IJIE Int. J. Indust. Entomol. 35(1) 58-62 (2017)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2017.35.1.58

[Note]

Comparative Evaluation of Conidia, Blastospores and Culture Filtrates from Entomopathogenic Fungi against *Tetranychus urticae*

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Abstract

The two-spotted spider mite (*Tetranychus urticae*) has sustained damage on more than 200 host plants worldwide. Many farmers have relied on chemical acaricides to control mite, but the abuse of acaricides has caused serious resistance to mite. To overcome this problem, microbial control using entomopathogenic fungi have been studied. Entomopathogenic fungi have been an important role against the control of pest, and most of their culture products have been demonstrated to have virulence against pest population. In this study, we evaluated and compared the virulence of culture filtrates, aerial conidia and blastospores of selected *Metarhizium anisopliae* 4-2 and *Beauveria bassiana* 2R-3-3-1, respectively, among two-spotted spider mite-pathogenic fungi. As a result, the virulence was confirmed in all treatments, and the accumulated mortality rates were between 77 and 100% within 7 days. Especially, treatment with the fungal culture filtrate alone exhibited quite high virulence, and combined treatment with aerial conidia or blastospores enhanced activity. However, the median lethal time of treatments was not significantly different. When two isolates were compared, *M. anisopliae* 4-2 showed higher virulence than *B. bassiana* 2R-3-3-1. These results suggest that the selected two fungal isolates and their culture products could be used effectively for the control of two-spotted spider mite.

Int. J. Indust. Entomol. 35(1), 58-62 (2017)

Received : 12 Sep 2017
Revised : 14 Sep 2017
Accepted : 15 Sep 2017

Keywords:
Blastospore,
Conidia,
Culture filtrate,
Entomopathogenic fungi,
Tetranychus urticae

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, has sustained damage on more than 200 host plants, including vegetables, fruit crops, food crops and ornamentals (Gatarayiha *et al*., 2010). To control this mite, the most commonly used method has been relied on chemical acaricides, but long-term overuse of these acaricides has resulted in resistance and has become a serious problem (Shin *et al*., 2017). Thus, there is a need to find alternative strategies to control two-spotted spider mites populations.

Entomopathogenic fungi have been an important role in the control of pest populations. These fungi are commonly harmless to humans and are known to no negative impact on the environment. Also, these fungi can effectively control sucking pests because they infect with penetration into the insect cuticle (Lacey *et al*., 2001). Since the use of entomopathogenic fungi, more than 170 pest control products have been exploited based on at least twelve species

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of fungi. For example, various entomopathogenic fungi, such as *Beauveria* sp., *Lecanicillium* sp., *Metarhizium* sp., and *Paecilomyces* sp., have been used to control diverse pests (de Faria and Wraight, 2007). The method of producing these fungi is divided into two ways: aerial conidia in solid medium and blastospores in liquid medium. Aerial conidia are more stable to unsuitable environmental conditions than blastospores, but production of blastospores through liquid culture is necessary to obtain a large amount of products in a short time. Moreover, entomopathogenic fungi produce various compounds and metabolites, and these products generally have diverse activities and functions. Especially, the extracellular cuticle-hydrolyzing enzymes (protease and chitinases) and toxins (destruxin of *M. anisopliae* and beauvericin of *B. bassiana*) produced by entomopathogenic fungi are highly related to insecticidal activity and these enzymes and toxins can be supplied by liquid culture (St. Leger *et al*., 1986; Petlamul and Prasertsan, 2012).

Recently, we reported several two-spotted spider mite-pathogenic fungal isolates (Shin *et al*., 2017). In this study, among them, we evaluated the virulence of selected two fungal isolates for various culture products (fungal culture filtrates, blastospores and conidia) against two-spotted spider mite. This study proposes the possibility of culture product by entomopathogenic fungi as biological agents against the two-spotted spider mite.

Materials and Methods

Two-spotted spider mite

Two-spotted spider mites were collected from a colony of a continuous culture in the Insect Ecotoxicology Laboratory, Chungbuk National University, Korea. The two-spotted mite population was raised and maintained on garden pea (*Pisum sativum* L.) at 25±1 °C with 70% relative humidity and 12:12 (L:D) photoperiod. To obtain fixed-age females for the bioassays, quiescent deutonymphs were collected and put on leaf discs. The newly emerged mites were used for the experiments 2 day later (Shin *et al*., 2017).

Fungal growth and production of culture filtrates

Entompathogenic fungi with high virulence to two-spotted spider mite (Shin *et al*., 2017) were cultured on potato dextrose agar (PDA, Difco, USA) plates for 2 weeks at 25 °C. Then, two-week-old cultures were used for each experiment and stock cultures of each isolate were stored at -70 °C until further use. 14-day-old fungal conidia were collected by scraping fungi from PDA plates and suspending the material in a 0.02% Tween-80 (Difco, USA) solution. The conidial suspension was strongly stirred and through a sterile fourth layer of cheese cloth to remove the mycelial debris. The conidial concentration was adjusted using a hemocytometer, and viability was determined on PDA with 0.05% benomyl (95% active ingredient, Sigma, USA). Then, 50 μL of the conidial suspension (9×10^5 conidia/mL) with more than 90% viability was inoculated in 300 mL of PDB (pH 5.6) in a 1000 mL flask. The samples were cultured at 25 °C in the dark with shaking at 150 rpm for two weeks. After two weeks, the samples were centrifuged at 10,000 × g for 20 min at 4 °C, and then the precipitated cells and the supernatants were separated. The precipitated cells washed two times with sterile distilled water and through a sterile double layer of cheese cloth to remove the mycelial debris. The supernatants were filtered through a membrane filter paper (Advantec No. 2, Advantec, Tokyo, Japan) to separate the mycelial and spore masses, and The culture filtrate re-filtered through a 0.2 μm membrane filter (28 mm syringe filter, Corning, New York, USA). All fungal culture filtrates were stored at -70 °C until further use.

Bioassay

Bioassays were performed to confirm mite control efficacy among fungal culture filtrates, blastospores and conidia. The conidia suspensions in 0.02% Tween-80 or fungal culture filtrate were adjusted to 1×10^8 conidia/mL using a hemocytometer. The blastospore suspensions were also adjusted by the same method. Bioassays were conducted in 60 mm petri dishes containing moist cotton wool, and a cut 25 mm circle leaf disk of garden pea was placed on the plate. Twenty two-spotted spider mite nymphs were placed on the leaf disks using a hair brush pen, and then 1 mL of suspensions containing each fungal culture filtrates, blastospores, conidia and their mixture were sprayed into each dish by an SD tower sprayer (Shin *et al*., 2011). Treatments were maintained at over 70% relative humidity in a chamber at 25±1 °C and 12:12 (L:D) photoperiod for 7 days. Two-spotted spider mites were checked daily for mortality, and bioassay was repeated three times.

Statistical analysis

The mortality data was analyzed by SPSS statistics 24 (IBM, USA). Median lethal time for treatments used probit analysis. Data were subjected to one-way analysis of variances (ANOVA) and comparisons among groups were performed with analysis of Duncan's multiple range test. Data were expressed as means ± standard error (SE) and statistical significance was set at the conventional $\alpha < 0.05$ level.

Results

The virulence of *M. anisopliae* 4-2 and *B. bassiana* 2R-3-3-1 against two-spotted spider mite was evaluated for activity specific to their fungal culture filtrates, aerial conidia and blastospores. The virulence was confirmed in all treatments (Fig. 1), and the accumulated mortality rates were significantly different in all treatments except fungal culture filtrate alone

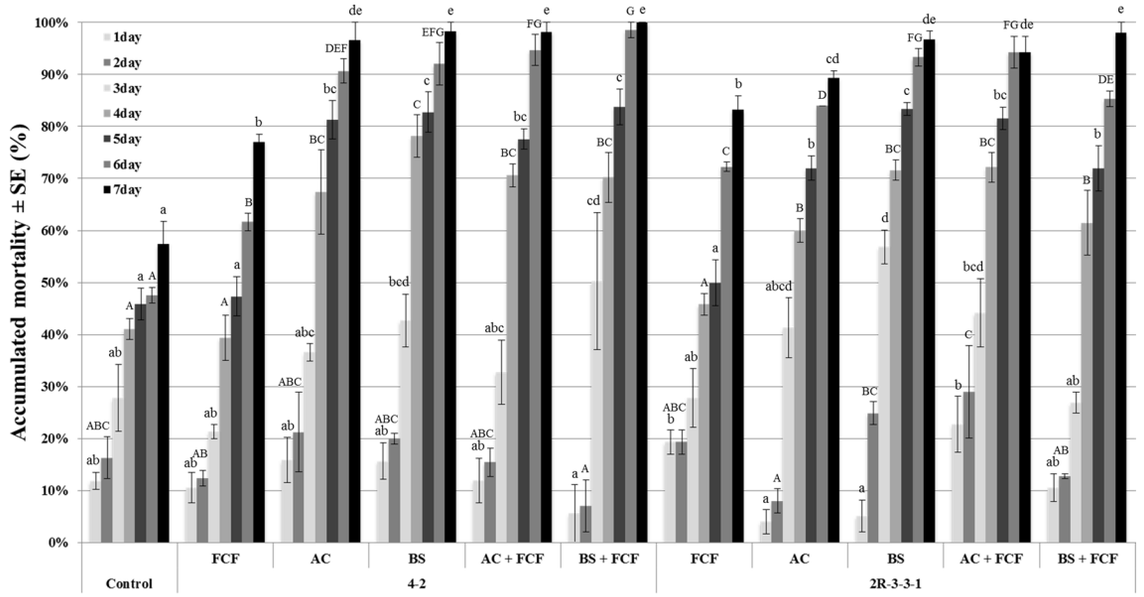

Fig. 1. Control efficacy of two-spotted spider mite by the fungal culture filtrates, blastospores and aerial conidia. Control, 0.02% tween-80; FCF, fungi culture filtrate; AC, aerial conidia; BS, blastospore. Values followed by different letters are significantly different among treatments by date (Duncan test, $P < 0.05$). Vertical bars correspond to the standard error.

Table 1. Evaluation of mortality and median lethal time against two-spotted spider mites

		Mortality ± SE (%)		LT50 (day)
		4 day	7 day	
Control		41 ± 2.1 a	57.4 ± 4.3 a	5.97
4-2	FCF	39.3 ± 4.3 a	77 ± 1.5 b	4.79
	AC	67.4 ± 8.1 bc	96.7 ± 3.3 de	2.85
	BS	78.1 ± 4.1 c	98.3 ± 1.7 e	2.69
	AC + FCF	70.6 ± 2.2 bc	98.2 ± 1.8 e	3.01
	BS + FCF	70.2 ± 4.8 bc	100 ± 0 e	3.02
2R-3-3-1	FCF	45.8 ± 2.1 a	83.3 ± 2.7 b	3.96
	AC	60 ± 2.3 b	89.3 ± 1.3 cd	3.52
	BS	71.6 ± 1.9 bc	96.7 ± 1.6 de	2.78
	AC + FCF	72.2 ± 2.9 bc	94.3 ± 3.1 de	2.49
	BS + FCF	61.4 ± 6.2 b	98 ± 2 e	3.35

Values are presented as mean ± SE. Means within the same column followed by the same letter are not significantly different using Duncan's multiple range test.

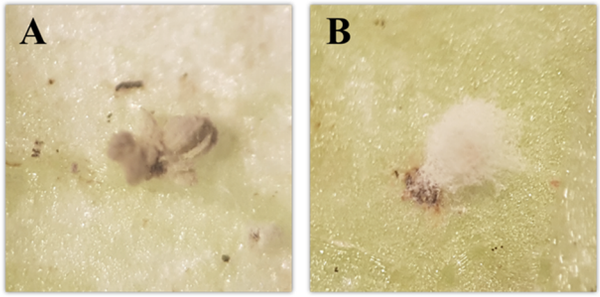

Fig. 2. Two-spotted spider mite infected with each isolates at 7 day after death. A, cadaver infected by *Metarhizium anisopliae* 4-2; B, cadaver infected by *Beauveria bassiana* 2R-3-3-1.

within 4 days. On the 7 day, the accumulated mortality rates were significantly different in all treatments and were between 77 and 100% (Table 1). Interestingly, treatment of the fungal culture filtrate alone exhibited quite high virulence with 77 and 83% for *M. anisopliae* 4-2 and *B. bassiana* 2R-3-3-1, respectively. Blastospores treatment alone showed the highest virulence in both fungi with 97 and 98% for *M. anisopliae* 4-2 and *B. bassiana* 2R-3-3-1, respectively. Combination treatments with fungal culture filtrates increased the mortality by both aerial conidia and blastospores at 7 post-treatment days when compared with all treatments alone. However, the median lethal time was not significantly different between the combined and single treatments (Table 1). When two isolates were compared, *M. anisopliae* 4-2 showed higher virulence than *B. bassiana* 2R-3-3-1 in all treatments except fungal culture filtrate alone. Cadavers from all treatments, except fungal culture filtrate alone, showed visible mycelia on the body surfaces (Fig. 2).

Discussion

In general, the control of spotted mites has been difficult because of their rapid reproduction and resistance development. Especially, chemical pesticides have caused many problems such as mite resistance development and environmental pollution. To overcome the problems of these chemical insecticides, the development of biological pesticides using entomopathogenic fungi has been developed. To produce successful and commercial biological agents, it is necessary to have high virulence to the target pests, mass production with rapid growth, and abundant sporulation (Feng *et al*., 1994). These needs are possible with blastospore production in liquid culture. The blastospores which are developed from hyphae in liquid culture could be obtained in 3-4 days. These blastospores are known for germinating more rapidly than aerial conidia, and these could be effective to control pests (Jackson *et al*., 1997; Vega *et al*., 1999). Culture filtrates of entomopathogenic fungi in liquid culture have insecticidal effect or feeding deterrence for pests (Kim *et al*., 2013). These filtrates have various enzyme activities, such as chitinase, lipase and protease, and are useful as pesticidal agents (Yoon *et al*., 2013). In this study, high virulence was showed on blastospores as well as culture filtrate, and the mixtures of blastospore and culture filtrate, which is the products of the liquid culture, showed synergistic effect (Fig. 1, Table 1). In this result, accumulated mortality of the control group tended to be slightly high. We supposed that humid condition caused high mortality in control group because the experimental insect, two-spotted spider mite, is very sensitive to humid condition. However, the treatment of fungal isolates and their culture products increased mortality clearly comparing controls (Table 1, Fig 1) and their death, except fungal culture filtrate along, was confirmed by fungi from the mycosis (Fig. 2).

Furthermore, it has been recently communicated that entomopathogenic fungi can play unexpected roles in nature such as fungal endophytes, antagonists of phytopathogens, beneficial rhizosphere microorganism and plant growth promoters. These effects of entomopathogenic fungi result from the production of various metabolites, such as antibiotics, compounds and enzymes (Vega *et al*., 2008). Secondary metabolites of entomopathogenic fungi are known to have varied insecticidal, anticancer, antimicrobial and antioxidant properties (Shin *et al*., 2017).

This study suggests that the liquid culture products, including blastospores and culture filtrates by the two fungal isolates, *M. anisopliae* 4-2 and *B. bassiana* 2R-3-3-1, would be powerful biological agent against two-spotted spider mite, and then we expect to be an excellent crop protection agent if the high insecticidal activity of the liquid culture products and unexpected roles of entomopathogenic fungi are applied together.

Acknowledgements

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (115086-2).

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