EFFECTS OF MYCOFUMIGATION USING *MUSCODOR ALBUS* AND *MUSCODOR ROSEUS* ON DISEASES OF SUGAR BEET AND CHRYSANTHEMUM

by

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

of

Master of Science

in

Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2004

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ABSTRACT

Methyl bromide-chloropicrin mixtures are the major chemicals used for soil disinfestation in the U.S. These chemicals are highly efficacious fumigants used for pre-plant soil fumigation in field and greenhouse production. They control soil-borne diseases, insects, nematodes and weeds. The 1996 International agreement to phase-out methyl bromide for soil fumigation will seriously reduce options for controlling soil-borne pathogens. Mycofumigation is the use of antimicrobial volatiles produced by fungi such as *Muscodor albus* and *Muscodor roseus* for the control of other microorganisms. A synthetic mixture of organic components, which were originally identified in *M. albus* gases, provided disease control equal to the formulated fungus in terms of reducing damping-off by *Aphanomyces cochlioides*, *Pythium ultimum*, and *Rhizoctonia solani*. The effect of growth substrates for *M. albus* and *M. roseus*, their placement and fumigation period on efficacy for control of sugar beet seedling diseases was studied. Optimal growth substrates for *M. albus* were barley, canola, oat, and stabileze while optimal growth substrates for *M. roseus* were oat and barley. A 1-week mycofumigation period provided better control of damping-off than direct planting for both *Muscodor* sp. Mixing substrates with soil was superior to placement in the upper 25 % of the pot in Rhizoctonia-infested soil, whereas placement the in the upper 25 % of the pot was superior in Aphanomyces- and Pythium-infested soil. Field experiments indicate that mycofumigation of Aphanomyces-infested soil, buried 20 and 30 cm deep in field soil, increased seedling establishment of sugar beets. A winter survival study confirmed that *M. albus* does not survive the winter when buried (0 to 45 cm) in field soil in Montana. Storage of *Muscodor* sp. stabileze in a starch /sucrose / silica formulation was effective at - 10 °C and 3 to 5 °C for 5 months. In chrysanthemum experiments mycofumigation with *M. albus* and *M. roseus* resulted in significantly decreased Verticillium stem colonization compared to the *V. dahliae* pathogen control.

CHAPTER 1

INTRODUCTION

Methyl bromide-chloropicrin mixtures are the major chemicals used for soil disinfestation in the U.S. These chemicals are highly efficacious fumigants used for pre-plant soil fumigation in field and greenhouse production (Raabe et al., 2002). They control soil-borne diseases, insects, nematodes and weeds. The phase-out of methyl bromide for soil fumigation, based on its nature to deplete the ozone layer, will seriously reduce options for controlling soil-borne diseases (www.nps.ars.usda.gov., 2002). Pre-plant soil fumigation with chloropicrin-methyl bromide combinations are most common and the main treatment against soil-borne diseases such as *Pythium ultimum*, *Aphanomyces cochlioides*, *Rhizoctonia solani*, and *Verticillium dahliae* (Raabe et al., 2002). Post-planting fungicide drenches are sometimes used for control of soil-borne diseases (Daughtrey et al., 1995). Soil solarization is effective for soil disinfestation in warm and sun intensive climates (Coelho et al., 1999). In addition, steam pasteurization has been widely used. This method is limited by the availability of steam generators (Forsberg, 1963; Daughtrey et al., 1995).

Current research on methyl bromide replacements includes developing management systems utilizing alternative chemicals (Duniway et al., 2000), biofumigation with *Brassica* spp. (Sarwar et al., 1998) and organic amendments/biological control (Cuester and Hoitink, 1999). At this time, these

disinfestation methods fit niches as alternatives for methyl bromide replacement, but none offer the broad-spectrum activity and efficacy of methyl bromide.

Mycofumigation is the use of antimicrobial volatiles produced by fungi such as *Muscodor albus* and *Muscodor roseus* for the control of other microorganisms (Jacobsen et al., 2004; Stinson et al., 2003). Mycofumigation using *Muscodor* sp. is a new concept of soil-fumigation with a broad application potential and it is critical to understand the effects of the mycofumigant fungi and their volatile organic compounds on plant pathogenic and beneficial organisms. The relatively non-toxic nature (www.cdc.gov/niosh/npg/npg.html) of the volatile gases of *M. albus* and *M. roseus* (Strobel et al., 2001) promise to increase worker safety during soil fumigation and may provide a healthier environment for humans, animals and plants compared to methyl bromide and most of its chemical alternatives, which are extremely toxic to humans and animals (www.ars.usda.gov, 2002; www.epa.gov/ozone/mbr/qa.html, 2004.)

Mycofumigant Fungi

Muscodor albus

M. albus, isolate 620, is an endophytic fungus isolated from small limbs of a mature *Cinnamomum zeylanicum* (cinnamon tree) west of La Ceiba, Honduras (Worapong et al., 2001). *M. albus* is characterized as a deuteromycetous (mycelia sterilia) endophytic species and is related to the ascomycetous group *Xylaria* due to 96 – 98 % homology of its 18S rDNA (2089 bp) (Worapong et al., 2001). Isolate 620 could not be named *Xylaria* sp. because no fruiting structures have ever been observed. *M.*

albus produces a whitish mycelium with 6-12 pie shaped sectors forming from the center of the culture after 4-6 weeks of growth on PDA (potato dextrose agar, Difco Laboratories, Detroit, MI) (Worapong et al., 2001). Hyphae are 1.1 – 1.7 µm in diameter, commonly grow by 90° angle branches, and intertwine and make rope-like strands (3.0 – 5.0 µm diameter). Under cultural conditions the fungus produces gases with a musty odor (Worapong et al., 2001).

Strobel et al., 2001, analyzed the volatiles produced by *M. albus* quantitatively and qualitatively using gas chromatography and mass spectroscopy GC/MS (Table 1.1). The identified volatile compounds fell into the chemical classes of alcohols, esters, ketones, acids, and lipids, all of which are considered to have low toxicity to mammals and higher plants (Strobel et al., 2001).

The influence of the volatiles emitted by *M. albus* on several fungi and bacteria was tested in *in vitro* assays (Strobel et al., 2001). The test organisms, grown on PDA, were exposed to the volatile compounds for 2 days, transferred to new PDA plates without exposure to the volatiles and observed for viability (Table 1.2). Most organisms tested were killed after exposure to volatiles of *M. albus*. A few organisms such as the fungi *Fusarium solani*, *Cercospora beticola*, and *Xylaria* sp. were only partially inhibited or not affected at all. Furthermore, beneficial soil organisms such as *Trichoderma* sp. and *Gliocladium* sp. were not inhibited by *M. albus* (Table 1.2).

Table 1.1: Gas chromatography / Mass spectroscopy analysis of the volatile compounds produced by *M. albus* (Strobel et al., 2001)

RT (min : s)	Total area (%)	*m / z*	Possible compound	M_r
3 : 45	0.33	114	Octane	114
4 : 19	0.93	58	Acetone	58
4 : 37	0.68	74	Acetic acid, methyl ester	74
5 : 56	7.63	88	Acetic acid, ethyl ester	88
6 : 51	0.31	102	Propanoic acid, 2-methyl, methyl ester	102
7 : 16	6.24	∗	Ethanol	46
8 : 03	2.07	116	Propanoic acid, 2-methylethyl ester	116
11 : 45	0.58	∗	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12 : 05	2.06	74	Isobutyl alcohol	74
12 : 50	22.24	∗	1-Butanol, 3-methyl-, acetate	130
14 : 57	1.53	∗	Propanoic acid, 2-methyl, 3-methylbutyl ester	158
15 : 28	22.99	∗	1-Butanol, 3-methyl-	88
16 : 08	0.29	138	†Furan, 2-pentyl-	138
18 : 53	0.29	142	†4-Nonanone	142
20 : 38	0.41	142	2-Nonanone	142
21 : 07	0.30	204	†Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4a*R*-*trans*)-	204
22 : 54	1.51	204	†Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S*-(1.alpha.,4.alpha.,7.alpha.)]	204
23 : 16	0.94	204	†Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204
25 : 20	3.63	204	†1H-3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-,[3*R*-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]	204
25 : 30	6.08	88	Propanoic acid, 2-methyl	88
26 : 04	0.48	204	Caryophyllene	204
27 : 55	0.34	204	†Naphthalene, 1,3,4a,5,6,8a-hexahydro-4,7-dimethyl-1-1-methylethyl)-,[(1*R*-(1.alpha.,4a.alpha.,8a.alpha.)]	204
28 : 34	0.36	204	†Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-	204
28 : 50	1.07	204	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1, 4-dimethyl-7-(1-methylethyenyl)-, [1*S*-(1.alpha.,7.alpha.,8a.beta.)] (common name: bulnesene)	204
28 : 57	3.24	204	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-,[1*R*-(1.alpha.,7.beta.,8a.alpha.)] (common name: valencene)	204
31 : 12	1.74	∗	Acetic acid, 2-phenylethyl ester	164
33 : 17	1.06	122	Phenylethyl alcohol	122
39 : 00	9.76	204	†Unknown	204

∗ No molecular-ion peak was observed in the spectrum of either the standard compound or the compound undergoing the analysis.
† Denotes that a spectrum and retention time of this component were observed and the substance matched to the most likely compound in the NIST database, but the data have not been confirmed by use of an appropriate standard compound by either retention time or MS. These compounds were not placed in the artificial mixture in the bioassay.
RT = retention time; *m / z* = molecular weight; M_r = observed molecular weight

Table 1.2: Influence of the volatile compounds of *M. albus* on several fungi and bacteria

Test organism	Growth in presence of *M. albus* after 2 days? (YES / NO)	Growth when transferred to a new plate after 2 days (YES/NO)
Aspergillus fumigatus	N	N
Bacillus subtilis	N	Y
Candida albicans	N	N
Cercospora beticola	Y	Y
Colletotrichum coccoides	N	N
Erwinia amylovora	N	N
Erwinia carotovora	N	N
Escherichia coli	N	N
Fusarium sambucinum	N	Y
Fusarium solani	Y	Y
Gliocladium sp.	Y	Y
Helminthosporium solani	N	N
Micrococcus luteus	N	N
Muscodor albus	Y	Y
Penicillium roqueforti	N	N
Phytophthora cactorum	N	N
Phytophthora cinnamomi	N	N
Phytophthora erythroseptica	N	N
Pythium debaryanum	N	N
Pythium ultimum	N	N
Rhizoctonia solani	N	N
Sclerotinia sclerotiorum	N	N
Streptomyces scabies	N	N
Trichoderma sp.	Y	Y
Ustilago hordei	N	N
Verticillium dahliae	N	N
Xylaria sp.	Y	Y

A synthetic biorational mixture of the volatiles emitted by *M. albus* was produced by using authentic standard chemicals obtained from Sigma-Aldrich (St. Louis, MO) and

synthesized propanoic acid esters (Strobel et al., 2001). The compounds of the mixture were adjusted to the same proportions as the compounds identified in the naturally occurring volatile organic compounds, VOCs, (Strobel et al., 2001) (Table 1.3).

Table 1.3: Components of *Muscodor albus* biorational mixture*

Chemical compound	**%** **
Octane	0.47
Acetone	1.36
Acetic acid, methyl ester	0.99
Acetic acid, ethyl ester	11.14
Propanoic acid, 2-methylethyl ester	3.03
Isobutyl alcohol	3.01
1-Butanol, 3-methyl-, acetate	32.47
Propanoic acid, 2-methyl, 3-methylbutyl ester	2.24
1-Butanol, 3-methyl-	33.58
2-Nonanone	0.60
Propanoic acid, 2-methyl	8.86
Caryophyllene	0.71
Phenylethyl alcohol	1.54

* The relative percent of each compound used in the biorational mixture was based on the gas chromatography and mass spectroscopy analysis of the volatiles produced by 10 day old cultures of *M. albus* grown on PDA (Strobel et al., 2001).
** The compounds of the mixture were adjusted to the same proportions as the compounds identified in the naturally occurring volatile organic compounds (Volume / Volume concentration) (Strobel et al., 2001).

<u>*Muscodor roseus*</u>

M. roseus, isolate A 3-5, is an endophytic fungus isolated from small limbs of a fern-leafed Grevillea (*Grevillea pteridifolia*) in the Northern Territory of Australia (Worapong et al., 2001). *M. roseus* is characterized as a deuteromycetous (mycelia sterilia) endophytic species and is related to the ascomycetous group *Xylaria* due to 96 –

98 % homology of its 18S rDNA (2055 bp) (Worapong et al., 2001). High similarity of the 18S rDNA also identified *M. roseus* as a close relative to *M. albus*. Similar to *M. albus*, no fruiting structures of *M. roseus* have ever been observed. *M. roseus* produces a pinkish felt-like mycelium with pie shaped sectors forming from the center of the culture after 4-6 weeks of growth on PDA (Worapong et al., 2001). Hyphae are 0.8 – 3.6 µm in diameter, intertwine, and make rope-like strands (Worapong et al., 2001). The formation of tight coils (20 µm diameter) of multiple loops of single fungal hypha is frequently observed (Worapong et al., 2001). When grown on PDA, the fungus produces gases with a musty odor similar to *M. albus* (Worapong et al., 2001).

The effects of the volatiles emitted by *M. roseus* were tested on different fungi and bacteria in *in vitro* assays (Ezra and Strobel, 2003). It was observed that the volatiles had anti-microbial properties which were not as active as those of *M. albus*. Therefore only an artificial mixture of *M. albus* was tested as part of this research. The use of the synthetic mixture of organic compounds found in volatiles produced by *M. albus* for control of sugar beet (*Beta vulgaris* L.) seedling diseases caused by *Aphanomyces cochlioides*, *Rhizoctonia solani* AG 2-2 and *Pythium ultimum* is described in detail in chapter 2. Current research addresses the potential of *M. albus* for control of soil-borne pathogens and nematodes, and for control of diseases of seeds. Different formulations of the fungus and application methods, such as in furrow application, are under observation.

Soil-Borne Pathogens

Soil-borne pathogens like *Aphanomyces cochlioides, Pythium ultimum*, *Rhizoctonia solani*, and *Verticillium dahliae* cause economically important yield losses by reducing seedlings emergence, damping-off, causing root rots, and therefore yield reduction (Berlanger and Powelson, 2000; Schneider and Whitney, 1986). The weakened plants are more susceptible to secondary infections. Unlike the seedlings diseases mentioned above, *Verticillium dahliae* can infect plants at any growth stage and will result in wilting, foliar chlorosis and necrosis (Berlanger and Powelson, 2000). Therefore for this study relevant soil-borne pathogens are described in the following pages in detail with emphasis on their biology.

Aphanomyces cochlioides

Aphanomyces cochlioides is the causal agent of Aphanomyces root rot and black root (Franc et al., 2001). *A. cochlioides* is characterized as an oomycete, reproduces asexually by short-living zoospores and sexually by oospores (thick-walled resting spores) (Dyer and Windels, 2003). The oospores are produced by mating of a homothallic, subspherical,and terminal oogonium and up to 5 antheridia (Schneider and Whitney, 1986). The zoospores develop in irregular massed on filamentous sporangia produced by oospores (Schneider and Whitney, 1986). Oospores overwinter in plant debris in the soil (Dyer and Windels, 2003). Aphanomyces root rot has a negative effect on the mature sugar beet. It causes wilting of the foliage and decay of the taproot. Aphanomyces black root is a form of post-emergence damping-off which occurs only in

the seedling phase and does not affect the seed or the initial stand establishment (Franc et al., 2001). *A. cochlioides* attacks the hypocotyls near the soil level and results in grayish, water-soaked lesions which in progress turn black and weaken the stem. Finally the seedlings will break at this point (Franc et al., 2001). Infection of the hypocotyls is initiated when overwintering oospores germinate and infect the plant directly. Disease also occurs by infection through motile zoospores, which like the oospores, require warm (soil temperature 18-32 °C) and wet soils (Franc et al., 2001). Recommended management strategies for *A. cochlioides* include crop rotation, well-drained soils, control of weed hosts, seed treatments with the fungicide Tachigaren (hymexazol) and the use of tolerant plant varieties (Schneider and Whitney, 1986; Franc et al., 2001).

Pythium ultimum

P. ultimum, the one of several Pythium species causing root rot, is characterized as an oomycete. Sexual reproduction is in form of mating of terminal or occasionally intercalary oogonia and 1 to 2 intercalary, barrel-shaped, usually monoclinous antheridia (Schneider and Whitney, 1986). The oospores germinate and infect plant roots directly (Schneider and Whitney, 1986). In contrast to *A. cochlioides, P. ultimum* can cause pre- and post-emergence damping-off and can also affect mature plants by continuous rotting of feeder roots. Young plants are infected in the late spring, early summer, when overwintering oospores germinate in warm soils (higher than 27 °C). Additional high soil moisture will favor pre-emergence damping-off by killing the seeds. The main dissemination factor would be through infested soils or through soil water (Franc et al.,

2001). *P. ultimum* can be controlled by reduction of high levels of soil moisture for long periods, like planting in raised beds, drainage promoting tillage, and avoidance of excessive irrigation (Franc et al., 2001). Initial stand establishment can be protected by seed treatments with the fungicides Tachigaren (hymexazol) or Ridomil (metalaxyl) (Franc et al., 2001).

<u>*Rhizoctonia solani*</u>

R. solani is commonly characterized as a deuteromycetous fungus (Fungi Imperfecti) since its basisiomycete perfect stage, *Thanatephorus* sp., is commonly not involved in the disease cycle. Two anastomosis groups (AG) of *R. solani* are found causing damping-off of sugar beet, AG-4 and AG 2-2. On mature sugar beets *Rhizoctonia solani* AG 2-2 can cause a blackish discoloration of the petioles, starting at the crown, which will begin as a permanent wilting and results in dark necrotic tissue. Also proceeding from the crown, the infection of the root will start as small dark brownish lesions, which will finally cover the entire root surface (Franc et al., 2001). Management of *R. solani* includes the use of resistant plant varieties, adequate soil drainage, crop rotation, and control of weed hosts (Franc et al., 2001).

<u>*Verticillium dahliae*</u>

Verticillium dahliae is the causal agent of Verticillium wilt of many plant species. *V. dahliae* is characterized as deuteromycete (Fungi Imperfecti) since no sexual stage is known (Berlanger and Powelson, 2000). The fungus multiplies through production of conidia on special hyphae, named phialides, which are produced in a whorl around each

conidiophore (Berlanger and Powelson, 2000). The soil-borne pathogen overwinters in plant debris in the soil as microsclerotia (masses of melanized hyphae) (Rowe and Powelson, 2002). The germination of the resting structures is triggered by root exudates (Rowe and Powelson, 2002). The fungal hyphae penetrate the root of the host plant and colonize the vascular tissue of its host plant (Rowe and Powelson, 2002). Characteristics of all host plants are stunted plants, premature foliar chlorosis, and necrosis and vascular discoloration in stems and roots. Symptoms of wilting are most visible on warm and sunny days. Once a plant is infected with *V. dahliae*, no cure is possible (Berlanger and Powelson, 2000). For the control of V. dahliae, crop rotation of 3 to 4 years and avoidance of host plants are recommended. The introduction of infested plant material should be avoided (Franc et al., 2001). Methyl bromide-chloropicrin mixtures are the major chemicals used for soil disinfestation in U.S. (www.ars.usda.gov., 2002).

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CHAPTER 2

CONTROL OF SUGAR BEET SEEDLING DISEASES WITH A SYNTHETIC MIXTURE OF VOLATILE COMPOUNDS PRODUCED BY *MUSCODOR ALBUS*

Introduction

Muscodor albus, isolate 620 (Worapong et al., 2001), an endophytic fungus obtained from *Cinnamomum zeylanicum* L. in Honduras, produces a mixture of volatile organic compounds (VOCs), which effectively inhibit a wide range of microorganisms (Ezra and Strobel, 2003; Jacobsen et al., 2004; Strobel et al., 2001). The antimicrobial use of the *Muscodor* species and their volatile compounds is named "mycofumigation" (Stinson et al., 2003; Zidack et al., 2001 and 2002). The VOCs produced include the following chemical classes: alcohols, esters, ketones, acids, and lipids, all of which are considered to have low toxicity to mammals and higher plants (Strobel et al., 2001).

In greenhouse assays with *M. albus* and its close relative *M. roseus* (Stinson et al., 2003; Worapong et al., 2001; Zidack et al., 2001), and in field experiments with *M. albus* (Grimme, unpublished), reduction of sugar beet (*Beta vulgaris* L.) diseases caused by the soil-borne pathogens *Aphanomyces cochlioides*, *Rhizoctonia solani*, and *Pythium ultimum* were shown. Reduction of disease was also recorded for Verticillium wilt of eggplants (*Solanum melongena* L.) caused by *Verticillium dahliae*.

In the U.S., soil-fumigation is highly dependent on the use of methyl bromide and methyl bromide-chloropicrin mixtures. Current research on methyl bromide alternatives

includes developing management systems utilizing alternative chemicals (Duniway et al., 2000), biofumigation with *Brassica* spp. (Sarwar et al., 1998), and organic amendments/biological control (Cuester and Hoitink, 1999). At this time these alternatives fit niches for a methyl bromide replacement, but none offer the broad-spectrum activity and efficacy of methyl bromide. Mycofumigation using *Muscodor* sp. is a new concept of soil-fumigation with a broad application potential and it is critical to understand the effects of the mycofumigant fungi and their volatile organic compounds on plant pathogenic and beneficial organisms. This study compares a synthetic mixture of components of *M. albus'* VOCs with the natural gas produced by this fungus while growing on stabileze formulation (Quimby et al., 1999). Synthetic mimics of natural pesticidal compounds are termed biorational pesticides. Examples include various strobilurin class fungicides that mimic strobilurin A produced by *Strobiluris tenacellus* (Pers. Ex Fr.) Sing. (Anke et al., 1977) and fludioxonil that mimics an antibiotic of *Pseudomonas* sp. (Leadbitter et al., 1994).

Materials and Methods

Muscodor sp. Formulations and Storage

The mycofumigant fungus culture of *M. albus*, isolate 620, used in the bioassay was obtained from Dr. Gary A. Strobel of the Department of Plant Sciences and Plant Pathology, Montana State University.

M. albus was grown and maintained in 10 cm Petri plates containing potato dextrose agar (PDA, Difco Laboratories, Detroit, MI). Fifteen agar plugs (5 mm

diameter) were transferred from 3-week-old Petri plate cultures maintained at 23 °C ± 1 °C into separate 2 liter flasks containing 1 liter autoclaved potato dextrose broth (PDB, Difco Laboratories, Detroit, MI), placed on a shaker for 2 weeks at 23 °C ± 1 °C and prepared using the stabileze formulation method (Quimby et al., 1999). This formulation was selected because it was efficacious in tests reported by Stinson et al. (2003). The stabileze formulation was dried for 48 hours under the hood, packed in plastic bags and stored at 4 °C for 2 weeks. This storage method has provided high *M. albus* viability in numerous studies for up to 6 months (Grimme, unpublished).

Artificial Mixture of Volatile Compounds of *M. albus* and Storage

The VOCs produced by *M. albus* were quantitatively and qualitatively determined using gas chromatography and mass spectroscopy GC/MS (Strobel et al., 2001). The synthetic biorational mixture was produced by using authentic standard chemicals obtained from Sigma-Aldrich (St. Louis, MO) and propanoic acid esters that were synthesized (Strobel et al., 2001). The compounds of the mixture were adjusted to the same proportions as the compounds identified in the naturally occurring VOCs (Ezra and Stobel, 2003; Strobel et al., 2001) (Table 2.1). The artificial mixture was stored in vials at -20 °C until being used.

Preparation of Plant-Pathogenic Fungi Inocula

R. solani inoculum was prepared as infested ground barley according to Ruppel et al. (1979). Inoculum production of *A. cochlioides* and *P. ultimum* was prepared by growing fungal cultures on 10 cm Petri plates containing PDA for 2 weeks at 23 °C ± 1

°C. The *R. solani* inoculum consisted of dormant mycelium, whereas the *A. cochlioides* and *P. ultimum* inocula consisted of mycelia, sporangia, and oospores (Stinson et al., 2003).

Table 2.1: Components of *Muscodor albus* biorational mixture*

Chemical compound	**%** **
Octane	0.47
Acetone	1.36
Acetic acid, methyl ester	0.99
Acetic acid, ethyl ester	11.14
Propanoic acid, 2-methylethyl ester	3.03
Isobutyl alcohol	3.01
1-Butanol, 3-methyl-, acetate	32.47
Propanoic acid, 2-methyl, 3-methylbutyl ester	2.24
1-Butanol, 3-methyl-	33.58
2-Nonanone	0.60
Propanoic acid, 2-methyl	8.86
Caryophyllene	0.71
Phenylethyl alcohol	1.54

* The relative percent of each compound used in the biorational mixture was based on the GC/MS analysis of the volatiles produced by 10 day old cultures of *M. albus* grown on PDA (13).
** The compounds of the mixture were adjusted to the same proportions as the compounds identified in the naturally occurring volatile organic compounds (Volume / Volume concentration) (Strobel et al., 2001).

Greenhouse Bioassays for Testing the Efficacy of *Muscodor albus* against Soil-Borne Pathogens

Greenhouse bioassays were designed to measure the efficacy of the gas producing fungus *M. albus* formulated as stabileze and the biorational mixture of the VOCs found in

gases produced by *M. albus* for control of *R. solani*, *A. cochlioides*, and *P. ultimum* on sugar beet (*Beta vulgaris* L.). All experiments were carried out under standardized greenhouse conditions at Montana State University, Plant Growth Center (PGC). The growing substrate (Bozeman silt loam **:** washed concrete sand **:** Canadian sphagnum peat, 1:1:1) used for all experiments was sieved (2 mm mesh opening) and autoclaved at 121 °C for 60 minutes. For soil infestation, 5 g of ground barley inoculum of *R. solani* were mixed thoroughly in 1 kg of soil. One 2-week-old colonized Petri plate of *A. cochlioides* or *P. ultimum* was chopped in small pieces (1 to 2 mm) and mixed with 6.4 kg of soil.

Stabileze formulation was used for mycofumigation by placing 0.5 g of the formulation in a 10 cm Petri plates prior adding of 60 cm^3 of pathogen-infested soil. Fumigation with the biorational mixture was done by adding 60 µl of the artificial mixture to plastic mini-cups, which were placed in the middle of 60 cm^3 of pathogen infested soil in Petri plates. For the pathogen controls, 60 cm^3 of infested soil was placed in Petri plates, and non-infested soil was filled in for the non-infested control. All Petri plates were sealed with two layers of Parafilm (Pechiney Plastic Packaging, Inc., Neenah, WI) and stored for one week at 23 °C ± 1 °C.

After the one-week fumigation period, the soil treatments were added as a top layer onto 400 cm^3 of soil in 10 cm square pots (10 x 10 x 8.6 cm). Twenty-five non-treated Beta 8754 sugar beet seeds were planted into each pot, covered with 80 cm^3 of pasteurized soil, and placed in a greenhouse with 22 °C day-temperature, 18 °C night-temperature, and a photoperiod of 16 hours. The pots of the treatments with the pathogens *A. cochlioides* and *P. ultimum* were placed in shallow saucers with standing water for 3 days

to favor infection (Stinson et al., 2003). For comparison purposes, pots of the non-infested control were also placed in saucers for the same time period. Percent healthy sugar beet seedling establishment was monitored 14, 21, and 28 days after planting.

The experimental design was completely randomized with five replications per treatment for all treatments and the non-infested control. Experiments of all pathogens were repeated two times with similar results. Statistical analysis of the data was conducted by analysis of variance using the general linear model procedure of the SAS program (Madden et al., 1982). The treatment means were separated using Fisher's least significant difference test (Swallow, 1984) at $P < 0.05$.

Results

In soil infested with *R. solani*, mycofumigation with live *M. albus* formulated as stabileze, and fumigation with the biorational mixture, resulted in significantly higher seedling survival at 28 days post planting ($P < 0.0001$) relative to the *R. solani* infested control (Table 2.2). Mycofumigation with the *M. albus* stabileze formulation and fumigation with the biorational mixture resulted in seedling survival statistically similar to the non-infested control (Table 2.2).

In *A. cochlioides*-infested soil, both mycofumigation with live *M. albus* and fumigation with the biorational mixture treatments resulted in higher seedling survival 28 days post planting ($P < 0.0001$) than that in the pathogen control (Table 2.2). *M. albus* formulated as stabileze resulted in seedling survival similar to the non-infested control

treatment while fumigation with the biorational mixture provided seedling survival similar to the live *M. albus* treatment (Table 2.2).

Table 2.2: Percent sugar beet seedling survival after 28 days following mycofumigation of *Rhizoctonia solani, Pythium ultimum, or Aphanomyces cochlioides* infested soil using live *Muscodor albus* (stabileze formulation) or biorational mixture of chemicals found in volatiles emitted by *M. albus*.

	Pathogen used to infest soil [1)]		
Treatment	*R. solani*	*P. ultimum*	*A. cochlioides*
Live *M. albus*	94.4 a	92.0 ab	88.8 ab
Biorational chemical mixture	89.2 ab	77.2 b	78.0 b
Pathogen infested control	71.2 c	2.8 c	0.8 c
Non-infested control	98.4 a	97.6 a	93.6 a

[1]) Soil was infested as follows: *R. solani*, 5 g of dry ground barley inoculum / kg soil; *P. ultimum* and *A. cochlioides*, one homogenized, completely colonized 10 cm Petri plate / 6.4 kg of soil. Means followed by the same letter are not significantly different at $P < 0.05$. Each value is the mean of two experiments each with five replications. There was no treatment-experiment interaction.

Mycofumigation of *P. ultimum*-infested soil using live *M. albus* in the stabileze formulation and the biorational chemical mixture, resulted in higher seedling survival ($P < 0.0001$) than that in the Pythium-infested control (Table 2.2). *M. albus* stabileze formulation resulted in similar seedling numbers as in the non-infested control. There were no significant differences in fumigation-efficacy between the *M. albus* stabileze formulation and the biorational chemical mixture treatment.

The *M. albus* treatment formulated as stabileze resulted in stunted sugar beet seedlings 14 days after planting, as described by Stinson et al. (2003), however no growth differences were observed 21 and 28 days after planting when compared to seedlings

grown in sterilized soils. No reduction of the growth was observed for seedlings grown in the biorational mixture fumigation treatment. This observation supports the suggestion that the stabileze formulation itself was affecting the seedling growth, not the *Muscodor* fungus (Stinson et al., 2003) or VOCs produced by the fungus.

Discussion

The chemical and beneficial biological properties of *Muscodor* sp. were described previously *in vitro* (Ezra and Strobel, 2003; Strobel et al., 2001) and *in vivo* (Stinson et al., 2003; Zidack et al., 2001 and 2002). The results for the live *M. albus* stabileze formulation were similar to those reported by Stinson et al. (2003) and fumigation with the biorational chemical mixture resulted in similar disease control over the 28-day test period. Comparison of both live formulations of *M. albus* and a biorational chemical mixture for soil disinfestation is an important step for the development of large-scale field and greenhouse sanitation strategies. On a broadcast basis the biorational mixture tested would be used at 1120 liters per hectare compared to 9363 kg/ha for the stabileze formulation of live *M. albus*. These rates compare to 701.2 liter/ha (highest label rate) for the widely used soil fumigant Vapam HL® (AMVAC, Los Angles, CA) or 392 kg/ha (high labeled rate) for the granular soil fumigant Basamid® (BASF Corp., Research Triangle Park, NC). The rates for the formulated products are for control of soil-borne fungi such as Rhizoctonia and Pythium. While the rates for the biorational chemical mixture or the stabileze formulation of *M. albus* reported herein are much higher than comparable registered chemical fumigants, we have not yet attempted to examine the

lowest efficacious rate of either the biorational mixture or stabileze formulation for use in soils. Ezra and Strobel (2003), report that the use of 0.26 µl of the biorational mixture per cubic centimeter of air space provided 100 % mortality of *R. solani* and *P. ultimum* in *in vitro* studies. Thus, it may be possible to reduce the rate of the biorational formulation by as much as 75 %, although a lesser reduction is likely in soil. The use of the biorational mixture eliminates all consideration of introducing a non-indigenous fungus into soils and the rates used appear to be within the realm of feasibility. In addition, the use of the biorational mixture eliminates the variability associated with live formulations of *M. albus*.

Mycofumigation with the fungus *M. albus* was effective in experiments involving *A. cochlioides*, *P. ultimum* and *R. solani* infested soils. The treatment of live *M. albus* formulated as stabileze, and the biorational chemical mixture resulted consistently in a significantly higher sugar beet seedling survival relative to the respective pathogen treatments. The biorational mixture treatment was statistically as effective against the tested soil-borne pathogens as the fungus stabileze formulation itself. These results support the *in vitro* results of Strobel et al. (2001), that the fungus mycelium was not responsible for microbial inhibition but the VOCs produced by *M. albus* were effective in killing or inhibiting the other microorganisms.

Since these tests were performed under controlled environmental greenhouse conditions, field experiments are necessary to confirm the results. The application of the *M. albus* biorational mixture in the field situation could be in form of soil drenches or as injection-application with plastic cover as it is practiced with methyl bromide fumigation.

Use rates could be reduced by using row fumigation. Future experiments will address the field use of both the biorational mixture and *M. albus* dried barley and other substrates at various rates. These experiments will provide information as to the practicality of mycofumigation or fumigation with a biorational mixture of *M. albus* generated gases.

Mycofumigation using *Muscodor* sp. is a new concept of soil-fumigation with a broad application potential. Due to the fact that methyl bromide and most of its chemical alternatives are extremely toxic to humans and animals, the relatively non-toxic nature of the volatile gases of *M. albus* promise to increase the worker safety during soil fumigation and may provide a healthier environment for humans, animals and plants (Stinson et al., 2003; www.ars.usda.gov/is/np/mba/jul02/status.htm.; www.epa.gov/ozone/mbr/qa.html. 2004)

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CHAPTER 3

DETERMINATION OF OPTIMAL SUBSTRATE AND PLACEMENT OF *MUSCODOR ALBUS* AND *MUSCODOR ROSEUS* FOR CONTROL OF SOIL-BORNE DISEASES OF SUGAR BEET

Introduction

Many fungal species are known to produce different concentrations of volatile organic compounds (VOCs) (Bjurman and Kristensson, 1992). Dennis and Webster (1971) described that species of *Trichoderma* emit VOCs with inhibiting effects on other fungi. Many wood inhabiting fungi generate volatile products, which have no or only little antimicrobial properties. *Muscodor albus*, isolate 620 (Worapong et al., 2001), and its close relative *M. roseus*, isolate A 3-5 (Worapong et al., 2001), were recently described as endophytic fungi isolated from *Cinnamomum zeylanicum* in Honduras and from *Grevillea pteridifolia* in Australia, respectively (Worapong et al., 2001). Both fungi produce volatile organic compounds (VOCs), which effectively inhibit or kill microorganisms including soil-borne plant pathogens (Strobel, et al., 2001, Stinson et al., 2003). 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, unpublished).

Stinson et al. (2003) introduced the biological management of soil-borne pathogens using mycofumigation. Mycofumigation is the use of antimicrobial volatiles

produced by fungi such as *M. albus* and *M. roseus* for the control of other microorganisms. Soil-fumigation with the mycofumigant fungi *M. albus* and *M. roseus* was effective in greenhouse assays (Stinson et al., 2003) and field experiments (Grimme, unpublished) controlling soil-borne pathogens such as *Aphanomyces cochlioides*, *Pythium ultimum*, and *Rhizoctonia solani* of sugar beet seedlings, and *Verticillium dahliae* of eggplants (*Solanum melongena* L.).

In vitro experiments showed that the growth media influences the bioactivity of the VOCs emitted by *M. albus* (Ezra and Strobel, 2003). Research reported in this paper was focused on finding the growth substrate for *Muscodor* sp. resulting in optimal control of soil-borne pathogens of sugar beet seedlings in greenhouse experiments. Seven growth substrates for the mycofumigant fungi *M. albus* and *M. roseus* were tested in greenhouse assays with *P. ultimum* and *R. solani*.
In addition, effects of pre-planting mycofumigation and placement of the mycofumigant fungi were examined using *M. albus* and *M. roseus* formulated as whole barley kernels, ground barley and stabileze (Quimby et al., 1999).

Materials and Methods

Muscodor sp. Formulations and Storage

Cultures of *M. albus* and *M. roseus* used in the bioassays were obtained from Dr. Gary A. Strobel of the Department of Plant Sciences and Plant Pathology, Montana State University. *M. albus* and *M. roseus* were grown and maintained in 10 cm Petri plates on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) at 23 °C ± 1 °C for 2 weeks.

Fifteen 5 mm diameter agar plugs were transferred into separate 2 liter flasks with 1 liter autoclaved potato dextrose broth (PDB, Difco Laboratories, Detroit, MI), and placed on a rotary shaker for 2 weeks at 23 °C ± 1 °C. Seed based formulations were prepared by adding 60 ml of colonized PDB to Mason jars containing 300 g of the following sterilized seeds: barley (*Hordeum vulgare* L.), canola (*Brassica napus* L.), millet (*Panicum miliaceum* L.), oat (*Avena sativum* L.), rye (*Secale cereale* L.), and wheat (*Triticum aestivum* L.). After growth on the substrates for 3 weeks at 23 °C ± 1 °C, colonized seeds were dried for 48 hours in a fume-hood. The inoculated seeds were ground using a Waring blender, resulting in particles ranging from 30 – 1500 µm with a mean particle size of 300 µm, and stored in plastic bags at 4 °C until used. For comparison purposes, *M. albus* and *M. roseus* were prepared using the stabileze formulation method (Quimby et al., 1999). The stabileze formulations were dried for 48 hours under a fume-hood, packed in plastic bags and stored at 4 °C until used.

Preparation of Pathogen Inoculum

R. solani inoculum was prepared as infested ground barley according to Ruppel et al., 1979. Inoculum production of *A. cochlioides* and *P. ultimum* was prepared by inoculating 10 cm Petri plates of PDA and incubating for 2 weeks at 23 °C ± 1 °C. The *R. solani* inoculum consisted of dormant mycelium, whereas the *A. cochlioides* and *P. ultimum* inocula consisted of mycelia, sporangia, and oospores (Stinson et al., 2003).

Greenhouse Bioassays for Testing the Influence of different Growth Substrates for *Muscodor* sp. on the Efficacy to Control *P. ultimum* and *R. solani* on Sugar Beet

Greenhouse bioassays were designed to measure the efficacy of different growth substrates colonized by *M. albus* and *M. roseus* to control damping-off caused by *R. solani* and *P. ultimum* on sugar beet (*Beta vulgaris* L.). The plant growth substrate (Bozeman silt loam **:** washed concrete sand **:** Canadian sphagnum peat, 1:1:1) used for this experiment was sieved (2 mm mesh opening) and autoclaved at 121 °C for 60 minutes.

For soil infestation, 5 g of ground barley inoculum of *R. solani* were mixed thoroughly in 1 kg of soil. For *P. ultimum* soil infestation, one 2-week-old and completely colonized Petri plate of the fungus was chopped in small pieces (1 to 2 mm) and mixed separately with 6.4 kg of sterilized soil.

For *M. albus* and *M. roseus* treatments, 325 g of infested soil was filled into 10 cm square pots (10 x 10 x 8.6 cm), then a layer of 2 g of *Muscodor* sp. colonized ground seed was added, followed by 50 g of infested soil. The pots were placed in 3.78 liter plastic bags, containing 100 ml of water, twist tied and placed in a dark room at 23 °C ± 1 °C for one week. After the one-week fumigation period, 25 non-treated Beta 8754 sugar beet seeds were planted into each pot and placed in a greenhouse with 22 °C / 18 °C day / night-temperature (photoperiod: 16 hours). Pots inoculated with the pathogen *P. ultimum* were placed in shallow saucers with standing water for 3 days to favor infection (Stinson et al., 2003). Pots of the non-infested control were also placed in saucers for the

same time period for comparison. Percent healthy sugar beet seedling establishment was monitored at 14, 21, and 28 days after planting.

Greenhouse Bioassays for Testing the Influence of Different Formulations and Placement of *Muscodor* sp. and Fumigation Period on the Bioactivity

Greenhouse bioassays were designed to evaluate the influence of different mycofumigant placement on the efficacy of the gas producing fungi *M. albus* and *M. roseus* for control of damping-off caused by *R. solani*, *A. cochlioides*, and *P. ultimum*. All experiments were carried out under standardized greenhouse conditions as described above. The plant growth substrate used for this experiment, containing equal parts of Bozeman silt loam, washed concrete sand, and Canadian sphagnum peat, was sieved and steam-pasteurized at 80 °C for 30 minutes.

For *A. cochlioides* soil infestation, one 2-week-old and completely colonized Petri plate of the fungus was chopped in small pieces (1 to 2 mm) and mixed separately with 6.4 kg of pasteurized soil. Soil was infested with *P. ultimum* and *R. solani* as previously described for the growth substrate experiment. The formulations of the mycofumigant fungi tested were *Muscodor* sp. colonized whole barley kernels, ground barley and stabileze. Placement of the *M. albus* and *M. roseus* treatments was 2g of inoculum mixed with the upper 25% of the soil volume (top layer) in each 10 cm square pot or thoroughly mixed with the whole volume of infested soil. The pots were then placed in 3.78 liter plastic bags, containing 100 ml of water, twist tied and placed in a dark room at 23 °C ± 1 °C for 1 week.

After the 1-week fumigation period, 25 non-treated Beta 8754 sugar beet seeds were planted into each pot of the fumigated soil treatments and placed in a greenhouse. The pots of the treatments with the pathogens *A. cochlioides* and *P. ultimum* were placed in shallow saucers with standing water for 3 days to favor infection (Stinson et al. 2003). Pots of the non-infested control were also placed in saucers for the same time period for comparison. Percent healthy sugar beet seedling establishment was monitored 14, 21, and 28 days after planting. To study the effect of pre-plant fumigation compared to direct planting, the experiment described above also had sugar beet seeds planted directly into the 10 cm pots after the soil was prepared and placed in the greenhouse. The sugar beet seedling establishment was monitored as described above.

The experimental design was completely randomized with five replications per treatment. Experiments for all pathogens were repeated two times. Statistical analysis of the data was conducted by analysis of variance using the general linear model procedure (Madden et al., 1982) of the SAS program. The treatment means were separated using Fisher's least significant difference test (Swallow, 1984) and evaluated at $P < 0.05$.

Results

In soil infested with *P. ultimum*, mycofumigation with live *M. albus* and *M. roseus* grown on different seeds resulted in significantly higher sugar beet seedling survival 28 days after planting ($P < 0.0001$) relative to the *Pythium*-infested control (Table 3.1). There was no statistical difference in seedling establishment between *M. roseus* formulated as ground millet and the Pythium-infested control. *M. albus*

formulated as barley, canola, millet, oat, and stabileze, and *M. roseus* formulated as barley and oat resulted in similar sugar beet seedling establishment as the non-infested control. Mycofumigation with *M. albus* formulated as barley, canola, millet, and stabileze, and *M. roseus* formulated as barley and oat resulted in the highest seedling establishment. The treatments were followed by *M. albus* formulated as oat, rye, and wheat and *M. roseus* formulated as rye, stabileze and wheat. The treatments *M. roseus* formulated as canola and millet resulted in the lowest seedling establishment compared to the other *Muscodor* sp. treatments (Table 3.1).

In *R. solani* infested soil, mycofumigation with *M. albus* formulated as stabileze resulted in similar seedling establishment as the non-infested control ($P < 0.0001$) (Table 3.1). *M. albus* grown on barley, oat, rye, and wheat and *M. roseus* grown on barley, canola, and formulated as stabileze resulted in significantly higher numbers of healthy seedlings ($P < 0.0001$) than the Rhizoctonia-infested control. *M. albus* grown on canola and millet and *M. roseus* grown on millet, oat, rye, and wheat resulted in seedling survival similar to the pathogen-infested control (Table 3.1).

In the treatments *M. albus* and *M. roseus* formulated as stabileze the germination of the seedlings was delayed for about 5 days compared to the other *Muscodor* sp. treatments. No other effect of the mycofumigant fungi on the seedlings was discovered.

Table 3.1: Percent sugar beet seedling survival after 28 days following mycofumigation using *Muscodor albus* or *M. roseus* grown on different grain substrates, of soil infested with *Pythium ultimum* or *Rhizoctonia solani* AG 2-2.

	Pathogen used to infest soil [1)]			
Treatment	*P. ultimum*		*R. solani*	
Non-infested control	79.6	a	86.0	a
Pathogen-infested control	13.6	f	30.8	ef
M. albus ground barley	68.0	ab	55.2	bc
M. albus ground canola	62.8	ab	47.2	cde
M. albus ground millet	65.2	ab	46.4	cde
M. albus ground oat	59.6	abc	56.8	bc
M. albus ground rye	52.8	bcd	53.2	bcd
M. albus ground wheat	50.8	bcd	49.2	cd
M. albus as stabileze	61.6	ab	68.8	ab
M. roseus ground barley	69.6	ab	54.8	bc
M. roseus ground canola	36.0	de	49.2	cd
M. roseus ground millet	23.6	ef	31.2	ef
M. roseus ground oat	60.8	ab	31.0	ef
M. roseus ground rye	55.2	bcd	40.8	cdef
M. roseus ground wheat	38.8	cde	36.8	def
M. roseus as stabileze	52.8	bcd	49.6	cd

[1)] Soil was infested as follows: *P. ultimum*, one homogenized, completely colonized 10 cm Petri plate / 6.4 kg of soil; *R. solani*, 5 g of dry ground barley inoculum / kg of soil. Means followed by the same letter are not significantly different at $P < 0.05$. Each value is the mean of two experiments, each with 5 replications.

In soil infested with *A. cochlioides*, none of the *Muscodor* sp. treatments was equal in seedling establishment to the non-infested control (Table 3.2). Mycofumigation using the fungi *M. albus* and *M. roseus* prior to planting of the sugar beet seeds resulted

in better control of *A. cochlioides* damping-off than planting at the same time the mycofumigant fungi were applied ($P < 0.0001$) (Table 3.2). Fifty % of the 1-week fumigated treatments in the placement experiment resulted in significant more sugar beet seedlings ($P < 0.0001$) than the pathogen control. Only 2 out of 12 of the directly planted treatments were significantly better than the Aphanomyces control (Table 3.2). Soil-fumigation of Aphanomyces-infested soil was more effective with *M. albus* than with *M. roseus* (Table 3.2). The fumigated treatments of *M. albus* formulated as whole barley kernels, ground barley and stabileze, applied in the top 25 % of the soil (top layer), resulted in significantly higher seedling numbers ($P < 0.0001$) than in the direct-planted experiment. *M. albus* whole barley kernel treatment, mixed throughout the infested soil and fumigated, resulted in significantly higher seedling establishment than the respective treatment in the direct-planted experiment (Table 3.2). *M. roseus* formulated as whole barley kernels and stabileze resulted in significantly better seedling establishment when applied as a top layer and the seeds were directly planted. No other significant differences between fumigation and direct planting were observed (Table 3.2).

The comparison of the effect of the placement of *Muscodor* sp., *M. albus* formulated as ground barley and stabileze, resulted after the fumigation period in significantly higher numbers of seedlings ($P < 0.0001$) when applied in the top 25 % than mixed throughout the infested soil. When formulated as whole barley kernels, *M. albus* resulted in similar seedling establishment when added as a top layer or mixed into the infested soil. *M. roseus*, formulated as ground barley, resulted in significantly higher seedling numbers when applied as top layer than mixed throughout the soil (Table 3.2).

When sugar beet seeds were planted directly into with *Muscodor* sp. treated, Aphanomyces-infested soil, the treatments *M. albus* formulated as whole barley kernel and as stabileze, mixed throughout the soil, and *M. roseus* stabileze formulation applied as a top layer, resulted in significantly ($P < 0.0001$) higher seedling establishment than the pathogen control (Table 3.2). *M. roseus* stabileze formulation was significantly more effective when applied as top layer than mixed throughout the infested soil (Table 3.2).

In *P. ultimum* infested soil, *M. roseus* formulated as whole barley kernel, applied as top layer and fumigated for 1 week was equal in seedling establishment to the non-infested control ($P < 0.0001$) (Table 3.2). Mycofumigation using the fungi *M. albus* and *M. roseus* prior to planting of the sugar beet seeds resulted in better control of *P. ultimum* damping-off than planting at the same time the mycofumigant fungi were applied ($P < 0.0001$) (Table 3.2). One-week fumigated treatments in the placement experiment resulted in significantly more sugar beet seedlings ($P < 0.0001$) than the pathogen control. Only 1/3 of the directly planted treatments were significantly better than the Pythium control (Table 3.2). Soil-fumigation of Pythium-infested soil was more effective with *M. albus* than with *M. roseus* (Table 3.2). The fumigated treatments of *M. albus* formulated as whole barley kernels, ground barley and stabileze, applied in the top 25 % of the soil (top layer), resulted in significantly higher seedling numbers ($P < 0.0001$) than in the direct-planted experiment. *M. albus* whole barley kernels treatment, mixed throughout the infested soil and fumigated, resulted in significantly higher seedling establishment than the respective treatment in the direct-planted experiment (Table 3.2).

Table 3.2: Percent sugar beet seedling survival after 28 days following mycofumigation using *Muscodor albus* or *M. roseus* formulated as barley kernels, ground barley, and stabileze, of soil infested with *Aphanomyces cochlioides*, *Pythium ultimum* or *Rhizoctonia solani* AG 2-2. The *Muscodor* sp. were added as a top layer (top 25 % of the soil) or completely mixed through the infested soil.

	A. cochlioides [1)]		*P. ultimum*		*R. solani*	
Treatment	fumigated	direct	fumigated	direct	fumigated	direct
Non-infested control	90.4 a	78.0 ab	75.6 abc	93.2 a	90.0 ab	93.2 a
Pathogen-infested control	0.4 j	8.8 hij	19.6 klmn	22.8 jklmn	40.4 ghi	18.0 j
M. albus kernel, top layer	50.8 cd	22.8 efghi	65.2 bcde	38.4 fghijk	51.2 efg	60.0 def
M. albus kernel, mixed	48.0 cd	27.2 efg	66.0 bcd	36.0 ghijkl	74.8 bcd	74.4 bcd
M. albus ground, top layer	35.2 de	15.6 fghij	65.6 bcd	16.8 lmn	50.8 fg	40.4 ghi
M. albus ground, mixed	10.8 ghij	12.8 fghij	14.4 mn	9.2 mn	77.2 abcd	88.8 ab
M. albus stabileze, top layer	60.4 bc	22.8 efghi	47.6 defh	27.6 ijklm	43.2 fgh	43.2 fgh
M. albus stabileze, mixed	33.6 de	35.2 de	18.0 lmn	34.4 hijkl	89.2 ab	69.2 cde
M. roseus kernel, top layer	1.2 j	25.2 efgh	76.8 ab	46.0 efghi	29.2 hij	42.4 fgh
M. roseus kernel, mixed	10.4 ghij	11.6 fghij	62.4 bcde	56.8 cdef	80.4 abc	30.0 hij
M. roseus ground, top layer	22.8 efghi	14.8 fghij	63.6 bcde	40.8 fghij	42.0 fgh	21.2 j
M. roseus ground, mixed	2.8 j	3.6 j	8.0 n	9.6 mn	54.4 efg	87.6 ab
M. roseus stabileze, top layer	4.4 j	29.2 ef	62.8 bcde	52.4 defgh	42.8 fgh	23.6 ij
M. roseus stabileze, mixed	4.4 j	7.2 ij	54.4 defg	47.6 defgh	85.6 abc	53.6 efg
$LSD_{0.05}$	17.81		19.32		18.19	

[1)] Soil was infested as follows: *P. ultimum* and *A. cochlioides*, one homogenized, completely colonized 10 cm Petri plate / 6.4 kg of soil; *R. solani*, 5 g of dry ground barley inoculum / kg of soil. Means followed by the same letter are not significantly different at $P < 0.05$. Each value is the mean of two experiments, each with 5 replications.

M. roseus formulated as whole barley kernels and ground barley resulted in significantly better seedling establishment when applied as a top layer and planted directly. No other significant differences between fumigation and direct planting were observed (Table 3.2).

The comparison of the effect of the placement of *Muscodor* sp., *M. albus* formulated as ground barley and stabileze, resulted after the fumigation period in significantly higher numbers of seedlings ($P < 0.0001$) when applied in the top 25 % than mixed throughout the infested soil. When formulated as whole barley kernels, *M. albus* resulted in similar seedling establishment when added as top layer or mixed into the infested soil. *M. roseus* formulated as ground barley resulted in significantly higher seedling numbers when applied as top layer than mixed throughout the soil (Table 3.2).

When sugar beet seeds were planted directly into with *Muscodor* sp. treated, Pythium-infested soil, the treatments *M. roseus* formulated as whole barley kernels and as stabileze, applied as top layer or mixed throughout the soil, resulted in significantly higher ($P < 0.0001$) seedling establishment than the pathogen control (Table 3.2). *M. roseus* ground barley formulation was significantly more effective when applied as top layer than mixed throughout the infested soil (Table 3.2).

In soil infested with *R. solani*, *M. albus* formulated as ground barley and stabileze and *M. roseus* formulated as whole barley kernels and stabileze, mixed throughout the infested soil and fumigated for 1 week was equal in seedling establishment to the non-infested control ($P < 0.0001$) (Table 3.2). Direct planting in with *M. albus* and *M. roseus* ground barley treated soil also resulted in seedling establishment equal ($P < 0.0001$) to

the non-infested control (Table 3.2). Mycofumigation using the fungi *M. albus* and *M. roseus* prior to planting of the sugar beet seeds resulted in better control of *R. solani* damping-off than planting at the same time the mycofumigant fungi were applied ($P < 0.0001$) (Table 3.2). Treatments of the 1-week fumigated treatments in the placement experiment resulted in significantly more sugar beet seedlings ($P < 0.0001$) than the pathogen control. When planted directly 75 % of the treatments were significantly better than the Rhizoctonia control (Table 3.2). Soil-fumigation of Rhizoctonia-infested soil was more effective with *M. albus* than with *M. roseus* (Table 3.2). The fumigated treatment of *M. albus* formulated as stabileze, mixed throughout the soil, resulted in significantly higher seedling numbers ($P < 0.0001$) than in the direct-planted experiment. All fumigated *M. roseus* treatments, except of *M. roseus* formulated as ground barley and mixed throughout the infested soil, resulted in significantly higher seedling establishment than the respective treatment in the direct-planted experiment (Table 3.2). *M. roseus* ground barley formulation, mixed throughout the soil and planted directly, resulted in significantly better seedling establishment than in the fumigated experiment. No other significant differences between fumigation method and direct planting were observed (Table 3.2).

The comparison of the effect of the placement of *Muscodor* sp., all fumigated *M. albus* and *M. roseus* treatments resulted in significantly higher numbers of seedlings ($P < 0.0001$) when mixed throughout the infested soil than applied in the top 25 % of the soil. Only *M. roseus* ground barley formulation resulted in no differences in the application method (Table 3.2).

When sugar beet seeds were planted directly into with *Muscodor* sp. treated, Rhizoctonia-infested soil, all treatments of *M. albus* and *M. roseus* mixed throughout the soil resulted in significantly higher ($P < 0.0001$) seedling establishment than when applied as top layer (Table 3.2). There was no difference between the placement of the treatments of the *M. albus* and *M. roseus* whole barley kernel formulation (Table 3.2).

Discussion

Mycofumigation is the use of antimicrobial volatiles produced by fungi such as *Muscodor albus* and *M. roseus* for the control of other microorganisms. It is known that many fungal species are producing low concentrations of VOCs (Bjurman and Kristensson, 1992). Also, the inhibitory properties of VOCs emitted by *Trichoderma* spp. are known and have been analyzed (Dennis and Webster, 1971). Wheatley et al. (1997) described that the composition of the growth substrate greatly influenced the properties of the VOCs produced by the fungal organism. Ezra and Strobel (2003) tested the VOC production of *M. albus* grown on different media and their antimicrobial properties. As described with *Trichoderma* spp. before, the medium composition greatly influenced the VOC composition of *M. albus*. When grown on enriched media like PDA, with dextrose and potato starch as carbon sources, the antimicrobial effect of the VOCs was higher than on low nutrient media like Water agar (HA) (Ezra and Strobel, 2003). It is known that imperfect fungi like *R. solani* grow well on grain kernels like barley (Ruppel et al., 1979). Therefore this study was conducted to compare and possibly enhance the effectiveness of *Muscodor* sp. by growing it on different substrates and by

changing their placement and fumigation period. Their efficacy was tested for control of seedling diseases caused by soil-borne pathogens such as *R. solani* AG 2-2, *A. cochlioides*, and *P. ultimum* on sugar beet. *M. albus* and *M. roseus* grown on barley, provided disease control equal to *Muscodor* sp. formulated as stabileze (Quimby et al., 1999) in terms of reducing damping-off by *P. ultimum* and *R. solani* AG 2-2. A decrease of healthy sugar beet seedlings could be observed at mycofumigation with *M. albus* of *A. cochlioides* infested soil and with *M. albus* and *M. roseus* of Pythium-infested soil when the mycofumigant fungi were applied as a mix. Previous observations (data not shown) suggest that environmental conditions like high moisture are not favorable for *Muscodor* sp. and can cause reduced growth. Considering the experimental design for the pathogens *Aphanomyces cochlioides*, and *Pythium ultimum*, it can be assumed that the effectiveness of *Muscodor* sp. was reduced by high moisture, which prevented the growth of the mycofumigant fungi and therefore the production of the antimicrobial volatiles. In Rhizoctonia-infested soil, the seedling establishment was significantly increased ($P < 0.0001$) when *M. roseus* was mixed throughout the infested soil. This observation was consistent for both fumigation periods and therefore suggests a chance for other application possibilities besides fumigation, e.g. application at time of planting, seed treatment, or subsequent application. In general, this experiment showed that there is a preference for the effectiveness of *M. albus* and *M. roseus* in regard of the pathogens. It could be observed that *M. albus* was more effective against *A. cochlioides* and *R. solani* AG 2-2 than *M. roseus*. *M. roseus* instead promoted a better seedlings establishment in *P. ultimum* infested soil. These observations were independent from the place of the

treatment. The fact that we are working with a live microorganism can cause differences in efficacy when results of different experiments are compared. Therefore we are trying to develop stable and effective formulations of *M. albus*. It was observed that *M. albus* was not effective in an environment with high pathogen pressure. This proposes the application of *M. albus* after the soil was disinfested with Basamid (www.infoventures.com/e-hlth/pesticide/dazomet.html), chloropicrin mixtures (infoventures.com/e-hlth/pestcide/chlorpcn.html) or other soil-fumigants to reduce pathogen pressure. *M. albus* might find its place also in the integrated pest management in combination with crop rotation, solarization, application of beneficial microorganisms like *Trichoderma* sp and other strategies. Compared to chemical soil-fumigation of standard treatment with dazomet (www.infoventures.com/e-hlth/pesticide/dazomet.html) or 1,3 dichlorpropene (http://www.cdpr.ca.gov/docs/empm/ pubs/tac/recomm/1,3-d_mb_recomm00.pdf), soil fumigation with *M. albus* does not need a waiting period before planting. Since the VOCs produced by *M. albus* are more selective in their toxicity (Strobel et al., 2001) they would be a less health hazard than the above-mentioned chemicals. *M. albus* is not phytotoxic to members of eight different plant families tested (Strobel et al., 2001), therefore the control of weeds is not expected (Stinson et al., 2003).

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CHAPTER 4

EFFICACY OF MYCOFUMIGATION WITH *MUSCODOR ALBUS* IN FIELD EXPERIMENTS, STORAGE SURVIVAL, AND WINTER SURVIVAL IN THE FIELD

Introduction

Muscodor albus, isolate 620 (Worapong et al., 2001), an endophytic fungus obtained from *Cinnamomum zeylanicum* (cinnamon tree) in Honduras, produces a mixture of volatile organic compounds (VOCs), which effectively inhibit or kill certain bacteria and plant pathogenic fungi (Strobel et al., 2001; Ezra and Strobel, 2003; Jacobsen et al., 2004). The antimicrobial use of *M. albus* and its volatile compounds is named "mycofumigation" (Stinson et al., 2003). The VOCs produced by this endophytic fungus include the following chemical classes: alcohols, esters, ketones, acids, and lipids, which are considered to have low toxicity to mammals and higher plants (Strobel et al., 2001). The antimicrobial volatiles have been proven non-toxic to members of more than eight plant families, including representatives from the Rosaceae, Asteraceae, Graminae, Cruciferae, Cucurbitaceae, Leguminaceae, Chenopodiaceae, and Solanaceae. Based on results previously reported, APHIS (permit # 52384) and the California Department of Agriculture have permitted the use of *M. albus* in field experiments in Montana, USA.

In greenhouse bioassays with *M. albus* and its close relative *M. roseus* (Worapong et al., 2001), reduction of sugar beet (*Beta vulgaris* L.) diseases caused by soil-borne pathogens such as *Aphanomyces cochlioides*, *Rhizoctonia solani*, and *Pythium ultimum*

were shown (Stinson et al., 2003). Soil-fumigation with the *Muscodor* sp. also resulted in disease reduction of Verticillium wilt of eggplant (*Solanum melongena* L.) (Stinson et al., 2003) and in reduction of potato diseases caused by *R. solani* and *Streptomyces scabies* (Zidack et al., 2002). In addition, *in vitro* experiments have demonstrated activity of *Muscodor* sp. against *Meloidogyne incognita* (Jacobsen et al., 2004). In *in vitro* plate assays, and in field (Grimme, unpublished) and greenhouse experiments it was demonstrated, that *M. albus* does not inhibit or kill beneficial fungi of the genera *Gliocladium* and *Trichoderma* (Strobel et al., 2001).

Materials and Methods

Muscodor sp. Formulations and Storage

The culture of *M. albus*, isolate 620 (Worapong et al., 2001), used in these experiments was obtained from Dr. Gary A. Strobel of the Department of Plant Sciences and Plant Pathology, Montana State University. *M. albus* was maintained on 10 cm Petri plates with potato dextrose agar (PDA, Difco Laboratories, Detroit, MI). Inoculum was produced by transferring 15 agar plugs (5 mm diameter) from 3-week-old Petri plate cultures, maintained at 23 °C ± 1 °C, into separate 2 liter flasks containing 1 liter autoclaved potato dextrose broth (PDB, Difco Laboratories, Detroit, MI), and placing the flasks on a rotary shaker for 2 weeks at 23 °C ± 1 °C. Sixty ml of the liquid culture were added to a Mason jar containing 300 g of sterilized barley. Subsequently *M. albus* was allowed to grow on the substrate for 3 weeks at 23 °C ± 1 °C, the barley was dried for 48 hours under a fume-hood. The inoculated grain was ground to a sawdust texture (30 –

1500 µm with a mean particle size of approximately 300 µm) using a Waring blender and stored in plastic bags at 4°C until used. *M. albus* was also prepared using the stabileze formulation method (Quimby et al., 1999). This formulation was selected because it was efficacious in tests reported by Stinson et al. (2003). The stabileze formulation was dried for 48 hours under a fume-hood, packed in plastic bags and stored at 4 °C until used.

Preparation of Plant-Pathogenic Fungi Inocula

R. solani inoculum was prepared as infested ground barley according to Ruppel et al. (1979). Inocula of *A. cochlioides* and *P. ultimum* were prepared by growing fungal cultures for 2 weeks at 23 °C ± 1 °C on 10 cm PDA Petri plates. The *R. solani* inoculum consisted of dormant mycelium, whereas the *A. cochlioides* and *P. ultimum* inocula consisted of mycelia, sporangia, and oospores (Stinson et al., 2003).

Field Bioassays for Testing the Efficacy of *Muscodor albus* against Soil-Borne Pathogens of Sugar Beet

Field bioassays were designed to measure the efficacy of the gas producing fungus *M. albus* formulated as ground barley for control of *A. cochlioides*, *R. solani*, and *P. ultimum* on sugar beet (*Beta vulgaris* L.). All experiments were carried out in a silt-loam soil at the Horticulture Farm at Montana State University, Bozeman, USA. The substrate used for preparing the pathogen inocula contained equal parts of sieved (2 mm mesh opening) Bozeman silt loam **:** washed concrete sand **:** Canadian sphagnum peat. The substrate was steam pasteurized at 80 °C for 60 minutes. Five gram of ground barley inoculum of *R. solani* was mixed thoroughly in 1 kg for soil infestation. For *A.*

cochlioides and *P. ultimum* inocula, one 2-week-old colonized Petri plate was chopped in small pieces (1 to 2 mm) and mixed with 6.4 kg of soil. The infested soils were packed in nylon bags (200 g / bag) and placed in field soil at different depths (0, 10, 20, and 30 cm). Five replications of the 3 pathogen treatments and a non-infested control were buried in the soil. *M. albus* formulated as ground barley was spread over the surface of the soil using a Scotts® AccuGreen® 2000™ fertilizer spreader (Scotts®, Marysville, OH) at a rate of 480 kg / hectare. Black plastic mulch (Mechanical Transplanter Company, Holland, MI) and drip tape (Aquapore porous pipe, Hummert International, Earth City, MO), as well as a 5 cm thick layer of loose soil were then applied with a mechanical bedder/mulch layer (Buckeye Tractor Co., Columbus Grove, OH) on top of the layer of *M. albus* colonized barley. Irrigation water was applied 1 day after bed preparation through drip tubes. The nylon bags were recovered from the field after a one-week fumigation period. The soil from the nylon bags was added as a top layer onto 175 g of pasteurized soil in 10 cm square pots (10 x 10 x 8.6 cm). Twenty-five non-treated Beta 8754 sugar beet seeds were planted into each pot and placed in a greenhouse at 22 °C / 18 °C day-/night-temperature (photoperiod: 16 hours). Pots inoculated with the pathogens *A. cochlioides* and *P. ultimum* were placed in shallow saucers with standing water for 3 days to favor infection (Stinson et al., 2003). Pots of the non-infested control were also placed in saucers for the same time period for comparison. Percent healthy sugar beet seedling establishment was monitored 14, 21, and 28 days after planting.

The experimental design was completely randomized with five replications per treatment. Experiments of all pathogens were repeated two times. Statistical analysis of

the data was conducted by analysis of variance using the general linear model procedure of SAS (Madden et al., 1982). The treatment means were separated using Fisher's least significant difference test (Swallow, 1984) at $P < 0.05$.

Winter Survival of *M. albus* in the Field

Field assays were designed to test the capacity of *M. albus* to survive the winter in field soil in Montana. *M. albus* was formulated as stabileze and ground barley. Of each formulation 1 g was added to 100 g of sieved and pasteurized soil, and placed in nylon bags. One gram of the soil samples infested with *M. albus* as ground barley or stabileze was added to 9 ml 0.1 % agar tubes and vortexed for 20 seconds. Five hundred µl of each sample were plated onto PDA plates, containing 10 ppm chloramphenicol (Sigma-Aldrich, St. Louise, MO) and 25 mg Rose Bengal (Sigma-Aldrich, St. Louise, MO). Forming of *M. albus* colonies was determined after incubation at 23 °C ± 1°C for10 days. The nylon bags were placed in October in field soil 0, 15, 30, and 45 cm below the soil surface. Five replications of the 2 *M. albus* formulations were buried in the soil. The nylon bags were removed the following April. One gram of each soil sample was added to 9 ml 0.1 % agar tubes and vortexed for 20 seconds. Five hundred µl of each sample were plated onto PDA plates, containing 10 ppm chloramphenicol (Sigma-Aldrich, St. Louise, MO) and 25 mg Rose Bengal (Sigma-Aldrich, St. Louise, MO). To suppress growth of other fungal species present in the soil samples, Petri plates containing the soil samples were inverted and stabilized with Parafilm (Pechiney Plastic Packaging, Inc., Neenah, WI) on top of PDA plates with 1-week-old *M. albus* cultures. The 1-week-old

M. albus cultures were prepared by spreading 500 µl of *M. albus* grown in PDB, as described before, on Petri plates containing PDA, followed by 1 week of incubation at 23 °C ± 1 °C. The double Petri plates were incubated for 2 weeks at 23 °C ± 1 °C. Subcultures of the growing fungal cultures were transferred onto PDA, incubated for 2 weeks at 23 °C ± 1 °C, and compared with the original *M. albus* culture.

Storage Survival of *M. albus* Stabileze Formulation

M. albus was grown as liquid culture for 3 weeks and then formulated as stabileze (Quimby et al., 1999). Nine Ziploc Freezer plastic bags were filled with 20 g of *M. albus* stabileze formulation. Three plastic bags were placed at 23 °C, 3 to 5 °C, and – 10 °C respectively. Colony forming units (CFUs) were determined by adding 1 g of stabileze formulation to 9 ml of PBS (Phosphate-buffered saline) buffer, vortexing the solution for 20 seconds and plating 200 µl onto PDA plates. The Petri plates were incubated for 10 days at 23 °C ± 1 °C and *M. albus* colonies were counted and adjusted to CFU / 1 g of stabileze formulation. The stabileze formulation was assayed monthly as described above. Three replications per plastic bag and two Petri plates per replication were prepared. The storage assay was repeated two times.

Development of semi-selective media for *M. albus* and *M. roseus*

To develop a method for detection of *Muscodor* sp. in soil samples, 5 mm agar plugs of 3-week-old cultures of *M. albus* and *M. roseus* were transferred on PDA Petri plates containing 1 ppm or 10 ppm of 1 of the following active ingredients of fungicides: azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC), tetraconazole

(Eminent 125 SL; Sipcam Agro Inc., Roswell, GA) or methyl 1-(butylcarbamoyl)-benzimidazol-2-ylcarbamate (Benlate®; DuPont, Wilmington, DE). The fungi were incubated at 23 °C ± 1 °C and vitality was monitored weekly for 3 weeks.

In another method, the development of nitrate-nonutilizing (nit) mutants of *M. albus* and *M. roseus* was investigated. Agar plugs (5 mm diameter) were taken from 3-week-old fungal PDA cultures of both fungi and transferred to plates (4 plugs / plate) with Czapek agar (Difco Laboratories, Detroit, MI) or PDA (Difco Laboratories, Detroit, MI) amended with 10 g, 20 g or 30 g / L of potassium chlorate (Correll et al., 1987). After 8-12 days at 23 °C ± 1 °C, plates were inspected for fast growing fan-like chlorate-resistant sectors (Correll et al., 1987).

Results

Mycofumigation with *M. albus* of *A. cochlioides*, and *P. ultimum* infested soil resulted in higher seedling establishment than the pathogen controls in the field bioassay when seedling numbers were averaged over all depths (Table 4.1). In the non-infested control treatment, orthogonal contrasts showed that sugar beet seedling establishment was significantly higher in 20 cm ($P < 0.0081$) and 30 cm ($P < 0.0019$) samples than in 0 cm samples. Furthermore, the number of seedlings was significantly higher in 30 cm ($P < 0.0310$) than in 10 cm samples. Seedling establishment of mycofumigated soil infested with *A. cochlioides* was statistically higher at the 20 cm depth than the Aphanomyces control. In *A. cochlioides* infested soil samples, orthogonal contrasts showed that sugar beet seedling establishment was significantly higher in 20 cm

($P < 0.0032$) and 30 cm ($P < 0.0003$) samples than in 0 cm samples. In soil infested with *R. solani*, no significant improvement of seedling establishment could be detected when pathogen infested soil was treated with *M. albus* (Table 4.1). In general mycofumigation improved the healthy seedling establishment of sugar beets in soils infested with *A. cochlioides*. The best response to mycofumigation appeared to be in the 20 and 30 cm samples in *A. cochlioides* and *P. ultimum* infested soil (Table 4.1).

In soil samples plated out on PDA 2 hours after infesting the soil with *M. albus* formulated as stabileze or ground barley, alive colonies of *M. albus* could be recovered. No *M. albus* colonies could be recovered from the field assays conducted to determine the potential of *M. albus* to survive over the winter in soil in Montana. Subcultures recovered from the soil samples were identified as *Trichoderma* spp.

Experiments to determine the storage survival of *M. albus* at different temperatures resulted in good storage efficacy of the fungus at 3 to 5 °C and - 10 °C (Figure 4.1). Viability of *M. albus* in stabileze formulation stored at 3 to 5 °C was significantly better ($P < 0.0001$) than storage at - 10 °C and 23 °C, whereas storage at - 10 °C resulted in significant more CFUs than at 23 °C. After 5 months of storage, 25 % CFUs could be recovered at - 10 °C and 50 % CFUs at 3 to 5 °C respectively. Storage of *M. albus* stabileze formulation at 23 °C resulted in nearly undetectable CFUs when plated on PDA. After 5 months, only about 1 % CFUs were recovered when the stabileze formulation was stored at 23 °C (Figure 4.1).

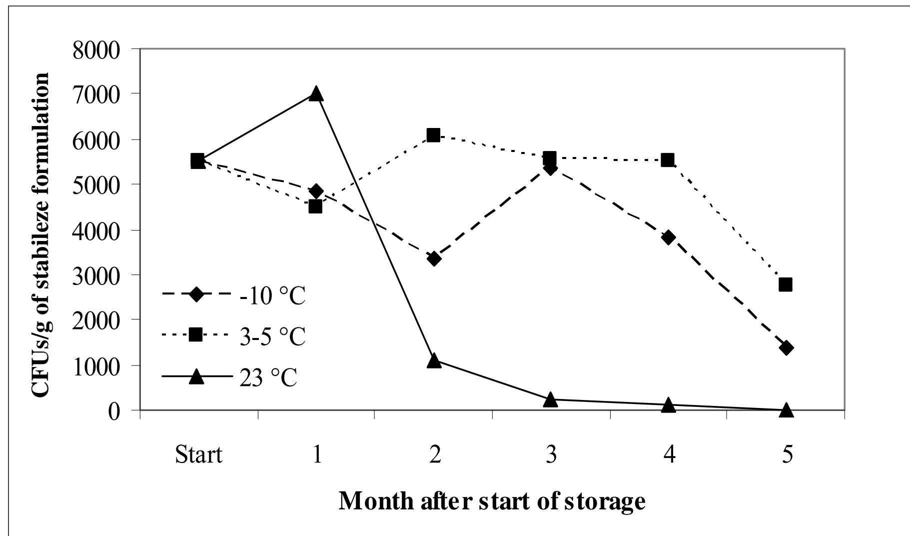
Table 4.1: Percent sugar beet seedling establishment in soil infested with *Aphanomyces cochlioides*, *Pythium ultimum*, or *Rhizoctonia solani*, buried from 0 to 30 cm in field soil and mycofumigated with *Muscodor albus* for 1 week.

	Percent seedling establishment at four soil depths									
	All Depths		0 cm		10 cm		20 cm		30 cm	
Treatment	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003
UTC	81	41	79	22	77	43	82	43	87	54
UTC + MF	78	55	43	55	86	26	91	70	91	70
Aphanomyces [1)]	26	30	64	57	29	26	8	22	5	14
Aphanomyces + MF [2)]	46	35	44	42	60	30	36	44	44	24
Pythium	16	40	24	36	20	46	14	38	15	41
Pythium + MF	33	46	45	30	11	50	34	42	34	61
Rhizoctonia	13	42	9	53	10	37	11	47	21	31
Rhizoctonia + MF	8	47	3	50	18	34	7	52	6	52
$LSD_{(0.05)}$	15	15	26	29	25	31	18	28	29	28

[1]) Soil was infested as follows: *R. solani*, 5 g of dry ground barley inoculum / kg soil; *P. ultimum* and *A. cochlioides*, one homogenized, completely colonized 10 cm Petri plate / 6.4 kg of soil. UTC = non-infested control

[2]) MF = Mycofumigation with *M. albus* for 1 week. *M. albus* formulated as ground barley was spread over the surface of the soil at a rate of 480 kg / hectare.

Figure 4.1: Monthly assayed colony forming units (CFUs) of *M. albus* when stored at 23 °C, 3 to 5 °C, and – 10 °C [1]



[1]) *Muscodor albus* was formulated as stabileze (Quimby et al., 1999) and stored in plastic bags at different temperatures.

The transfer of *Muscodor* sp. on selective media resulted in good vitality and growth of *M. albus* on PDA containing either 1 or 10 ppm tetraconazole (Eminent 125 SL; Sipcam Agro Inc., Roswell, GA). On media containing either 1 or 10 ppm azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) growth of *M. albus* was limited, whereas the subcultures on PDA containing 1 or 10 ppm methyl 1-(butylcarbamoyl)-benzimidazol-2-ylcarbamate (Benlate®; DuPont, Wilmington, DE) showed no fungal growth. *M. roseus* showed only limited growth on PDA containing azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC), whereas no growth on the other media was observed.

Muscodor sp. were very tolerant to chlorate. On PDA or Czapek agar amended with 10 g, 20 g or 30 g / L of potassium chlorate no development of fan-like chlorate-resistant sectors were observed.

Discussion

Mycofumigation using *Muscodor* sp. is a new concept for soil fumigation. Effectiveness of *M. albus* and its close relative *M. roseus* to control soil-borne pathogens of sugar beet, like *A. cochlioides*, *P. ultimum*, and *R. solani*, has previously been shown by Stinson et al. (2003). Since these tests were performed under controlled environmental greenhouse conditions, field experiments were necessary to confirm the results. Results reported in this article suggest that seedling emergence of sugar beet can be significantly improved in Aphanomyces-infested soils in depths of 20 to 30 cm. This would suggest that there are water-soluble antimicrobial compounds produced by *M. albus* that are reducing the viability of the pathogen inoculum. The data from the field assay also suggest that there was very high pathogen pressure in the infested soil samples. Dosages of *M. albus* may need to be raised to be effective in the field. Another conspicuous observation of this experiment was, that *Trichoderma* spp. were colonizing the area besides the *M. albus* colonies without being affected in growth by the VOCs produced by the mycofumigant fungus. This observation confirms the *in vitro* results of Strobel et al. (2001), that beneficial fungi of the genera *Trichoderma* and *Gliocladium* are not affected by *M. albus*. It would be of interest to investigate the changes of the microflora caused by *M. albus*, which obviously favor those beneficial microorganisms.

Therefore the proton transfer reaction-mass spectrometry (PTR-MS) can be used to monitor the VOCs emitted by *M. albus* (Ezra et al., 2004), and other beneficial fungi. *M. albus* might suppress the growth of several pathogens in the soil and increase the availability of nutrients for *Trichoderma* spp. The introduction of a foreign microorganism in a given environment and its effects on present microorganisms, especial on beneficial microorganisms, is very critical. It is also important to avoid establishment of introduced organisms like *M. albus* in the soil and their accumulation in the new environment. Adaptation and competition of the new microorganism in the introduced environment over a long period of time could not only affect pathogenic but also beneficial microorganisms. The winter survival study of *M. albus* showed that the mycofumigant fungus is not able to survive in field soil over the winter. Since no fruiting structures of *Muscodor* sp. could be observed to date (Worapong et al., 2001), it is unlikely that the mycelium will survive over a long time in the soil like other fungi with resistant overwintering structures. This observation was made in Montana, USA, where the soil temperatures can be as low as- 2 to -7 °C (Station at Horticulture Farm, Bozeman, MT) in the winter. Survival experiments in soils with warmer temperatures than in Montana should be performed before proposing the concept of mycofumigation for other regions.

The storage efficacy of the formulation of a live microorganism is critical for its success in practical application and its value as biological control agent. *M. albus* stabileze formulation stored at 3 to 5 °C provides as much as 50 % survival after a

storage period of 5 months. No special storage or dehydration of the stabileze formulation before use is necessary.

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CHAPTER 5

MYCOFUMIGATION AS AN ALTERNATIVE TO METHYL BROMIDE FOR CONTROL OF SOIL-BORNE DISEASES OF *CHRYSANTHEMUM* SP.

Introduction

Methyl bromide-chloropicrin mixtures are the major chemicals used for soil disinfestation in U.S. cut flower production (Raabe et al., 2002). The chemical is a highly efficacious fumigant used for pre-plant soil fumigation in field and greenhouse production (Carpenter et al., 2000). It controls soil-borne diseases, insects, nematodes and weeds (Raabe et al., 2002). The phase out of methyl bromide in 2005 for soil fumigation will reduce options for controlling soil-borne diseases (www.nps.ars.usda.gov).

In 2001, California was the leading state for floriculture crops with an overall value of $1.02 billion, and $292 million attributed to the cut flower production (www.nass.usda.gov/oh/florrpt.htm). *Chrysanthemum* sp. are among the most important pot and cut flowers produced in California. It is estimated that half of the total chrysanthemum production will be in greenhouses, 60 % of which use methyl bromide-chloropicrin mixtures (Raabe et al., 2002). The other 50 % of the chrysanthemums are produced in outdoor beds, which are also traditionally treated with methyl bromide–chloropicrin mixtures. Field-grown chrysanthemums can be alternatively treated with 1,3-D plus chloropicrin at a similar cost to methyl bromide (Raabe et al., 2002).

However, the treatment is not as effective as methyl bromide for broad-spectrum pest control and losses of up to 7.5 % occur (Carpenter et al., 2000).

Vascular wilts caused by *Verticillium dahliae* and *V. albo-atrum* effect many plant species including *Chrysanthemum* sp. and can cause severe yield losses (Agrios, 1997). Commercial chrysanthemum growers start their production with either rooted or non-rooted cuttings obtained from specialist propagators (Dole and Wilkins, 1999). In general only indexed pathogen-free cuttings are used. Cuttings are especially susceptible to soil-borne diseases, and planting in infested soil often results in devastating losses (Dole and Wilkins, 1999). *V. dahliae* is of special importance. This fungus produces microsclerotia, which can survive in the soil up to 15 years (Agrios, 1997). Six to 50 microsclerotia per gram of soil are enough to cause 100 % infection of plants. Both Verticillium species produce mycelia within perennial hosts or plant debris, which serve as overwintering structure (Agrios, 1997).

Root rot caused by *Pythium ultimum* is another important disease of chrysanthemums resulting in significant yield losses. Disease severity varies considerably depending on soil moisture, temperature and other environmental factors. Plants are seldom killed but become stunted and develop stem and root lesions (Agrios, 1997).

The control measures for Verticillium wilts and Pythium root rot are very similar. Preplant soil fumigation with chloropicrin - methyl bromide combinations are most common and the main treatment against the diseases (Raabe et al., 2002). Post planting fungicide drenches are sometimes used for Pythium root rot control (Daughtrey et al.

1995). In addition, steam pasteurization has been widely used. This method is limited by the availability of steam generators (Forsberg, 1963 and Daughtrey et al. 1995).

Current research on methyl bromide replacements includes developing management systems utilizing alternative chemicals (Duniway et al., 2000), biofumigation with *Brassica* spp. (Sarwar et al., 1998) and organic amendments / biological control (Cuester and Hoitink, 1999). At this time these alternatives fit niches as alternatives for methyl bromide replacement, but none offer the broad-spectrum activity and efficacy of methyl bromide (Zidack et al., 2001).

The fungi *Muscodor albus* (Worapong et al., 2001) and *M. roseus* (Worapong et al., 2001) produce volatile gases toxic to a wide range of plant pathogens, including pathogens of the genera *Aphanomyces*, *Pythium*, *Rhizoctonia*, and *Verticillium* and are currently being evaluated at Montana State University as methyl bromide fumigation alternatives (Zidack et al., 2001). These gases have been chemically characterized as esters, alcohols, acids and lipids, none of which are considered to be toxic to animals and have been proven non-toxic and non-pathogenic to a wide range of plant species (Strobel et al., 2001). The use of gas-producing fungi to control soilborne plant diseases has been named "mycofumigation" (Stinson et al., 2003).

Using sugar beet and eggplant pathosystems under greenhouse conditions, we have demonstrated that mycofumigation is effective in controlling soilborne diseases (Stinson et al., 2003; Grimme, unpublished). In greenhouse pot assays, soil fumigation using *Muscodor* sp. grown on barley significantly reduced disease incidence of *V. dahliae*

in eggplants. Damping-off and root rot diseases of sugar beet caused by *P. ultimum*, *R. solani*, and *A. cochlioides* were also effectively controlled through mycofumigation.

These alternative chemicals are toxic and can cause worker exposure problems (www.nps.ars.usda.gov). The non-toxic nature of the volatile gases of *M. albus* and *M. roseus* (Strobel et al., 2001) promise to increase the worker safety during soil fumigation. The focus of this research was to test mycofumigation for disinfesting soil in beds used for growing *Chrysanthemum* x *grandiflorum* Kitam. (*Chrysanthemum* x *morifolium*). Mycofumigation was tested on soils infested with *V. dahliae* and *P. ultimum.* Initial studies to develop treatment parameters were performed in individual pots and information gathered was used to design mycofumigation experiments on infested beds under greenhouse conditions.

Two formulations of the gas producing fungi were used in all greenhouse experiments. The first was barley (*Hordeum vulgare* L.) colonized with *Muscodor* sp., which was dried and ground; the second used the stabileze (starch, silica powder, sucrose and corn oil mix) formulation process (Quimby et al., 1999). These formulations have provided excellent disease control in greenhouse and laboratory experiments.

Materials and Methods

The greenhouse experiments were designed to measure the efficacy of the gas producing fungi *M. albus* and *M. roseus* for control of *V. dahliae,* and *P. ultimum* under standard conditions for *Chrysanthemum* sp. production. All experiments were carried out under standardized greenhouse conditions at the Montana State University, Plant Growth

Center. The growing substrate used for all experiments was a 1:1 mixture of sieved PGC Mix (Bozeman silt loan **:** washed concrete sand **:** Canadian sphagnum peat, 1:1:1) and Sunshine Mix®, which was steam pasteurized at 80 ºC for 60 minutes.

Muscodor sp. Formulations and Storage

The cultures of *M. albus*, isolate 620 (Worapong et al., 2001) and *M. roseus*, isolate A 3-5 (Worapong et al., 2001), used in these experiments were obtained from Dr. Gary A. Strobel of the Department of Plant Sciences and Plant Pathology, Montana State University. *Muscodor* sp. were maintained on 10 cm Petri plates with potato dextrose agar (PDA, Difco Laboratories, Detroit, MI). Inoculum was produced by transferring 15 agar plugs (5 mm diameter) from 3-week-old Petri plate cultures, maintained at 23 °C ± 1 °C, into separate 2 liter flasks containing 1 liter autoclaved potato dextrose broth (PDB, Difco Laboratories, Detroit, MI), and placing the flasks on a rotary shaker for 2 weeks at 23 °C ± 1 °C. Sixty ml of the liquid culture were added to a Mason jar containing 300 g of sterilized barley (*Hordeum vulgare* L.). Subsequently *M. albus* was allowed to grow on the substrate for 3 weeks at 23 °C ± 1 °C. The inoculated barley was then dried for 48 hours under a fume-hood. The inoculated grain was ground to a sawdust texture (30 – 1500 µm with a mean particle size of approximately 300 µm) using a Waring blender and stored in plastic bags at 4 °C until used.

Preparation of *V. dahliae* and *P. ultimum* Inocula

V. dahliae was grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) at 23 ˚C ± 1 ˚C for 2 weeks. Ten 5 mm agar plugs of the *Verticillium* culture were transferred into Mason jars containing 300 g of sterilized barley for inoculum production and incubated for three weeks at 23 ˚C ± 1 ˚C. The incubated barley was dried for 48 hours under a fume-hood and ground in a Waring blender to produce a homogenous inoculum formulation. Inoculum of *P. ultimum* was prepared by growing fungal cultures for 2 weeks at 23 °C ± 1 °C on 10 cm PDA Petri plates. The *P. ultimum* inoculum consisted of mycelia, sporangia, and oospores (Stinson et al., 2003).

Preliminary Experiments

Four different chrysanthemum varieties were obtained from Yoder Brothers, Inc., Barberton, OH, to determine a Verticillium wilt and Pythium root rot susceptible *Chrysanthemum* x *grandiflorum* Kitam. (*Chrysanthemum* x *morifolium*) cultivar. The varieties obtained were “Bright Golden Anne”, “Dark Pilar”, “Snowdon”, and “Vero”. The growing substrate was infested with 2.5 g, 3.5 g or 5.0 g Verticillium inoculum per kilogram soil. For infesting the soil with *P. ultimum*, 0.75, 1.0 or 1.5 of full-grown petri dish culture(s) of the pathogen were chopped in small pieces and incorporated in 6.4 kg growing substrate. The infested substrates were filled in 10 cm pots (375 g / pot), the chrysanthemum cuttings were planted into the filled pots (1 cutting / pot) and placed in a greenhouse with 22 °C / 18 °C day / night temperature (photoperiod: 16 hours).

In addition, one plate of a Verticillium culture was mixed in 100 ml of a 1 % methyl-cellulose solution (Sigma-Aldrich, St. Louis, MO). For this treatment the roots of the cuttings were washed, then directly dipped into the pathogen solution and planted into non-infested soil.

To determine any negative effects of the mycofumigant fungi on chrysanthemum cuttings, stabileze formulations of the *Muscodor* sp. were incorporated into pathogen-free growing substrate (2 g / 375 g soil). The growing substrate was filled in pots as described above. The chrysanthemum cuttings were planted and placed in a greenhouse. Five replications / treatment were prepared for all treatments and the non-infested control.

Results of Preliminary Experiment

Stunting and wilting of the cuttings caused by both pathogens could be observed after a growing period of 4 weeks. Overall the Verticillium rate of 5 g inoculum / kg soil, the dipped treatment and the inoculum of 1 and 1.5 petri dish(es) of Pythium / 6.4 kg soil resulted in moderate to severe plant disease symptoms (Table 5.1). The mycofumigant fungi seemed to reduce the growth of the chrysanthemum cuttings during the first 3 weeks after planting, but no differences in growth or phenotype could be observed after 8 weeks of cultivation compared to the non-infested control plants. Based on this observation, the stabileze formulation was not used in subsequent experiments. The mycofumigant fungi grown on barley were used in the remainder of the experiments reported here. This formulation has shown no adverse effects on members of 8 tested plant families examined to date.

After a cultivation period of seven weeks 1-2 mm discs of the lower part of the plant stems were cut and placed on PDA medium plus 10 ppm chloramphenicol to confirm that the stunting and yellowing of the Verticillium treated plants was caused by *V. dahliae*. After an incubation period of five days at 22 °C the stem discs were examined under a dissecting microscope for Verticillium structures. A stem disc was rated as positive (+) if the "typical" verticillate conidiophores of the pathogen were observed fruiting from the chrysanthemum stems.

Table 5.1: Disease evaluation of *Chrysanthemum* sp. based on stunting and wilting of the plants

	***Verticillium dahliae* inoculum/kg soil**				***Pythium ultimum* # petri dishes/6.4 kg soil**			**UTC**
Variety	2.5 g	3.5g	5g	dipped	0.75	1.0	1.5	-
“Bright Golden Anne”	+	++	++	+++	+	++	++	-
“Dark Pilar”	-	+	+	+	+	+	+	-
“Snowdon”	-	-	+	+	+	++	++	-
“Vero”	-	+	+	+	+	+	+	-

- = no symptoms; + = mild symptoms; ++ = moderate symptoms; +++ = severe symptoms
UTC = non-infested control

The variety "Bright Golden Anne" showed the highest susceptibility to *V. dahliae* and *P. ultimum* compared to the other varieties and was used for all experiments proposed in this research project.

<u>Greenhouse Pot Assay</u>

The experimental design for the greenhouse pot assay was based on the results of the preliminary experiments. *V. dahliae* and *P. ultimum* inoculum and the mycofumigant fungi were incorporated at different rates into pasteurized growing substrate as described above. Treatments and dosages for this experiment are listed in Table 5.2. The prepared pots were placed in plastic bags with 100 ml water added. The growing substrate in the 10 cm pots was fumigated for one week at 22 °C. The plastic bags were removed after the fumigation period and the pots were placed in a greenhouse (25 pots / m^2) under standardized conditions. One rooted chrysanthemum cutting was planted into each pot. The experimental design was completely randomized with ten replications per treatment for all treatments and the non-infested control. Plant cultivation followed normal production conditions for chrysanthemum. The plants were watered and fertilized (Peter's 15-16-17 Peat Lite Special Fertilizer and Calcium Nitrate 15.5-0-0; Hummert International, Earth City, MO) using drip irrigation.

The presence of *V. dahliae* in the pathogen control plants was determined seven weeks after planting. The three lower leaves of the plants were cut, surface sterilized in 10 % sodium hypochlorite solution for 30 seconds, the petioles cut in small pieces and placed on water agar. After an incubation period of 10 days at 22 °C verticillate growth was determined using a dissecting microscope.

All chrysanthemum plants were harvested after a cultivation period of 12.5 weeks. The length of the stem, the number of flowers on the top 1/3 of the stem, the diameter of the terminal flower and disease symptoms were evaluated following the

guidelines of the DEFRA EC Marketing Standards for Cut Flowers (Horticultural Marketing Inspectorate, UK, 2001).

After harvesting, stem discs of *V. dahliae* infested plants were cut from the lower 2 to 5 cm of the stems. The stems were surface sterilized and cut as described in the preliminary experiments. The discs were placed on water agar and incubated for one week at 22 °C. After the incubation period the stem discs were examined under a dissecting microscope for Verticillium structures. The stem disc cuttings were rated for plant stem colonization by Verticillium using a colonization index (CI) calculated by the following formula:

$$CI = \frac{\Sigma\,(\text{Number of discs in each severity class x class number})}{(\text{Mean number of discs per treatment x number of colonization classes})} \text{ x } 100$$

Colonization severity classes used were: 0 = no colonization; 1 = mild colonization with few Verticillium structures; 2 = moderate colonization, Verticillium structures present on 50 % of the disc; 3 = severe colonization, Verticillium structures covering the disc to 100 %.

Table 5.2: Treatment set-up for the development of parameters for the control of Verticillium wilt and Pythium root rot in *Chrysanthemum* sp. pot culture

Treatment formulation	***Rate of* Muscodor *sp. / 375g soil***	**Pathogen**	**Amount of pathogen inoculum**
M. albus/barley	2.0 g	Verticillium	5 g/kg soil
M. albus/barley	2.5 g	Verticillium	5 g/kg soil
M. albus/barley	3.0 g	Verticillium	5 g/kg soil
M. roseus/barley	2.0 g	Verticillium	5 g/kg soil
M. roseus/barley	2.5 g	Verticillium	5 g/kg soil
M. roseus/barley	3.0 g	Verticillium	5 g/kg soil
Pathogen control	-	Verticillium	5 g/kg soil
M. albus/barley	2.0 g	*Muscodor albus* control	
M. roseus/barley	2.0 g	*Muscodor roseus* control	
M. albus/barley	2.0 g	Pythium	1 plate[1]/6.4 kg soil
M. albus/barley	2.5 g	Pythium	1 plate/6.4 kg soil
M. albus/barley	3.0 g	Pythium	1 plate/6.4 kg soil
M. roseus/barley	2.0 g	Pythium	1 plate/6.4 kg soil
M. roseus/barley	2.5 g	Pythium	1 plate/6.4 kg soil
M. roseus/barley	3.0 g	Pythium	1 plate/6.4 kg soil
Pathogen control	-	Pythium	1 plate/6.4 kg soil
Non-inoculated control	-	-	-

[1]) Pythium inoculum: one 100 mm petri dish culture, two weeks old.

Greenhouse Flat Assay

The MSU Plant Growth Center provided 12 cm deep "dyna-flats™" (Dimensions: 35 cm wide, 50 cm long; Hummert International, Earth City, MO) for simulation of raised bed cultivation of chrysanthemum. Soil was infested with the same pathogens and equal amounts of inoculum as described in materials and methods for the greenhouse pot assay. The same chrysanthemum cultivar ("Bright Golden Anne") was used. Based on the information obtained from the greenhouse pot assay and preliminary experiments, the dosages of mycofumigant formulations for the flats was adjusted to 2 g / 375 g of soil. Treatments and dosages of this experiment are listed in Table 5.3.

Table 5.3: Mycofumigant dosages for raised bed cultivation of *Chrysanthemum* sp. and evaluation of disease control efficacy

Treatment formulation	***Rate of* Muscodor *sp./375g soil***	**Pathogen**	**Amount of pathogen inoculum**
M. albus/barley	2.0 g	Verticillium	5 g/kg soil
M. roseus/barley	2.0 g	Verticillium	5 g/kg soil
Pathogen control	-	Verticillium	5 g/kg soil
M. albus/barley	2.0 g	Pythium	1 plate[1]/6.4 kg soil
M. roseus/barley	2.0 g	Pythium	1 plate/6.4 kg soil
Pathogen control	-	Pythium	1 plate/6.4 kg soil
Non-inoculated control	-	-	-

[1]) Pythium inoculum: one 100 mm petri dish culture, two weeks old.

The formulations were incorporated into optimally moisturized soil in the flats, covered with white plastic tarps for temperature control and fumigated for one week. After the fumigation period, the plastic was removed and eight rooted chrysanthemum cuttings were planted per flat (40 plants / m²) spaced 15 x 18 cm (Dole and Wilkins, 1999; Escher et al., 1982). The experiment was designed completely randomized with four replications per treatment and the non-infested control. Plant cultivation followed normal production conditions for chrysanthemum. The plants were watered and fertilized (Peter's 15-16-17 Peat Lite Special Fertilizer and Calcium Nitrate 15.5-0-0; Hummert International, Earth City, MO) using drip irrigation. As described in the greenhouse pot assay, the chrysanthemum plants were harvested after a cultivation period of 12.5 weeks. The length of the stem, the number of flowers in the top 1/3 of the stem, the diameter of the terminal flower, and disease symptoms were evaluated (Horticultural Marketing Inspectorate, UK, 2001).

After harvesting, stem discs of *V. dahliae* infected plants were cut from the lower 2 to 5 cm of the stems. The stems were surface sterilized and cut as described above. The discs were placed on water agar and incubated for one week at 22 °C. After the incubation period the stem discs were examined under a dissecting microscope for Verticillium structures. The stem disc cuttings were rated for plant stem colonization by Verticillium using a colonization index (CI) calculated by the following formula:

$$CI = \frac{\Sigma\,(\text{Number of discs in each severity class x class number})}{(\text{Mean number of discs per treatment x number of colonization classes})} \times 100$$

Colonization severity classes used were: 0 = no colonization; 1 = mild colonization with few Verticillium structures; 2 = moderate colonization, Verticillium structures present on 50 % of the disc; 3 = severe colonization, Verticillium structures covering the disc to 100 %.

All experiments were designed completely randomized and repeated two times. The statistical analysis of the data was conducted by the analysis of variance using the general linear model procedure of the SAS program (Madden et al., 1982). The treatment means were separated using Fisher's least significant difference test (Swallow, 1984) and evaluated at $P < 0.05$.

Results

Mycofumigation Greenhouse Pot Assay

Verticillium dahliae

In soil infested with *V. dahliae*, mycofumigation with different dosages of *M. albus* and *M. roseus* resulted in significantly longer stems in the treatment *M. albus*, 2.5 g / 375 g soil compared to the non-infested control (Table 5.4). All other treatments resulted in stem length similar to the non-infested control plants. There was no significant difference in stem length within the other *Muscodor* sp. treatments. In the non-infested control and all other treatments, plants produced an equal number of flowers

per stem. Significant differences were observed in the developmental stage of the terminal flowers. The pathogen control, the non-infested control and *M. roseus*, 3 g / 375 g soil, resulted in significantly faster development of terminal flowers compared to the treatment *M. albus* 2.5 g / 375 g of soil.

Picture 5.1: Chrysanthemums after 12.5 weeks of cultivation (from left to right) Non-infested control, *V. dahliae* control, *M. albus* 2.0 g, 2.5 g, and 3.0 g

Within all other treatments, no significant differences were observed. The non-infested control as well as the treatment with *M. albus*, 3 g / 375 g soil, resulted in equal flower diameter. The treatments with *M. albus* 2.0 g, 2.5 g and *M. roseus* 2.5 g resulted in significantly smaller flower diameters, compared to the other treatments. Mycofumigated soil with only *M. albus* or *M. roseus* without the pathogen resulted in similar stem length and flower characteristics as the respective treatments with infested soil (data not shown).

In general the plants of the *V. dahliae* control resulted in thinner and more brittle stems (Picture 5.1) compared to all other treatments (data not shown). The plants appeared more asymmetrically developed with uneven flower development.

Table 5.4: Effects of *Muscodor* sp. on floricultural characteristics of *Chrysanthemum* x *grandiflorum* grown in *Verticillium dahliae* infested soil

Treatment		**Stem length (cm)**		**Number of flowers**		**Terminal flower development [1]**		**Diameter of terminal flower (cm)**	
M. albus	2.0g	69.58	ab	6.75	a	3.80	ab	10.03	c
M. albus	2.5g	70.64	a	7.15	a	3.50	b	10.07	c
M. albus	3.0g	69.13	ab	7.35	a	3.70	ab	10.66	ab
M. roseus	2.0g	70.38	ab	6.90	a	3.80	ab	10.18	bc
M. roseus	2.5g	68.05	ab	7.05	a	3.75	ab	9.45	d
M. roseus	3.0g	69.48	ab	7.45	a	3.90	a	10.15	bc
Verticillium control		68.15	ab	7.00	a	3.90	a	10.11	bc
Non-infested control		67.48	b	7.65	a	3.85	a	10.85	a
$LSD_{0.05}$		3.08		0.90		0.35		0.56	

Means followed by the same letter are not significantly different at $P < 0.05$.
[1)] Flower development: 1 = completely closed, no color; 2 = flower starts to open, shows color; 3 = flower ¾ open; 4 = flower completely open.

Determination of *Verticillium dahliae* Colonization

Mycofumigation with *M. albus* and *M. roseus* resulted in decreased Verticillium stem colonization in the stem discs compared to the *V. dahliae* pathogen control (Table 5.5). In the treatment *M. roseus*, 3 g / 375 g, colonization by the pathogen was significantly lower compared to the pathogen control. No significant differences were observed within the other treatments.

Table 5.5: Effects of *Muscodor* sp. on Verticillium colonization in stem disc cuttings from *Chrysanthemum* x *grandiflorum* grown in *Verticillium dahliae* infested soil

Treatment		Colonization severity (CI) [2]	
M. albus	2.0g	44.38[1]	ab
M. albus	2.5g	37.19	ab
M. albus	3.0g	33.75	abc
M. roseus	2.0g	45.31	ab
M. roseus	2.5g	42.81	ab
M. roseus	3.0g	24.69	bc
Verticillium control		58.44	a
Non-infested control		9.06	c
$LSD_{0.05}$		25.72	

[1]Means followed by the same letter are not significantly different at $P < 0.05$.
[2] Colonization Index was based on a scale of 0-3, with 0 = no colonization; 1 = mild colonization with few Verticillium structures; 2 = moderate colonization, Verticillium structures present on 50 % of the disc; 3 = severe colonization, Verticillium structures covering the disc to 100 %. Colonization index was calculated using the following formula:

$$CI = \frac{\Sigma(\text{ Number of discs in each severity class x class number})}{(\text{ Mean number of discs per treatment x number of colonization classes})} \times 100$$

<u>*Pythium ultimum*</u>

In soil infested with *P. ultimum*, mycofumigation with different dosages of *M. albus* and *M. roseus*, resulted in significantly shorter plants in the treatment *M. roseus*, 2.0 g, compared to the non-infested control (Table 5.6). There was no significant difference in stem length within the other *Muscodor* sp. treatments and the pathogen control. The non-infested control produced significantly more flowers compared to the treatments *M. roseus*, 2 g and 2.5 g. All other treatments resulted in equal numbers of

flowers. Furthermore, the development of the terminal flower was equal for all treatments. Treatment *M. roseus*, 2.5 g, resulted in significantly larger diameter of the terminal flowers compared to the treatments *M. roseus*, 2 g, *M. albus*, 2 g, and to the pathogen control. The *P. ultimum* control resulted in significantly smaller flowers

Picture 5.2: Effects of *Muscodor* sp. on Chrysanthemums (from left to right) non-infested control, *M. albus* 2.0 g, *M. roseus* 2.0 g

compared to all other treatments. No other significant differences were observed within the other *Muscodor* sp. treatments and the non-infested control.

Soil mycofumigated with *M. albus* or *M. roseus* without presence of the pathogen showed no negative effects on the chrysanthemum culture and resulted in similar stem length and flower characteristics compared to the non-infested control (Picture 5.2) (data not shown).

Table 5.6: Effects of *Muscodor* sp. on floricultural characteristics of *Chrysanthemum* x *grandiflorum* grown in *Pythium ultimum* infested soil

Treatment	**Stem length (cm)**		**Number of flowers**		**Terminal flower development [1]**		**Diameter of terminal flower (cm)**	
M. albus 2.0g	64.73	ab	6.85	ab	3.60	a	10.20	cd
M. albus 2.5g	64.83	ab	6.70	ab	3.75	a	11.10	ab
M. albus 3.0g	63.23	ab	6.80	ab	3.65	a	11.10	ab
M. roseus 2.0g	62.14	b	6.35	b	3.89	a	10.45	bc
M. roseus 2.5g	62.88	ab	6.20	b	3.70	a	11.25	a
M. roseus 3.0g	64.48	ab	6.80	ab	3.90	a	10.60	abc
Pythium control	65.28	ab	6.75	ab	3.85	a	9.56	d
Non-infested control	67.48	a	7.65	a	3.85	a	10.85	abc
$LSD_{0.05}$	5.22		1.12		0.37		0.74	

Means followed by the same letter are not significantly different at $P < 0.05$.
1) Flower development: 1 = completely closed, no color; 2 = flower starts to open, shows color; 3 = flower ¾ open; 4 = flower completely open

Mycofumigation Greenhouse Flat Assay

Verticillium dahliae

The mycofumigation greenhouse flat assay resulted in significantly longer chrysanthemum stems in the *Muscodor* sp. treatments compared to the non-infested control (Table 5.7). There were no significant differences in stem length within the *Muscodor sp*. treatments and the *V. dahliae* pathogen control. No differences between the pathogen control and the non-infested treatment were detected.

The treatments *M. albus*, *M. roseus* and the non-infested control produced significantly more flowers per stem compared to the pathogen control. No differences in the number of flowers could be determined within the *Muscodor* sp. treatments, whereas

the non-infested control resulted in significantly more flowers compared to the *M. roseus* treatment. The treatment with *M. albus* resulted in significantly faster development of the terminal flower compared to the non-infested control. No significant differences were observed between the *Muscodor* sp. treatments and the pathogen control. There was no significant difference in diameter of the terminal flower within all treatments.

Table 5.7: Effects of *Muscodor* sp. on floricultural characteristics of *Chrysanthemum* x *grandiflorum* grown in *Verticillium dahliae* infested soil

Treatment		**Stem length (cm)**		**Number of flowers**		**Terminal flower development [1]**		**Diameter of terminal flower (cm)**	
M. albus	2.0g	88.01	a	6.41	ab	3.86	a	11.13	a
M. roseus	2.0g	89.06	a	6.27	b	3.69	ab	10.70	a
Verticillium control		86.89	ab	5.77	c	3.70	ab	10.51	a
Non-infested control		84.70	b	6.84	a	3.61	b	10.83	a
$LSD_{0.05}$		3.19		0.48		0.21		0.64	

Means followed by the same letter are not significantly different at $P < 0.05$.
[1)] Flower development: 1 = completely closed, no color; 2 = flower starts to open, shows color; 3 = flower ¾ open; 4 = flower completely open

Determination of *Verticillium dahliae* Colonization

Mycofumigation with *M. albus* and *M. roseus* resulted in decreased Verticillium stem colonization in the stem discs compared to the *V. dahliae* pathogen control (Table 5.8). The treatments *M. albus* and *M. roseus* resulted in low stem colonization similar to the non-infested control. No significant differences in *Verticillium* infection were observed within the *Muscodor* sp. treatments.

Table 5.8: Effects of *Muscodor* sp. on disease severity in stem disc cuttings from *Chrysanthemum* x *grandiflorum* grown in *Verticillium dahliae* infested soil

Treatment		Colonization severity (CI) [2]	
M. albus	2.0g	25.15[1]	b
M. roseus	2.0g	22.85	b
Verticillium control		54.10	a
Non-infested control		6.60	b
$LSD_{0.05}$		24.12	

[1]Means followed by the same letter are not significantly different at $P < 0.05$.
[2] Colonization Index was based on a scale of 0-3, with 0 = no colonization; 1 = mild colonization with few Verticillium structures; 2 = moderate colonization, Verticillium structures present on 50 % of the disc; 3 = severe colonization, Verticillium structures covering the disc to 100 %. Colonization index was calculated using the following formula:

$$CI = \frac{\Sigma(\text{Number of discs in each severity class x class number})}{(\text{Mean number of discs per treatment x number of colonization classes})} \times 100$$

Pythium ultimum

In the mycofumigation greenhouse flat assay similar plant stem length was observed in the *Muscodor* sp. treatments and the *P. ultimum* infested soil of (Table 5.9). The chrysanthemum stems of the non-infested control was significantly shorter compared to the other treatments. The *M. roseus* treatment and the non-infested control produced significantly more flowers per stem compared to the *M. albus* treatment and the pathogen control. No significant differences of number of flowers were observed within the *M. albus* treatment and the non-infested control. Treatment *M. roseus*, 2 g, resulted in significantly faster development of the terminal flower compared to the non-infested control. There were no significant differences between the *M. albus* treatment and the pathogen control, compared to the non-infested control. Furthermore no significant

differences between the *Muscodor* sp treatments and the pathogen control were detected. *M. roseus*, 2 g, resulted in terminal flowers with significantly larger diameter compared to the pathogen control and the non-infested control. *M. albus* resulted in terminal flowers with equal diameter to the terminal flowers in the pathogen control and the non-infested control. No significant differences within the *Muscodor* sp. treatments were observed.

Table 5.9: Effects of *Muscodor* sp. on floricultural characteristics of *Chrysanthemum* x *grandiflorum* grown in *Pythium ultimum* infested soil

Treatment	**Stem length (cm)**		**Number of flowers**		**Terminal flower development [1]**		**Diameter of terminal flower (cm)**	
M. albus 2.0g	88.26	a	6.02	b	3.73	ab	11.12	ab
M. roseus 2.0g	89.16	a	6.61	a	3.87	a	11.64	a
Pythium control	87.75	a	5.69	b	3.79	ab	10.71	b
Non-infested control	84.70	b	6.84	a	3.61	b	10.83	b
$LSD_{0.05}$	2.78		0.56		0.21		0.59	

Means followed by the same letter are not significantly different at $P < 0.05$.
[1)] Flower development: 1 = completely closed, no color; 2 = flower starts to open, shows color; 3 = flower ¾ open; 4 = flower completely open

Discussion

Mycofumigation using *Muscodor* sp. is a new concept of soil-fumigation with a broad application potential. Due to the fact that methyl bromide and most of its chemical alternatives are extremely toxic to humans and animals, the non-toxic nature of the volatile organic gases of *M. albus* and *M. roseus* promise to increase the worker safety during soil fumigation and may provide a healthier environment for humans, animals and

plants. Another benefit of the *Muscodor* sp. is that they do not produce spores or other permanent structures, so an unintended dispersal of the fungi in the natural environment is unlikely (Stinson et al., 2003).

The results shown in this report indicate that the fungi *M. albus* and *M. roseus* are not phytotoxic to chrysanthemum. The results of mycofumigation in the greenhouse experiments involving the soil-borne pathogen *V. dahliae* show that *Muscodor* sp. can effectively reduce the colonization severity of *V. dahliae* and support the observations made by Stinson et al. (2003), using eggplants.

The lack of more severe disease symptoms in the experiments could be due to the fact that the chrysanthemum cultures were cultivated under optimal conditions for the plants. More severe disease symptoms including stunting, flower deformation, and root damage are expected under more stressed cultivation conditions.

Since there were no significant differences in the tray experiment between the *Muscodor* sp. treatments and the *V. dahliae* pathogen control in regard of stem length, terminal flower development and diameter (Table 5.7), it needs to be evaluated, if there would be a more significant effect under more stressed conditions. Nevertheless, there are trends to an increased number of flowers with a greater diameter of the terminal flower.

The stunting and root rot symptoms expected in experiments conducted with *Pythium ultimum* were not observed. The lack of infected and damaged roots or the alteration of the plant phenotype could be due to a low infection level and / or

unfavorable conditions for the pathogen. It would be necessary to repeat this experiment with a higher inoculation level to get more obvious results.

Future experiments will give way to a better understanding about an optimal growing substrate, application method, and an optimal dosage for *Muscodor* sp.

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