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SELECTION OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN ISOLATES FOR MANAGEMENT OF *MYZUS PERSICAE* (SULTZAR) (HOM.: APHIDAE) BASED ON VIRULENCE AND GROWTH RELATED CHARACTERISTICS

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ABSTARCT

Isolates of the entomopathogenic fungus *Beauveria bassiana* originated from Jordan were evaluated for their efficacy against the green peach aphid, *Myzus persicae* under laboratory and greenhouse conditions. Efficacy evaluation involved testing all isolates at a concentration of 1×10^7 conidia/mL followed by concentration dependent and greenhouse bioassays for the top virulent isolates. Growth characteristics related to virulence were evaluated for high, intermediate and low virulent isolates. Results showed that three isolates namely: BAU004, BAU018 and BAU019 were highly virulent to the aphid in the laboratory causing more than 75% infection. In the greenhouse, the three isolates caused infection from 41.3 to 46.5%. For the growth characteristics, isolate BAU019 produced more spores than the other highly virulent ones including the commercial isolate GHA. Highly virulent isolates also showed faster hyphal growth than low virulent isolates. These findings indicate that isolates BAU004, Bau018 and BAU019 might be developed as commercial microbial insecticides for safe and effective control of green peach aphid.

Keywords: *Beauveria Bassiana*, *Myzus Persicae*, Isolates, LC_{50}

1. INTRODUCTION

Entomopathogenic microbes such as fungi, bacteria and viruses can be developed as microbial insecticides to play a major role in Integrated Pest Management (IPM) and organic farming. Unlike chemical insecticides, these natural products are considered more safe for humans, less hazardous to the environment, less disruptive to natural controls and well not be eventually rendered ineffective due to resistance development (Hajek and Leger, 1994). The widespread fungus *Beauveria bassiana* (Balsamo) Vuillemin has been the focus for commercial development for many years (Goettel *et al*., 1997). Currently, this microbial control agent is registered for commercial use in Europe, The US and other parts of the world. *B. bassiana* has many characteristics that made it convenient for commercial development. It exhibit wide host ranges including many key pest species for economically important crops, can be produces on inexpensive artificial media and has long shelf life (Hajek and Leger, 1994; Goettel *et al*., 1997). Moreover, the infection process with *B. bassiana* involves the adherence of infective propagules to the host insect followed by direct penetration through the cuticle. This route of entry eliminates the need for the infective propagule ingestion by the host which is crucial for other entomopathogenic microbes such as bacteria and viruses. Therefore, *B. bassiana* could be more appropriate for management of insect pests with piercing sucking mouth parts which are unlikely to ingest microbes upon their feeding (Wraight and Carruthers, 2010). Aphids including the Green Peach Aphid, *Myzus persicae* (sultzar) feed by sucking the plant sap from the vascular bundles, have soft bodies that are less heavily sclerotized compared to other insects, sluggish and slow moving making them excellent candidates for microbial

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control by entomopathogenic fungi. The green peach aphid is widespread aphid species in the temperate and tropical parts of the world. It is highly polyphagous attacking many vegetable and fruit tree crops. In spite the commercial introduction of several predators and parasitoids for green peach aphid management, chemical insecticides continue to play a major role in green peach aphid control particularly in the tropical areas of the world (Abdel-Wali *et al*., 2007). Due to intensive insecticidal applications, green peach aphid populations had developed resistance to many insecticides including relatively new classes such as the neonicotinoids (Foster *et al*., 2002; Puinean *et al*., 2010). Therefore, safe and ecofriendly control measures such microbial control agents are required for effective green peach aphid management.

It is well known that isolates of anentomopathogenic fungus exhibit different efficacies against the same insect pest species. Therefore, one of the key factors that might lead to control failure when using microbial control agents is the inability to identify strains active at low doses (Leger and Wang, 2010). Therefore, this study evaluated 32 *B. bassiana* isolates for their efficacy against green peach aphid in the laboratory and greenhouses. Moreover, virulence related growth characteristics for isolates with different efficacies were determined and discussed in relation to isolate activity against green peach aphid.

2. MATERIALS AND METHODS

2.1. Experimental Material

The entomopathogenic fungal isolates evaluated in the current study were isolated using the *Galleria* baiting method (Zimmermann, 1986) from soil samples collected from different areas in Jordan. After isolation, the isolates were cultured on *B. bassiana* selective medium consisting of Sabouraud's dextrose agar (SDA) amended with 1% wt/v yeast extract (SDAY), 0.55% wt/v Dodine and 0.005% wt/v chlortetracycline (Chase *et al*., 1986) and then stored as dry conidia in a refrigerator at 4°C. In total, 33 *B. bassiana* isolates were tested consisting of 32 Jordanian isolates in addition to the commercially available green peach aphid isolate (BotaniGard®, Bio Works, USA). Before the bioassay was conducted, all the isolates were passed through *Galleria melonella*, re-isolated from the infected insects and cultured on the selective medium for 2 wk in the dark at 24±2°C. Conidia were harvested with a spatula and stored at 4ºC until used. Shortly before the bioassay, number of conidia per unit weight of each isolate was determined by suspending six 0.1g samples taken at random in 100 mL sterile distilled water and 0.1% v/v Tween 80 and agitated on a rotary shaker at 125 rpm for 3 h. After agitation, number of conidia was determined using a haemacytometer. The viability of *B. bassiana* conidia was checked by adding 200µl of each conidial suspension to 2 mL of Sabouraud's dextrose broth amended with 1% wt/v yeast extract (SDY) and 0.1% v/v Tween 80. After incubation for 20 h. at 24°C in darkness germination was assessed by counting 100 spores in four different fields of view of a haemacytometer (total of 400 spores).

Green peach aphid culture was established from adults and nymphs collected from naturally infested sweet pepper plants *Capsicum annum* L. The aphid was cultured in a controlled greenhouse compartment at 25 ± 5°C and a 16-h photoperiod on potted pepper plants, *C. annum*. The plants were grown in 15 cm diameter pots filled with a mixture of 1:1 sand and peat moss. When adult aphids were required for the experiments they were collected from plants using a camel hair brush after gentle propping to ensure that their piercing mouth parts were not damaged during the process.

2.2. Bioassay with Fungal Isolates

Evaluation of the virulence of the fungal isolates was started by a screening test for all isolates at the same concentration against the aphid adult stage.A suspension of each fungal isolate was prepared by suspending dry conidia in sterile distilled water with 0.1% v/v Tween 80. The concentration of the conidia for each suspension was then adjusted to 1×10^7conidia/mL. Ten adult aphids were aspired from infested plants in the culture and immobilized by placing in a refrigerator for 5 min. The insects were then contained inside a double petri dish cage with a fresh tomato leaflet as described by Al-mazra'awi and Ateyyat (2009). While still immobilized, the insects were consistently sprayed with each tested isolate using a potter spray tower (Burckard Scientific, UK). To prevent carryover effect among isolates, the potter tower was cleanedwith 70% ethanol and sterile distilled water between spraying sessions. Sterile distilled water plus Tween 80 was used as a control. The sprayed aphids were kept in a growth chamber at temperature of 24±2°C, 65±10% RH and 16:8 hr. photoperiod for 2 d. After the incubation period, the treated aphids were collected from the cages and surface sterilized in 70% ethanol for 15 s. followed by 0.3% NaOCL with 0.05% Tween 80 for 3 min and lastly, two rinses of sterile distilled water. The surface sterilized aphids were then placed in 90 mm diameter water agar plates and incubated 24±2°C, 65±10% RH and 16:8 hr. photoperiod for 5-6 d. After the incubation period, all

treated aphids were examined under the microscope for the presence of *B. bassiana*. There were five replicates for each tested isolate as well as the control.

Based on the efficacy of the screening test will all isolates, the four most virulent isolates and the commercial isolate (GHA) were selectedfor concentration dependent evaluation. Four concentrations ranging from 1×10^6 to 1×10^8 for each tested isolate were prepared and infection rates in Green peach aphids were evaluated as above. There were 3 replicates for each isolate as well as the control and the whole experiment was repeated twice.

2.3. Greenhouse Bioassay

The top three most virulent isolates based on the results from the previous bioassay and the reference green peach aphid isolate were selected for evaluation under greenhouse conditions. A suspension of each selected isolate was prepared as above and the concentration of the suspension was adjusted to 8.0×10^8 conidia/mL. Potted tomato plants at 4-5 leaf stage and infested with the aphid were used in the trials. The tomato plants were grown in 15 cm diameter pots filled with a mixture of 1:1 sand and peat moss. The plants were irrigated and fertilized as required. The plants were sprayed with the conidial suspension of the selected isolates until run off. Control treatment involved spraying infested plants with sterile distilled water with 0.1% Tween 80.The plants were kept in a controlled greenhouse inside meshed cages for 2 d. Temperature and RH were monitored inside the cages using shaded temperature/humidity probes (Hycal, Elmonte, CA, USA). After the incubation period, 10 randomly selected aphids were collected from the plants and treated as above to evaluate the infection rates. There were 3 replicate plants for each isolate as well as the control and the experiment was repeated twice.

2.4. Growth Characteristics Related to Virulence

Virulence related growth characteristics including spore production, speed of conidial germination and hyphal growth were studies for selected high, intermediate and low virulent isolates based on the bioassay results. High virulent isolates are those resulted in more than 70% infection rates. Intermediate and low virulent isolates are those resulted in 40-60% and less than 25% infection rates, respectively. Two isolates were selected to represent each virulence category. Therefore, the high, intermediate and low virulent isolates were BAU018 and BAU019, BAU005 and BAU021, BAU003 and BAU026, respectively.

To evaluate spore production, 0.2 mL conidial suspension of each isolate was inoculated on a SDAY plate. After incubating at 22°C for 14 d, five discs (4-mm diameter) were randomly removed from the culture using a sterile cork borer and placed in 10 mL sterile distilled water amended with 0.01% Tween 80. The discs were agitated at 110 rpm for 3 h on a rotary shaker to suspend the conidia. Conidial concentration in three aliquots of 0.1 mL of 10-fold serial dilutions of the aqueous suspensions was determined using a haemacytometer. The mean conidial yield per square centimeter was calculated for each isolate. Each plate served as a replicate and there were 5 plates for each isolate.

The speed of conidial germination was determined by placing suspended conidia in SDY broth as described previously, but germination was assessed bihourly starting 12 h after inoculation and ending after 24 h. The time required for 50% germination to occur (TG_{50}) was calculated. There were 5 replicates for each isolate.

To evaluate relative hyphal growth, 0.2 mL conidial suspension of each isolate was inoculated on SDAY plate. After incubating at 22°C for 72 h, mycelium disks, 6 mm in diameter, were cut off using a sterile cork borer and were placed in the center of freshly prepared SDAY plates. The diameter of the growing colony (exceeding the 6 mm diameter of the discs) was measured daily until sporulation (14 d) on a pre-marked line with a vernier caliper. Each plate served as a replicate and there were 5 plates for each isolate.

2.5. Statistical Analysis

Infection ratedata were arcsine square root transformed to meet the assumption of the ANOVA. Screening and greenhouse bioassays, conidia production and hyphal growth data were subjected to one way ANOVA. If the F-value was significant, means were separated using student-Newman-Keul (SNK) test. The concentration dependent and speed of germinationdata were analyzed using probit analysis. Type 1 error was set at 0.05% for all tests. When transformed, data were returned to the original scale for presentation in the tables and figures. Statistical analysis was done using SAS software version 9 (SAS, 2002).

3. RESULTS

Results of the screening test that involved evaluating infection rates for 33 *B. bassiana* isolates against the green peach aphids showed significant differences among the isolates ($F_{32,132} = 17.9$ $p<0.001$).

Table 1. Infection rates of green peach aphid adults sprayed with a suspension of different *B. bassiana* isolates and plated on water agar medium

Isolate	Infection rate (Mean ± S.E.)[a]
BAU018	84.0±4.00a
BAU004	82.0±3.74a
GHA	80.0±4.47a
BAU019	76.0±5.09a
BAU016	76.0±5.09a
BAU021	58.0±5.38b
BAU007	54.0±4.09bc
BAU025	52.0±4.89bcd
BAU005	48.0±7.35bcde
BAU027	48.0±5.38bcde
BAU014	44.0±6.78bcde
BAU011	36.0±5.10bcdef
BAU029	36.0±8.12cdef
BAU030	34.0±6.00cdef
BAU001	34.0±5.10cdef
BAU008	32.0±3.74cdefg
BAU013	30.0±6.32cdefg
BAU024	30.0±7.07cdefg
BAU031	28.0±4.90cdefg
BAU034	26.0±7.48efg
BAU015	26.0±2.45efg
BAU032	26.0±5.10efg
BAU003	22.0±5.83fg
BAU022	20.0±5.48fg
BAU033	20.0±3.16fg
BAU002	18.0±6.63fg
BAU028	18.0±3.74fg
BAU010	18.0±3.74fg
BAU009	16.0±6.00g
BAU026	14.0±4.00g
BAU012	14.0±2.45g
BAU023	14.0±2.45g
BAU017	12.0±3.74g

[a]Means within column with different letters are significantly different at 0.05 level using Student-Newman-Keul (SNK) test

Table 2. Lethal Concentration (LC_{50}) for green peach aphid adults exposed to 4 concentrations of different *B. bassiana* isolates in the lab

		95% Confidence Limits (CL)[a]	
Isolate	LC_{50} (conidia/mL)	Lower CL	Upper CL
BAU004	4.0×10^6	1.78×106	9.33×106
BAU016	5.9×10^6	2.69×106	1.45×107
BAU018	1.0×10^6	3.24×105	2.51×106
BAU019	3.2×10^6	1.32×106	7.59×106
GHA	7.9×10^5	3.63×105	1.91×106

[a]Isolates with overlapping 95% confidence levels are not significantly different at 0.05% level

Table 3. Infection rates of GPA nymphs treated with *B. bassiana* isolates under greenhouse conditions

Isolate	Mean mortality % ± (S.E.)[a]
GHA	48.3a ± (4.46)
BAU018	46.5a ± (3.99)
BAU019	43.1ab ± (3.50)
BAU004	41.3ab ± (3.34)
BAU016	30.7b ± (3.7)

[a]Means within column with different letters are significantly different at 0.05 level using Student-Newman-Keul (SNK) test

The isolates BAU004, BAU016, BAU018, BAU019 and GHA caused more than 70% infection which was significantly higher than the rest of the tested isolates. These isolates were considered highly virulent to the adult stage were selected for further evaluation against the aphid (**Table 1**). The isolates BAU021, BAU 007, BAU025, BAU 005, BAU027 and BAU015 caused infection ranging from 40-60% and were considered moderately virulent to the aphid. The rest of the tested isolates resulted in less than 40% infection rate and were considered poorly virulent to the aphid (**Table 1**).

Based on the previous test, he isolates BAU004, BAU016, BAU018, BAU019 and GHA were evaluated against the green peach aphid at four concentrations: 1×10^5, 1×10^6, 1×10^7 and 1×10^8 spore/mL. Results showed no significant differences among the isolates BAU019, BAU018, BAU04 and GHA. The LC_{50} for these four isolates ranged from 7.9×10^5 to 5.9×10^6. However, isolate GHA was significantly more virulent to green peach aphid than isolate BAU016 (**Table 2**).

Evaluation of the isolates under greenhouse condition showed significant effect due to isolate ($F_{4,20}$ = 3.55p<0.024). Treatment of green peach aphid adults with isolates of *B. bassiana*in the greenhouse resulted in 30.7 to 48.3% infection rates. The highest infection rate was achieved using the isolate GHA followed by BAU018, BAU019 and BAU004 with no significant differences among them. The least virulent isolate was BAU016 which caused significantly lower infection rate compared the BAU018 and GHA isolates (**Table 3**).

Studying virulence related growth characteristics for highly virulent isolates compared to less virulent ones showed significant differences in conidia production ($F_{5,24}$ = 45.8 p<0.001), daily hyphal growth ($F_{5,294}$ = 5.5 p<0.001) but not in the speed of conidia germination (**Table 4**). The highly virulent isolates BAU019 and BAU018 produced significantly more spores than the rest of the less virulent isolates. However, isolate BAU019 significantly outperformed isolate BAU018 in spore production.

Table 4. Virulence related characteristics for highly, moderately and poorly virulent isolates of *B. bassiana* evaluated against the green peach aphid

Isolate	Conidia production (cm^{-1}) ± (S.E.)[a]	Mean daily growth (mm) ± (S.E.)[a]	Speed of conidial germination GT_{50}[b] (hr) (95% CL)[c]
BAU019	$2.2x10^7$a±(1.59×10^6)	2.6a±(0.07)	14.6 (13.5-15.5)
BAU018	$1.4x10^7$b±(1.51×10^6)	2.4ab±(0.08)	13.9 (13.0-4.6)
BAU026	$6.9x10^6$c±(8.16×10^5)	1.9bc±(0.06)	15.1 (13.9-16.1)
BAU005	$6.3x10^6$c±(1.09×10^6)	2.1abc±(0.04)	15.2 (14.0-16.1)
BAU021	$3.2x10^6$c±(4.69×10^5)	1.8c±(0.06)	15.0 (13.9-15.8)
BAU003	$2.9x10^6$c±(4.20×10^5)	1.6c±(0.06)	15.1 (14.1-15.9)

[a]Means within column with different letters are significantly different at 0.05 level using Student-Newman-Keul (SNK) test.
[b]GT_{50}: Time by which 50% of conidia had germinated
[c]GT_{50}'s within with overlapping 95% Confidence Limits (95% CL) are not significantly different at 0.05 level

There were no significant differences in spore production among intermediate and low virulent isolates (**Table 4**). Similarly, mean daily growth of hyphae in high virulent isolates was significantly longer than low virulent isolates. No significant differences were found among high virulent and intermediate virulent isolates in daily hyphae growth. Again, isolate BAU019 hyphal growth was significantly longer than the isolates BAU026, BAU021 and BAU003 (**Table 4**). For the speed of conidial germination, no significant different were found among all the isolates under investigation (**Table 4**).

4. DISCUSSION

It is well known for entomopathogenic fungal isolates of the same species to exhibit different biological and ecological differences when challenged against the same insect species. Therefore, one of the first important steps in the development of an effective microbial control agent is careful evaluation and selection of the appropriate isolate based on virulence against the target pest. *B. bassiana* is not an exception in this route of development as many studies involved evaluation of this fungal species against insect pests for further use as a biological control agent (Liu *et al*., 2003; Quesada-Moraga *et al*., 2006). Although *B. bassiana*is known to be able to infect the green peach aphid (Alongkorn *et al*., 2013), very little is known about the virulence of its different isolates against this economically important insect pest. Todorova *et al*. (2000) evaluated the pathogenicity of 10 *B. bassiana* isolates against two insect pests, the Colorado potato beetle (*Leptinotarsa decemlineata* Say) and the green peach aphid (*M. persicae*) under laboratory conditions. Six out of the ten tested isolates were found highly virulent to the two pests. The current study evaluated 33 different *B. bassiana* isolates under laboratory and greenhouse conditions where the green peach aphid is considered a major pest for many greenhouse crops.

B. bassiana might be an excellent candidate for the development as a microbial control agent against the green peach aphid. Aphids possess piercing sucking mouth parts by which they suck plant sap from the conductive tissues. This feeding behavior might result in avoidance of the ingestion of many microbial control agents such as bacteria and viruses which need to be ingested to infect their hosts. On the contrary, entomopathogenic fungi including *B. bassiana* cause infection by direct penetration through their host cuticle which makes them excellent candidates as microbial control agents against pests with piercing sucking feeding behavior (Wraight and Carruthers, 2010). Furthermore, *B. bassiana* is considered safer and ecofriendly than chemical insecticides (Goettel *et al*., 1997), has wide host range attacking a variety of important pests, can be cultured on relatively inexpensive media and have long shelf life (Hajek and Leger, 1994).

Several methods have been used to describe isolate variation within a species of entomopathogenic fungi. These include morphological characteristics of spores and colonies, extracellular protein profiles, pathogenicity and growth or nutrient requirements. The differences in efficacy are attributed to many factors such as the production of bioactive compounds and other physiological growth related characteristics. Evaluation of growth characteristics related to virulence showed that the highly virulence isolates such as BAU019 out performed all other isolates in spore production on artificial media. Moreover, all highly virulent isolates showed faster hyphal growth than low virulent ones. The speed of hyphal growth might be one of the factors affecting the differences in virulence among these tested isolates. The faster hyphal growth usually results in faster colonization of the infected insects leading to increased virulence. These finding coincide with previous report regarding efficacy of *B. bassiana* isolates against the

tarnished plant bug (*Lygus lineolaris*) (Liu *et al*., 2003). *B. bassiana* isolates that produced larger conidia, had higher spore production and faster spore germination and hyphal growth rate over a wide range of temperatures were generally the most virulent to tarnished plant bug (*Lygus lineolaris*) than the less virulent isolates (Liu *et al*., 2003).

It was clear from the obtained results that the highly virulent isolates were more efficacious under laboratory conditions compared to the greenhouse conditions. This variation might be due to environmental factors, particularly relative humidity that greatly influence the efficacy of *B. bassiana*. Increasing relative humidity up to 90% or more improves the efficacy of *B. bassiana* under greenhouse conditions (Shipp *et al*., 2003). Temperature is another important factor playing a key role in fungi growth and spread (Orozco-Avitia *et al*., 2013). Moreover, the conidia used in the greenhouse bioassay were unformulated conidia with no additives that might improve efficacy such UV protectants and other inert ingredients used in commercial formulations.

5. CONCLUSION

In the current study, a screening bioassay procedure starting with many *B. bassiana* isolates was carried out to identify the most virulent isolates against the green peach aphid. The study identified three isolates (BAU018, BAU019 and BAU004) as the most promising ones for further development as microbial insecticides against the green peach aphid. When developed as microbial insecticides, these isolates could be used as control tactics in organic farming and Integrated Pest Management program for environmentally safe control of the green peach aphid.

6. ACKNOWLEDGEMENT

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