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Biological control of *Aphis fabae* and *Bemisia tabaci* by the native isolates of *Beauveria bassiana* in Kerman province

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In this research, we isolated the pathogenic strain, KB512, of entomopathogenic fungi *Beauveria bassiana* from soil and insects, which produced aerial and submerged conidia and blastospores in the laboratory conditions. We investigated the best conditions for the production and utilisation of the spore suspension to spray *Aphis fabae* and *Bemisia tabaci*, which are two important pests in Kerman province (Iran). The pathogenicity test was carried out with direct spray. To bioassay the isolates, three concentrations of the spore suspension were prepared as follows: 1×10^8, 1×10^7 and 1×10^6 conidia/ml. The pests were sprayed by aerial conidial suspension, which was prepared by 0.01% Tween-80 in distilled water, and the controls were sprayed by 0.01% Tween-80 in distilled water. After spraying the pests, the plates were incubated at 25 ± 1°C and 80% of relative humidity. Then the treated pests were monitored every day for the fungal growth and mortality. After nine days, mortality in the insects treated with 1×10^8 conidia/ml was 42.4% and 34% for *Aphis fabae* and *Bemisia tabaci*, respectively. Nowadays, biological control methods, especially the use of microbial agents, are believed to be environmentally friendly and safe for non-target organisms. Therefore, such biological agents can be mass produced to control the pests.

Keywords: *Aphis fabae*; *Bemisia tabaci*; *Beauveria bassiana*; biological control; entomopathogenic fungi

Introduction

The increasing of the population throughout the world requires more staple food; however, each year pests destroy and waste about one-fourth of the agricultural products. Due to human requirements and the big amount of agricultural wastes, it is necessary to control the pests by an eco-friendly approach. According to the recent findings, the most effective and quick way to control pests is the use of chemical insecticides. Unfortunately, chemical synthetic insecticides are harmful for both humans and environment; therefore, researchers are pursuing alternative methods, which are considered to be less harmful, in order to protect agricultural products; one of them is biological control. Biopesticides are made of bacteria, viruses, nematodes, fungi and protistes agents. Apart from these, fungi have an important role in controlling the population of insects. So far, more than 700 species of

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entomopathogenic fungi have been recognised in the Hyphomycetes class. Among them, *Beauveria bassiana* is one of the most useful fungi in this regard.

Entomopathogenic hyphomycete fungi have great potential as biological control agents against insects and as one component within integrated pest management systems. They are being developed worldwide for the control of many important agricultural pests. Some of them are already available commercially for the control of various species of thrips and aphids.

White flies (Hemiptera: Aleyrodidae) are pest insects that affect a wide diversity of crops worldwide. Although more than 1200 species have so far been described, only *Trialeurodes vaporariorum* (Westwood), known as the "greenhouse white fly" and *Bemisia tabaci* (Gennadius), known as the "sweet potato white fly" have significant economic impact, resulting in substantial losses in global agricultural production. Even at low densities, whiteflies characteristically cause damage to the plants, both direct (debilitation as a result of sap extraction) and indirect (transmission of viruses and facilitation of the development of saprophytic fungi, e.g. *Limacina* spp. and *Capnodium* spp.). White flies feed by piercing the tissues of plants and sucking the sap directly from the vascular bundles. Consequently, they are not susceptible to many common insect pathogens, including bacteria and viruses, which are normally transmitted via host feeding on contaminated foliage. Most of the entomopathogenic fungi, on the other hand, infect their hosts by direct penetration into the body wall. Surveys have revealed that they are among the most important natural enemies of whiteflies, and various species have been registered or are under development as microbial control agents. *Bemisia tabaci*, originally obtained from interceptions on plants in trade, were cultured under quarantine conditions in Perspex cages (60 cm × 60 cm × 80 cm) on greenhouse poinsettia (*Euphorbia pulcherrima* cv. LiloPink) at 23 ± 1°C, 16:8 h light:dark (L:D) regime and an artificial dawn and dusk. The black bean aphid (*Aphis fabae*) is a true bug in the order, Hemiptera. It has specialised piercing and sucking mouthparts, which are used to suck plant juices. This insect is found throughout the Western Europe, Asia, Africa, North and South America. It is found on sugar beet, bean, potato, sunflower and tomato. Furthermore, it colonies more than 200 species of cultivated and wild plants. Among the latter, it prefers *Papaver somniferum, Arctium tomentosum, Chenopodium album, Atriplex rosea, Matricaria recutita* and *Cirsium arvense* (Brown 1998; Faria and Wraight 2001).

As a result of infestation by the black bean aphid, the leaves of sugar beet become swollen and rolled and the development is ceased. The roots develop poorly and the sugar content is becomes lower. Damage to flowers hinders the seed formation. Honeydew is produced and sooty moulds develop on the residue. In some other plants, although the leaves do not become distorted, growth is affected and flowers abort due to the action of toxic saliva. This aphid is also the vector for certain plant virus diseases. More studies with the aim of better controlling of these aphids in the region, was our purpose in this research.

Materials and methods

Source of conidia

Aerial conidia were produced on YPG agar medium, consisting of 0.2% yeast extract, 1% pepton, 2% glucose and 1.5% agar. Blastospores were produced in either YPG liquid culture or in a variety of media that contained 0.2% yeast extract,

1% pepton and 2% of one of the following carbohydrates: glucose, manitol, maltose, sorbitol, fructose, *N*-acetyl-D-glucosamin (GLCNAC), glycerol or trehalose. The isolates of *B. bassiana* were cultured on oatmeal agar (OA) and complete agar (CMA) that contained 0.001 g $FeSO_4$, 0.5 g KCl, 1.5 g K_2PO_4, 0.5 g $MgSO_4$ 7 H_2O, 6 g $NaNO_3$, 0.001 g $ZnSO_4$, 1.5 g casein, 0.5 g yeast extract, 10 g glucose, 2 g peptone, 20 g agar and 1000 ml distilled water. The viability of the conidia was assessed in Sabouaud Dextrose Agar plus 1% Yeast Extract (SDAY) (Uma Devi et al. 2001) (Figure 1).

Production of conidial suspension

The aerial conidia were obtained from three-week-old sporulating cultures grown at 25 ± 1°C on Petri dishes of semisynthetic medium (0.39 g KH_2PO_4, 1.42 g Na_2HPO_4 12 H_2O, 0.6 g $MgSO_4$ 7 H_2O, 1.0 g KCl, 10 g glucose, 0.7 g NH_4NO_3, 5.0 g yeast extract, 15 g agar and 1000 ml distilled water). The conidia were harvested directly from the surface of these cultures by scraping. Then they were suspended in sterile distilled water by shaking in 250 ml flasks containing 0.01% Tween-80 and glass beads (3 mm diameter). The suspensions were then adjusted to defined concentrations using a haemocytometer slide (Luz and Fargues 1997) (Figure 2).

Source of insect

Healthy *Aphis fabae* and *Bemisia tabaci* colonies were obtained from the Insect Pathology Collection at Bahonar University of Kerman and were cultured on Sabouraud Dextrose Agar (SDA) to recheck their healthiness. These colonies were

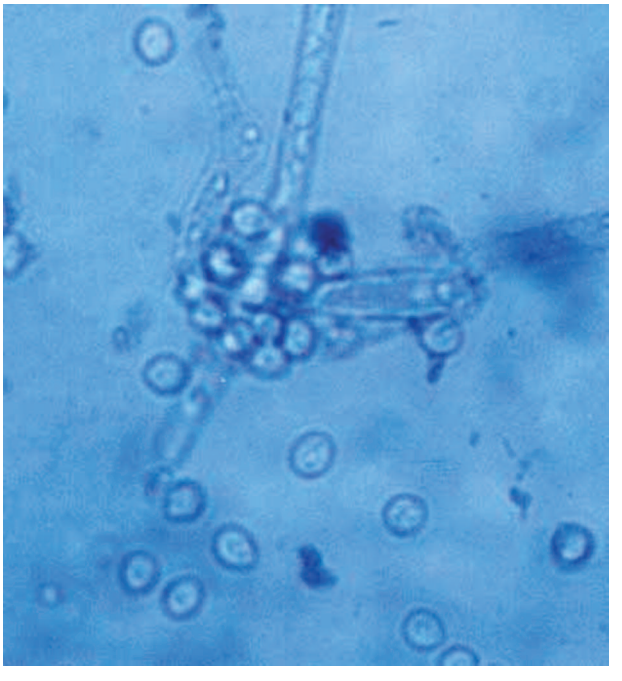

Figure 1. Conidiophore and conidia of *B. bassiana* on SDAY medium.

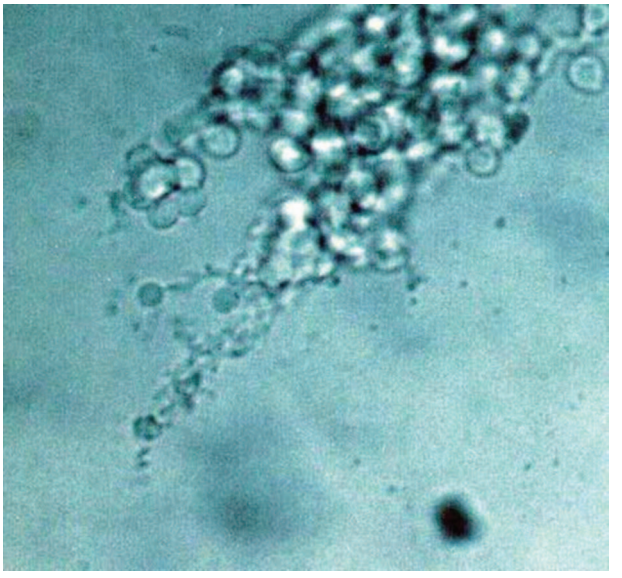

Figure 2. Aerial conidia of *B. bassiana* on semisynthetic medium.

used in bioassay tests to investigate the pathogenicity of *B. bassiana* (Chase et al. 1986).

Isolation from soil

Twenty soil samples were collected randomly from under the stump fruit orchards in Kerman country region using an auger to a depth of 10–20 cm. The collection tool was surface-disinfected by sodium hypochlorite between the samples to avoid cross-contamination. The soil samples were placed in a refrigerator at 5°C until processing. Soil plating dilution method was used to isolate the fungus from the soil; 5 g of soil was suspended in 50 ml sterile distilled water, and the sample was shaken for 30 min on a rotary shaker to release propagules from the soil matrix. A semi-selective medium, oatmeal agar amended with chlortetracycline and incubated in a liquid state between 25°C and 27°C. At the time of analysis, 1 ml of the extracted soil solution was distributed in two Petri dishes, and the molten oatmeal agar was poured over it. Homogenisation was done by manually rotating the dishes gently in a horizontal plane. Then the dishes were sealed with Parafilm® and incubated at 25°C for 2–7 days. After checking with stereomicroscope, individual colonies were transferred into PDA plates, and subsequently, the isolates were mounted in *lacto phenol blue* for identification (Figure 3). Initial classifications were based on the microscopic examination of morphological traits such as conidiophores and conidia using an Olympus research microscope (Strasser et al. 1996).

Morphological properties of the fungus on PDA

Species within the genus *Beauveria* are typically distinguishable from the other fungi by their morphological characteristics. They are filamentous fungi that freely produce colourless (hyaline) aerial conidia from conidiogenous cells on the mycelia.

This characteristic places them within the Moniliaceae (having hyaline conidia) Hyphomycetes. Aerial conidia are initially produced as terminal swellings formed on the conidiophore. The next conidium grows laterally, half way up the first neck of the conidiophore, towards the opposite direction and is pushed upwards by the sympodial growth. The resulting denticulate rachis, with denticles equally wide as the rachis, is characteristic of *Beauveria* spp. Colonies of *B. bassiana* grow relatively slowly and can appear powdery or wooly, with colours ranging from white to yellow, and occasionally pinkish. Aerial hyphae are septate, smooth, hyaline, with about 2 μm wide (Figure 4). Submerged hyphae are similarly structured but larger (1.5–3 μm). Conidiogenous cells, arising from short swollen stalk cells, are often found in dense clusters or whorls. They consist of a globose base and the characteristic denticulate rachis. The aerial conidia are hyaline, smooth and relatively thin walled.

Figure 3. Clone of *B. bassiana* on PDA medium.

Figure 4. Phialid of *B. bassiana* on PDA medium.

They vary morphology from oval to spherical depending on the species, and occasionally by cultural conditions.

Fungal isolates

Beauveria isolates were isolated from soil, by using soil dilution plating technique. The isolates were grown and stored on Potato Dextrose Agar (PDA) in the laboratory condition at 25°C. Then they were cultured in 500 ml Erlenmeyer flasks containing sterile Potato Dextrose Broth (PDB) and were maintained in an incubator shaker for 3 days at 24°C and 150 rpm. The contents were filtered through eight layers of cheesecloth to separate mycelia from broth. The harvested conidia were washed with sterile distilled water and stored at −20°C until use (Abdo et al. 2006).

Blastospore culture

Beauveria isolates were cultured in 100 ml PDB in 250 ml Erlenmeyer flasks on a 300 rpm rotary shaker incubated at 28°C in a constant temperature chamber. Blastospore concentrations were measured microscopically using a haemocytometer at 2–4 or 7 days post-inoculation. For each experiment, two blastospore counts were made for each flask on each sampling date and three replicates (Hegedus et al. 1992).

Bioassay tests

Inoculation of *Aphis fabae* and *Bemisia tabaci* adults was carried out in 9-cm Petri dishes. The bottom of each dish was lined with filter paper moistened with distilled water and the edges were sealed with Parafilm®. Groups of 10 rearing adult insects were stored at 4°C for 15 min. The adult insects were sprayed by 10 ml of 1×10^8 spore suspension with constant pressure (0.7 kg/cm^2), while the control adult insects received 0.01% Tween-80. The stock solution was diluted and counts of the conidia were made using an improved Neubauer haemocytometer. For every insect stage, a new stock solution was prepared since it is difficult to get all insect stages at one time. After application, the adults were placed in separate screen cages, fed with fresh untreated *Altheae officinalis* pads and kept for 16 days at 23°C, 54% RH and a photoperiod of 12:12 h (L:D). To ensure high humidity for conidia germination, all dishes were sprayed with a mist of distilled water on alternate days. The bioassays were repeated three times. The adults were examined for mortality every 48 h for 16 days. The dead insects were removed and incubated at 25°C and 90% relative humidity to check for infection by direct visual observation. The assessment of disease symptoms and insect survival was done at 5, 7 and 9 days after treatment.

Each insect on a slide was stained with one droplet (≈2 µl) of Lacto fuchsine stain (1 mg acid fuchsine/ml lactic acid) just before observation at 500×. The conidia were immediately stained red, unlike the non-treated insects and debris (Tucker and Talbot 2001).

Results

Aphis fabae and *Bemisia tabaci* are the key pests in the world and cause many damages to our products; however, we can control them with *Beauveria bassiana*, a fungus which causes a disease known as the White Musculine disease in insects.

When spores of this fungus come in contact with the cuticle (skin) of susceptible insects, they germinate and grow directly through the cuticle to the inner body of their host. The fungus proliferates throughout the insect's body, produces toxins and drains nutrients of the insect and eventually kills it. Therefore, unlike bacterial and viral pathogens of insects, *Beauveria* and other fungal pathogens infect the insect through contact and do not need to be consumed by their host to cause infection. Once the fungus has killed its host, it grows back out through the softer portions of the cuticle, covering the insect with a layer of white mould called as white muscadine disease. This downy mould produces millions of new infective spores that are released to the environment. Among the advantages of this fungus are its persistence and its reproduction in the soil. The success of fungal entomopathogens as biological control agents depends not only on their high efficacy against insect pests but also on low virulence against non-target insects. The use of such biological control agents to control insect pests might also have an effect on beneficial insects, such as the natural enemies of insect pests. Collembolan represents one of the most abundant arthropod groups in soil. We also tested its safety on non-target insects and the result showed that it is safe and secure.

The effect of entomopathogenic fungi, *Beauveria bassiana* (Balsamo), on non-target insects, such as natural enemies, *Coccinella septempunctata* L. (Col., Coccinellidae), *Chrysoperla carnea* (Stephens) (Neur., Chrysopidae) and *Dicyphus tamaninii* (Hem., Miridae) as well as on the beneficial soil insect, *Heteromurus nitidus* (Collembola: Entomobryidae) were studied by Thungrabeab and Tongma (2007). The experiments were conducted on conidial suspensions at a concentration of 1×10^8 conidia/ml. The results showed that *B. bassiana* is non-pathogenic to natural enemies and beneficial soil insects. The GHA strain of *B. bassiana* had no negative effect on fish (Collins et al. 1994) or birds (Althouse et al. 1997). The potential for resistant *Bemisia tabaci* populations to develop as a consequence of intensive use of chemical insecticides has stimulated studies on integrated pest management strategies in which biological control may play a significant role. The importance of predators and parasitoids has been discussed elsewhere (Gerling 1990). Although extensive research about the biological control of white fly by parasitoids and predators has been conducted, entomopathogenic fungi can also be considered as potential biological control agents. It was observed that by the day three post-parasitisation (after parasitoid egg hatch), *B. tabaci* nymphs were immune to infection by *B. bassiana* strain GHA (Poprawski et al. 2000). Bioassays of *B. bassiana* strain GHA against the second, third and fourth instars of *B. tabaci* reported by Wraight (1997) showed no direct relationship between instar and LC50. In our study, *Beauveria* was also isolated from two out of the 20 soil samples using the dilution plate method and a semi-selective medium. Initial classifications were done based on conidial shape. *Beauveria bassiana* was inoculated on *Aphis fabae*

Table 1. Mortality of *Aphis fabae* and *Bemisia tabaci* at 5, 7 and 9 days post-inoculation with 1×10^8 conidia/ml suspension of *Beauvaria bassiana*.

	Mortality %		
	Day 5	Day 7	Day 9
Aphis fabae	20.57	26.11	34.16
Bemisia tabaci	26.59	31.83	42.4

and *Bemisia tabaci* using a 1×10^8 conidia/ml suspension of the fungus in the laboratory conditions. Mortality after 5, 7 and 9 days was 26.59%, 31.83% and 42.4% for *Aphis fabae* and 20.57%, 26.11% and 34.16% for *Bemisia tabaci*, respectively, (Table 1).

Wraight and Ramos (2002) found that *B. bassiana* affects on a wide variety of insect groups (beetles, caterpillars, thrips, aphids, etc.). However, few have investigated the effect of chemicals commonly used to control white flies on *B. bassiana*. Hassan et al. (2002) reported that Luprofezin is relatively harmless on *B. bassiana*. This study has confirmed that direct mixing of Luprofezin with *B. bassiana* is a viable IPM option.

Almeida et al. (1997) found that *B. bassiana* isolates from termites were more virulent than those obtained from soil samples. We also did this test and found that high virulence and pathogenicity are essential for *B. bassiana* to control termites.

Poprawski et al. (2000) reported that the third instars of *Bemisia tabaci* were highly susceptible to infection by *B. bassiana* on cucumber plants. We also did this test on cucumbers and tomatoes and gained the same results.

The improvement of mass production technologies continues to be of critical importance, although optimisation of biphasic fermentation techniques providing high yields of stable conidia has been a major recent advance. Emerald BioAgriculture Corporation (formerly Mycotech) of Lansing, Michigan, USA, has an installed capacity for producing 5×10^{18} conidia of *B. bassiana* per year, an amount sufficient for treatment of 200,000 ha at a rate of 2.5×10^{13} conidia/ha (Bradley et al. 1992). The average yield is 1×10^{10} conidia/g of a proprietary substrate occupying approximately 1 l of fermenter space (Bradley et al. 1994). The isolated of *B. bassiana* as the active ingredient in the mycoinsecticide products developed by Mycotech for whitefly control was strongly influenced by the greater conidia mass-production potential of this fungus relative to that of *P. fomosoroseus* (Wraight et al. 1998). Since it reduces the consumption of toxins in the province, if the toxins are not used, the soil and water will remain poisonous and consequently the disadvantages for human health will be diminished. Conidia of entomopathogenic fungi formulated in pure vegetable oils and adjuvant oils are claimed to be more resistant to UV radiation than unformulated conidia (Alves et al. 1998). Therefore, it is suggested to be used it in greenhouse products and in IPM, along with safe toxins.

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