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Mortality of western flower thrips, *Frankliniella occidentalis*, under influence of single and mixed fungal inoculations

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The entomopathogenic fungus *Metarhizium anisopliae* is proved to be a biological control agent for Western flower thrips (WFT) *Frankliniella occidentalis*. Moreover, to enhance fungal activity and sustainability, the study of relationship of *M. anisopliae* with entomopathogenic fungi *Beauveria bassiana* and *Lecanicillium muscarium* in combination with antagonistic fungus *Trichoderma viride*, was conducted. *T. viride* was emerged under high humidity conditions that required for entomopathogenic fungi. The results demonstrated that the principal possibility of using the tank mixtures of different species of entomopathogenic fungi for control of WFT gave a good result for mass production. In addition, formulation of *T. viride* in combination with *M. anisopliae* could possible perform for mass production to be used as broad spectrum mycopesticide.

Key words: biological control, entomopathogenic fungi, antagonistic fungus, western flower thrips

Introduction

Western flower thrips (WFT), *Frankliniella occidentalis* Perg. (Thysanoptera: Thripidae) remains one of the most important pest of protected agriculture in the USA and world-wide (Mound, and Marullo, 1996; Mound, 1997; Berry, 1998; Moritz *et al*., 2001). This insect is identified by growers as a primary pest throughout many industrial and developmental countries, where pesticides are applied every 3-7 days in some crops (Skinner *et al*., 2003). WFT feeds on a wide range of greenhouse ornamentals, causing cosmetic

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damage that reduces crop value and transmitting virus diseases, which can destroy an entire crop, if this pest is not controlled (Moritz *et al.*, 2004; Robb and Parrella, 1995). Its cryptic behavior, rapid reproductive rate and potential to develop resistance to insecticides which makes WFT particularly difficult to control. Several predatory mite species are used to combat WFT in the aboveground parts of the plants (Van den Meiracker and Sabilis, 1999; De Courcy Williams, 2001; Jacobson *et al.* 2001). However, a large portion of WFT population pupates in the soil, which is generally overlooked as an environment to target for management (Berndt *et al.*, 2004; Deligeorgidis and Ipsilandis, 2004; Wiethoff *et al.*, 2004).

Several biological control agents exist for use against the above and below ground stages of WFT. Nematodes and predatory mites are available commercially. Various entomopathogenic fungi species have been studied for WFT management, and formulations based on *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium muscarium* have significantly reduced WFT populations in greenhouse vegetable and floral crops under research conditions (Brownbridge, 1995; Butt and Brownbridge, 1997; Bradley *et al.,* 1998; Shipp *et al.*, 2002; Ugine *et al.*, 2005, 2007). Such products have many desirable traits – they leave no toxic residues, and are generally harmless to beneficial insects and pose minimal risk to human and the environment (Dent, 1999; Goettel *et al.*, 2001). Despite these positive aspects, in commercial greenhouses, fungi have provided inconsistent results for WFT control. Jacobson *et al*. (2001) reported that glasshouse population of WFT were reduced by 87% with three consecutive high volume sprays or low volume mist applications of Naturalist-L or BotanyGard WP, both *B. bassiana*-based products, applied at 6-days intervals. Effect of the entomopathogenic fungi, *M. anisopliae, L. lecanii*, and *B. bassiana*, on mortality and injury level of WFT was investigated using special bioassay in laboratory under favorable physical conditions. *M. anisopliae* showed the highest level of activity (Gouli *et al.,* 2008). However, enhance of mycopesticides activity and efficacy of application technology remained very important part of research connected with WFT control. There are numerous possibilities for solution of this problem. First of all, the management of optimal humidity and temperature are the most important factors for fungal activity. Other possibility is linked with creation of genetic modification of fungal strains with defined biological properties. Additional possibility is connected with combination of different species of fungi which can show synergetic effects. The first possibility connected with creation of optimal physical conditions for fungal activity has two principal limitations. Temperature and humidity management can be realized only in greenhouses. However, high humidity required for effective

action of entomopathogenic fungi stimulates activity of phytopathogenic fungi. This shortcoming can overcome using combined application of entomopathogenic and antagonistic fungi. Unfortunately, there is not sufficient information about a relationship between these two groups of fungi. Genetic modification of fungal strains will give a great future, but, at the present time, we do not have sufficient information regarding ecological consequences associated with application of transgenic species. This circumstance does not provide reliable application of the genetically modified fungi under natural conditions. Therefore, combination of different species of fungi can increase efficacy of WFT control. At the present time, there is information about susceptibility of WFT to several entomopathogenic fungi including *B. bassiana, M. anisopliae, L. muscarium* and some others (Gouli *et al*., 2008). Theoretically, the combined application of different species of insect pathogens can provide raising the efficacy of pest control. Entomopathogens have different types of relationship *in vivo* including independent development, antagonism, synergism and others. There is restricted information about results of mixed fungal inoculation of insects. Several experiments were conducted for establishment of the effect of two fungal pathogens attacking a single host. *B. bassiana* and *M. flavoviridae* were applied together against the migratory grasshopper, *Melanoplus sanguinipes*. The results of mixed fungal inoculations have depended on temperature regimes (Inglis *et al.,* 1997, 1999). However, nowadays, the information related to the fungal mixed infections is very limited, and this problem demands special additional research. Possibility for using two or more fungal species has a practical importance from two points of view. First of all, there is possibility to enhance the efficacy of pest control. Second significance connected with practical needs. Usually, at the same time the growers have the problems with complex of pests, such as thrips, aphids, whiteflies, mites *etc.* However, each entomopathogenic fungus has its own target specialization; for example, *L. muscarium* is more effective against whiteflies and aphids, *B. bassiana* and *M. anisopliae* against thrips and mites. Additional problem is associated with possibility of combined application of entomopathogenic and antagonistic fungi. This possibility is pressing problem from two points of view. In the first place, the combined application of entomopathogenic and antagonistic fungi gives a possibility to conduct simultaneous control of complex plant pests. In the second place, the antagonistic fungi can suppress phytopathogenic microorganisms in period of application of mycoinsecticides when it is necessary to support high humidity level. Combined application of mycopesticides can solve the problem of plant protection from the pest complex using tank mix to apply them simultaneously.

Entomopathogenic fungus *M. anisopliae* has demonstrated the higher activity to WFT compared with *B. bassiana* and *L. muscarium* (Gouli *et al.*, 2008). Due to this circumstance, it is of interest to conduct the study of relationship of the entomopathogenic fungus *M. anisopliae* with antagonistic fungus *Trichoderma viride*, as well as entomopathogenic fungi *B. bassiana* and *L. muscarium* under *in vitro* and *in vivo* conditions. Antagonistic fungus *T. viride* was included as potential means for suppression of phytopathogenic microorganisms in case of humidity increase for improving the efficacy of fungal entomopathogens.

Materials and methods

Insects

Second instar WFT were aspirated from an even-age insect colony reared on bean plants, *Phaseolus vulgaris* cv. Royal Burgundy, at constant temperature 22º C, 90% R.H., 16:8 (L:D) light regime. The WFT colony is permanently supporting under laboratory conditions according special technology.

Fungi

The fungi used in this experiment (Table 1) were cultivated on ¼ Sabouraud dextrose agar supplemented with 1% yeast extract without antibiotics for 15 days at 24ºC to receive optimal sporulation. The fungal strains were characterized according to their growth rate in case of single and double opposite cultures. For estimation of growth rate of single cultures subsequent method was used. Ten microliters of a conidial suspension with titer 1 x 10^6 conidia per ml was pipetted onto a 10-mm diameter disc of filter paper located in the center of each of four Petri dishes containing 20 ml of media. Growth rate was estimated for 6 strains of entomopathogenic fungi and 3 strains of antagonistic fungus *T. viride*. Three strains were selected for experiments connected with mixed cultivation and mixed inoculation of WFT. Following double mixed cultures were used in these experiments: *M. anisopliae* with *B. bassiana*, *M. anisopliae* with *L. muscarium*, and *M. anisopliae* with *T. viride*. Three different variants of initial inoculum locations were used for cultivation of fungi together. According to the first variant, 10-mm diameter discs of filter paper with fungi were located in the opposite parts of the Petri dishes. Second variant included one fungus on 10mm diameter disc and opposite fungus on right-angled filter paper of 10 x 30mm size. Two right-

angled filter papers of the same size were used in third variant. Distance between opposite fungi in all experiments constituted 40 mm. Colony growth was marked on the dish bottom at the outer edges of the fungi at 2, 3, 5, 10, 15, and 20 days after inoculation and measured from the center of the Petri dish to the mark. Mature conidia for insect inoculation were collected in 0.01% Tween-80, agitated for 2 min using a Vortex-Genie-2 to disrupt conidial aggregates, and filtered through eight layers of cheesecloth.

Table 1. Entomopathogenic fungi used for the experiments.

Fungal species	Strain	Original host	Isolation date	Collection location and collector
Metarhizium anisopliae	ERL-49	*Heliothis zea* (Lepidoptera; Noctuidae);	Fall, 1983	Gainesville (Florida, USA) L.A. Lacey
	ERL-259	*Tenebrio molitor* (Coleoptera, Tenebrionidae)	1994	Taiwan, B.L. Parker
	ERL-1171	Avocado plantation soil	May, 2001	California, USA, S. Gouli, M. Brownbridge
Lecanicillium muscarium	ERL-65	*Frankliniella occidentalis* (Thysanoptera: Thripidae);	March, 1992	Bellegarde (France) M. Brownbridge B. Parker, M. Skinner
	ERL-132	*Adelgid tsugae* (Homoptera, Adelgidae)	Fall, 2005	Mt. Tom State Reservation, Holyoke, Massachusetts S. Gouli
Beauveria bassiana	ERL-1170	Unidentified thrips from avocado plantation soil	May, 2001	California, USA S. Gouli M. Brownbridge
Trichoderma viride	ERL-1348	Soil from avocado plantation	May, 2001	California , USA S. Gouli, M. Brownbridge
	ERL-1346	Hemlock tree	June, 2005	Mt. Tom State Reservation, Holyoke, Massachusetts, S. Gouli
	ERL-1347	Maple tree	June, 2006	Burlington Vermont , USA, S. Gouli

Fungal application

Bean leaves, *P. vulgaris* cv: Royal Burgundy were used for experiment as the fodder plant for test insects. Fresh standard leaves 70 x 40 mm in size were sprayed with 2 ml of conidial suspensions using a Potter precision spray tower.

Each group of insects included 20 WFT larvae on individual bean leaves in the containers used for colony thrips rearing. Concentrations of conidia 5.0 x 10^6, 2.5 x 10^6, and 1.25 x 10^6 per ml were used for the trials. Both water and 0.01% Tween 80 controls were included in each test. Each treatment was replicated eight times within a trial. Treated leaves were put in plastic containers providing satisfactory temperature condition (20^0C ± 2^0C) and humidity (85-90%) for the plant and insect material throughout the trial. Mortality readings were taken at 7 days after treatment.

Data analysis

All statistical analyses were performed using SAS® (SAS Institute, 1990) and plotted using SPSS (SPSS Inc., 2005). An □ = 0.05 was used for all statistical analyses, including mean separation tests. Data on fungal growth were analyzed as a completely randomized design. Rates of colony growth (millimeters per 24 hours) over the 20 days experiment were then calculated and the data reanalyzed using GLM of analysis of variance. The effect of suspension concentration for each fungus or fungi tested at all concentrations was determined with LSD and Turkey test for P = 0.05.

Results and discussion

Synchronous inoculation of entomopathogenic and antagonistic fungi on standard medium showed significantly differences in growth rate (millimeter per day) at the same temperature (Table 2). Growth rate of *T. viride* exceeded the one of the entomopathogenic fungi more than 10 times. There were not differences among the entomopathogenic fungal strains in their growth rate ($P < 0.0001$, $F = 137.43$, $df = 30.93$). Strains ERL-49, *M. anisopliae*, and ERL-65, *L. muscarium* grew significantly better than the others. Strain ERL-1348, *T. viride* showed the best results among all three tested cultures. The strains being high speed of growth were selected for estimation of their interaction at combined cultivation and for estimation of possibility of their combined application against WFT.

Table 2. Average (±SE) growth rate (millimeter per day) of entomopathogenic and antagonistic fungi.

Fungus	Strain	Radial growth rate (mm/day)
Metarhizium anisopliae	ERL-49	1.55±0.02
	ERL-259	1.0±0.01
	ERL-1171	1.4±0.01
Lecanicillium muscarium	ERL-65	1.45±0.02
	ERL-132	1.25±0.01
Beauveria bassiana	ERL-1170	1.15±0.01
Trichoderma viride	ERL-1348	12.7±0.08
	ERL-1346	12.0±0.07
	ERL-1347	10.0±0.06

Cultivation of fungus *M. anisopliae* together with *L. muscarium* or *B. bassiana* did not show significantly advantage to other fungal culture, but the growth rate of each culture was decreased to 30% - 35% relative to single cultivation (Table 3).

Table 3. Average (±SE) radial cross-growth rate (millimetre per day) of fungus *Metarhizium anisopliae,* strain ERL-49, under condition of cross-cultivation with antagonistic and entomopathogenic fungi.

Opposite fungus	Location of starting inoculum	Radial growth rate, mm/day	
		M. anisopliae	**Opposite fungus**
	O & O	1.0±0.01	0.9±0.01
Beauveria bassiana ERL-1170	O & II	0.95±0.01	0.75±0.008
	II & II	1.0±0.01	0.9±0.01
	O & O	1.0±0.01	1.0±0.01
	O & II	0.9±0.01	0.85±0.009
Lecanicillium muscarium ERL-65	II & II	1.0±0.01	0.95±0.01
	O & O	0	12.7±0.08
Trichoderma viride ERL-1348	O & II	0	12.7±0.08
	II & II	0	12.7±0.08
	O & O	1.55±0.02	1.55±0.02
Metarhizium anisopliae ERL-49 (control)	O & II	1.55±0.02	1.55±0.02
	II & II	1.55±0.02	1.55±0.02

T. viride completely suppressed growth and development of entomopathogenic fungi *L. muscarium* and *B. bassiana in vitro*. This circumstance can significantly influence on survival rate and persistence of entomopathogenic fungi in case of combined application.

Mortality rates for the different fungi were determined to be normally distributed by plotting the residuals of the model (Fig. 1). Bars show mean mortality rates achieved with different fungi and combinations tested.

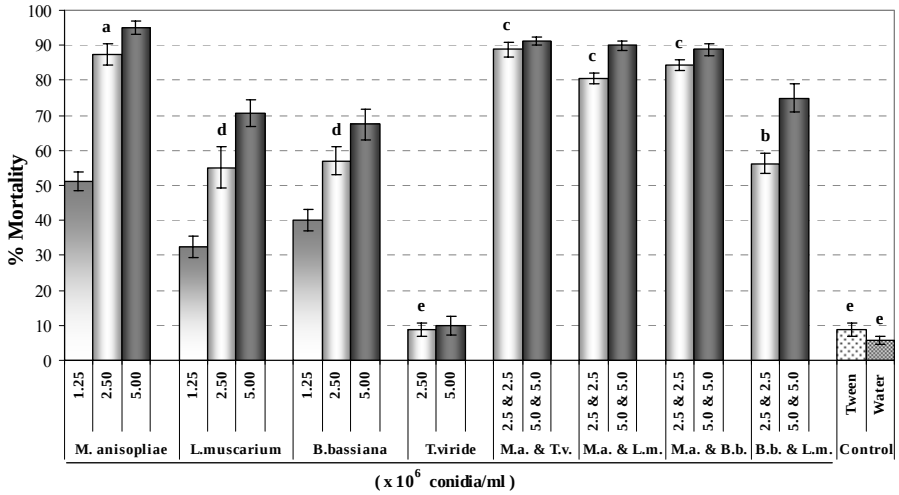

Fig. 1. Western flower thrips mortality in single and mix fungal bioassays. (Note: M.a. – *Metarhizium anisopliae*; L.m. – *Lecanicillium muscarium*; B.b. – *Beauveria bassiana*, and T.v. – *Trichoderma viride*).

The individual concentration attained mortality rates that was different statistically with LSD and Tukey tests. Fungus *M. anisopliae* 1080 was different from other fungi and combination tested and gave the highest one. The combination of fungi were higher in mortality rate than the fungus alone. However, the concentration of fungi was the double (2.5 + 2.5), so they can only be compared with the $5x10^6$ of fungus alone. The fungal combinations are higher in insect mortality than *B.bassiana* and *L. muscarium* at concentration $5x10^6$. *M. anisopliae*1080 gave better result than the others.

There were significant differences in mortality for the different fungi and suspensions used ($P<0.001$) and also there was a significant interaction between the two main effects (fungus used and suspension). There were differences in mortality for all tested fungi depending on the concentrations used, except for combination *B. bassiana* + *M. anisopliae*, *M. anisopliae* + *L. muscarium*; *M. anisopliae* + *T. viride*, and for *T. viride* alone. In these ones there were no differences in mortality at different concentrations.

It is showed that *T. viride* could suppress advantage over entomopathogenic hyphomycetes during their joint cultivation *in vitro*. This phenomenon is occurred both by the growth rate of the fungus and by its fermentative activity. It is necessary to assume that the entomopathogenic fungi during the joint application with the antagonist under the actual conditions would be in suppressed state and the expected action on target pest would not be achieved. It is completely understandable that under natural or hot-houses conditions, the complex of abiotic and biotic factors would act additionally. If the influence of abiotic factors including temperature, humidity and solar radiation can forecast from the known by portion probability with respect to the majority of the forms of hyphomycetous fungi, then biotic factors, such as the microbicidal properties of plants and the activity of epiphytic microflora frequently prove to be unpredictable. Nevertheless, on the basis of represented data, it is possible to draw the conclusion that the joint application of a fungal antagonist and a fungal entomopathogen can negatively affect the effectiveness of the latter. However, there are possibilities to avoid such an effect. First of all, it is necessary to ensure the direct contact of entomopathogenic fungus with the target pest on the basis of the optimization of spraying technology (Ugine *et al*., 2007). In this case, entomopathogen would immediately obtain advantage and would realize its potential. This is confirmed by the experiments described above on the joint application of a entomopathogenic fungus *M. anisopliae* together with the antagonist *T. viride*. The presence of antagonist did not affect the insect mortality caused by the entomopathogen. This result could be explained by the fact that in the laboratory the optimum contact of insects with pathogen was ensured. Under certain conditions, it is possible to use an antagonistic fungus on the second day after the application of mycoinsecticide. Thus, the diagram of optimum use can include treatment of plants by entomopathogenic fungus with a maximally high humidity, and then, after 24 hours, when entomopathogen enters productive interaction with the host. In addition, the experiments carried out on the joint application of entomopathogenic fungi make it possible to formulate a possibility in principle of using the tank mixtures of different forms of entomopathogenic fungi in the case of control of the complex of insect pests.

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