGGNAG  429
            |||||||||||||||||||||||||||||||||||||| ||||||| |||||||||| ||
Sbjct  363  CAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACGGTTGACGTAGGCCCTTAAAGGTAG  422

Query  430  TGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCGGA  489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  TGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCGGA  482

Query  490  GGGACTCTTGCCNTAAAACCCCCAAATTTTTAAAGGTTGACCTCGGATCAGGTAGGAATA  549
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  GGGACTCTTGCCGTAAAACCCCCAAATTTTTAAAGGTTGACCTCGGATCAGGTAGGAATA  542

Query  550  CCCGCTGAACTTAANCATATCAA-AAGGCGGAGGA  583
            ||||||||||||| |||||||| ||| |||||||
Sbjct  543  CCCGCTGAACTTAAGCATATCAATAAG-CGGAGGA  576
```

APPENDIX D

Verticillium dahliae DNA SEQUENCE

DEFINITION *Verticillium dahliae* internal transcribed spacer region sequence

ORGANISM *Verticillium dahliae*

ORIGIN

```
      1 AGGCTCGTGG TGACAGCGGA GGGATCATTA CCGAGTATCT ACTCATAACC CTTTGTGAAC
     61 CATATTGTTG CTTCGGCGGC TCGTTCTGCG AGCCCGCCGG TCCATCAGTC TCTCTGTTTA
    121 TACCAACGAT ACTTCTGAGT GTTCTTAGCG AACTATTAAA ACTTTTAACA ACGGATCTCT
    181 TGGCTCTAGC ATCGATGAAG AACGCAGCGA AACGCGATAT GTAGTGTGAA TTGCAGAATT
    241 CAGTGAATCA TCGAATCTTT GAACGCACAT GGCGCCTTCC AGTATCCTGG GAGGCATGCC
    301 TGTCCGAGCG TCGTTTCAAC CCTCGAGCCC CAGTGGCCCG GTGTTGGGGA TCTACGTCTG
    361 TAGGCCCTTA AAAGCAGTGG CGGACCCGCG TGGCCCTTCC TTGCGTAGTA GTTACAGCTC
    421 GCATCGGAGT CCCGCAGGCG CTTGCCTCTA AACCCCCTAC AAGCCCGCCT CGTGCGGCAA
    481 CGGTTGACCT CGGATCAGGT AGGAATACCC GCTGAACTTA AGCATATCAT A
```

ANALYSIS

emb|AJ865691.1| *Verticillium dahliae* 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain MAT-756 Length=514

Score = 950 bits (514), Expect = 0.0
Identities = 514/514 (100%), Gaps = 0/514 (0%)
Strand=Plus/Plus

```
Query  14   CAGCGGAGGGATCATTACCGAGTATCTACTCATAACCCTTTGTGAACCATATTGTTGCTT  73
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CAGCGGAGGGATCATTACCGAGTATCTACTCATAACCCTTTGTGAACCATATTGTTGCTT  60
```

```
Query  74   CGGCGGCTCGTTCTGCGAGCCCGCCGGTCCATCAGTCTCTCTGTTTATACCAACGATACT  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CGGCGGCTCGTTCTGCGAGCCCGCCGGTCCATCAGTCTCTCTGTTTATACCAACGATACT  120

Query  134  TCTGAGTGTTCTTAGCGAACTATTAAAACTTTTAACAACGGATCTCTTGGCTCTAGCATC  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCTGAGTGTTCTTAGCGAACTATTAAAACTTTTAACAACGGATCTCTTGGCTCTAGCATC  180

Query  194  GATGAAGAACGCAGCGAAACGCGATATGTAGTGTGAATTGCAGAATTCAGTGAATCATCG  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GATGAAGAACGCAGCGAAACGCGATATGTAGTGTGAATTGCAGAATTCAGTGAATCATCG  240

Query  254  AATCTTTGAACGCACATGGCGCCTTCCAGTATCCTGGGAGGCATGCCTGTCCGAGCGTCG  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATCTTTGAACGCACATGGCGCCTTCCAGTATCCTGGGAGGCATGCCTGTCCGAGCGTCG  300

Query  314  TTTCAACCCTCGAGCCCCAGTGGCCCGGTGTTGGGGATCTACGTCTGTAGGCCCTTAAAA  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTTCAACCCTCGAGCCCCAGTGGCCCGGTGTTGGGGATCTACGTCTGTAGGCCCTTAAAA  360

Query  374  GCAGTGGCGGACCCGCGTGGCCCTTCCTTGCGTAGTAGTTACAGCTCGCATCGGAGTCCC  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCAGTGGCGGACCCGCGTGGCCCTTCCTTGCGTAGTAGTTACAGCTCGCATCGGAGTCCC  420

Query  434  GCAGGCGCTTGCCTCTAAACCCCCTACAAGCCCGCCTCGTGCGGCAACGGTTGACCTCGG  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCAGGCGCTTGCCTCTAAACCCCCTACAAGCCCGCCTCGTGCGGCAACGGTTGACCTCGG  480

Query  494  ATCAGGTAGGAATACCCGCTGAACTTAAGCATAT  527
            ||||||||||||||||||||||||||||||||
Sbjct  481  ATCAGGTAGGAATACCCGCTGAACTTAAGCATAT  514
```