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Isolation and efficacy of the endophytic fungus, *Beauveria bassiana* (Bals.) Vuillemin on grapevine aphid, *Aphis illinoisensis* Shimer (Hemiptera: Aphididae) under laboratory conditions

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Abstract

The endophytic fungus, *Beauveria bassiana* has been found to occur endophytically in hundreds of plant species tissues and has been inoculated for establishment in different plants. It has negative effect on piercing-sucking insects in grapevine plants. Thus, the present study aimed to detect the endophytic fungus, *B. bassiana* throughout ITS genes analysis in the grapevine plant, *Vitis venifera* cv. Taify and assess its potential for controlling the grapevine aphid, *Aphis illinoisensis* Shimer (Hemiptera: Aphididae). The obtained 5 isolates were DNA sequenced for ITS region. Data analysis showed that there were 2 different isolates (accession number: MN900613 for isolate Bb-Taif1 and MN900614 for isolate Bb-Taif2). The values of LC_{50} were 6.041×10^4, 3.199×10^5 spores/ml while those of LC_{90} were 6.13×10^6 and 7.474×10^7 spores/ml after 3 days of treatment by Bb-Taif1 and Bb-Taif2, respectively. These values showed that the virulence of Bb-Taif1 isolate was higher in its efficacy than the Bb-Taif2 isolate. Further investigations are needed to evaluate its efficacy on other piercing sucking or chewing insect pests throughout conidia spraying, soil inoculation, and/or root immersion for grapevine plants.

Keywords: Endophytic fungi, Grapevine, *Beauveria bassiana*, Identification, ITS genes, *Aphis illinoisensis*, Bioassay

Background

The fungal endophytes (living within plant tissues) play important roles in plant community ecology (Wani et al. 2015). The study of endophytic microorganisms is defined as "endophytology" (Unterseher et al. 2012). They are called endophytes because they colonize the healthy plant tissues in some periods of their life cycle without causing damage for the plants (Azevedo et al. 2000). The distinction between pathogenic and endophytic microorganisms depends on the stage of the interaction of the microorganism with the host plant (Strobel et al. 2004). Many authors have proposed that the studies of endophytic fungi can be called "endophytism" (Suryanarayanan 2013), and others define these fungi as "mycoendophytes" (Rai et al. 2014). These endophytic fungi have negative effects on different insect pests and plant pathogens by producing toxic compounds or by modifying the host plant defense response to enable pest and pathogen resistance (Backman and Sikora 2008; Saikkonen et al. 2010). There are different beneficial effects of these endophytic fungi on host plants as plant growth promoting, increasing and induction of plant defense mechanisms, disease severity

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reduction, and producing of different anti-herbivore products (Yang et al. 2016).

The important group of endophytic microorganisms is the entomopathogenic fungi of the order Hypocreales, known to cause infections in insects. *Beauveria* spp. are common terrestrial entomopathogens, infecting numerous insect and arachnid taxa in various regions of the world (Vega 2008). *Beauveria bassiana* has been recognized to occur endophytically in hundreds of species in plant tissues and has been inoculated for establishment in different valuable crops and vegetable plants (Vega 2008; Vidal and Jaber 2015; and Gonzalez et al. 2016). The lethal effect of these endophytic fungi on insects is shown by eating the plant parts in chewing insects or by oral ingestion of plant sap in sucking insects (Batta 2018).

Thus, there is much interest in how to detect and use endophytic fungi specially *Beauveria* and *Metarhizium* with insect pathogenic capabilities to induce plant tolerance to insect pests (Vega 2008). These fungi are globally distributed and commercially available as biological control agents (Lacey et al. 2015). Many investigations were carried out on the inoculation of 13 plant species belonging to 15 families with various entomopathogenic fungi such as *B. bassiana*. These studies have examined seed dressing, seed soaking, foliar sprays, mycosed insects placed in the soil, soil drenching, and fungal plugs placed in the soil (Vega 2018). However, the endophytic *B. bassiana* present in grapevine has negative effect on piercing-sucking insects, while it reduces infestation rate and growth of the vine mealy bug, *Planococcus ficus* (Signoret) in young plants and induces high reduction of infestation with grape leafhopper, *Empoasca vitis* (Goethe) (Rondot and Reineke 2018).

Till now, there is no negative impact of the presence of endophytic *B. bassiana* on various host plants in a range of investigations (Zimmermann 2007; Klieber and Reineke 2016). Endophytic *B. bassiana* has been reported for maintaining systemic protection against various piercing-sucking insect pests or for inhibiting insect development (Rondot and Reineke 2018). Moreover, presence of endophytic *B. bassiana* reduces disease symptoms induced by various fungal pathogens (Cosoveanu et al. 2014; Jaber 2015).

The detection and identification of endophytic fungi is important to determine their distribution in host plants and their beneficial effects. Detection of endophytic microorganisms is characteristically attained through various techniques, including isolation of fungal material directly from plant tissues to growth media (culture-dependent), microscopy, and molecular recognition of endophytic DNA from different plant materials, using PCR (culture-independent) (McKinnon et al. 2017). Another recent technique is the fluorescence microscopy, which can be used to detect endophytes inside plant tissues (Pacifico et al. 2019). In general, the internal transcribed spacer-1 (ITS1), the internal transcribed spacer-2 (ITS2), and 5.8 S regions of the nuclear ribosomal DNA are commonly utilized for molecular identification of *B. bassiana* (Mondal and Baksi 2018).

Grapevine (*Vitis vinifera* L. cv. Taify) is the second most important economical fruit in Saudi Arabia and it is consumed as table grapes, grape juice, or raisins. This cultivar has been reported as the best quality for chemical composition comparing to other cultivars cultivated in Saudi Arabia (Fahmi et al. 2012). The grapevine trees are infested with different insect pests such as lepidopterous insects, mealy bugs, leafhopper, thrips, scale insects, and aphids. The grapevine aphid, *Aphis illinoisensis* (Shimer) is a grapevine pest infesting young terminal shoots, the lower surface of young leaves (Blackman and Eastop 2006) and fruit clusters causing drops of some grape berries (Pfeiffer and Schultz 1986). It is one of such invasive species recorded since the 2000s in Southern Europe and in the North African countries (El-Gantiry et al. 2012).

The present study aimed to detect and assess the potential of endophytic fungus, *B. bassiana* in the grapevine, *V. venifera* cv. Taify for controlling the grapevine aphid, and *A. illinoisensis* under laboratory conditions.

Material and methods

Sample collection and plant material

Healthy leaves from 3-year-old grapevine plants (*Vitis venifera* cv. "Taify"), cultivated in 2 locations at Taif, Saudi Arabia [21° 9′ N, 40° 35′E (location-1), and 20° 56′ N, 40° 49′ E (location-2)], were collected approximately 1.5–2.0 m above the ground. Five leaves per plant (5 distant plants from each location) were sampled in September of 2019. To isolate *B. bassiana* endophytes, each plant leaf was washed with distilled water. Then, the surface was sterilized by immersion in 3% sodium hypochlorite for 4 min, and rinsed twice in sterile distilled water for 1 min (Martini et al. 2009).

Fungi isolation

After sterilization of leaves surface, 3 leaf discs (1 cm in diameter) were obtained from each leaf. Leaf discs were placed on selective medium of EPF (DOC2-PDA medium), which prepared as follow: 2 mg crystal violet, 0.2 g $CuCl_2$, 39 g Potato Dextrose Agar (PDA) dissolved in 1 liter distilled water, while pH was adjusted to 4.0 with HCl and autoclaved for 20 min at 120 ° C (Shin et al. 2010). Plates were incubated at 20 ° C with a 12:12 h light: dark photoperiod. After 10-14 days, leaf discs were examined visually for the presence of endophytic *B. bassiana* that grew from internal plant tissues of surface sterilized leaf discs with white dense mycelia and creamy color at the edge (Humber 1997).

Genomic DNA isolation

For final detection of endophytic *B. bassiana*, mycelia samples were analyzed with molecular techniques. DNA was extracted from fungal tissues, using the fungi/yeast genomic DNA extraction mini Kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer's instructions. The extracted DNAs were stored at − 20 °C for using as templates for PCR.

PCR for ITS region and sequencing

The PCR amplification was carried out to amplify a partial region of ribosomal DNA (ITS) containing 18S gene (partial sequence), internal transcribed spacer-1 (complete sequence), 5.8S gene (complete sequence), internal transcribed spacer-2 (complete sequence), and 28S gene (partial sequence). The amplification was done using specific primers (F: 5-GGAAGTAAAAGTCGTAA-CAA-3 and R: 5-TCCTCGCTTATTGATATGA-3) (Sayed et al. 2018). PCR amplification was carried out in a final volume of 50 μl by mixing 2 μl of DNA with 1 μl of each primer (10 pmol), 25 μl of M7123 green master mix (Promega, USA), and 22 μl autoclaved double distilled water. The conditions of PCR amplification were 3 min at 94 °C followed by 35 cycles of 40 s at 94 °C, 40 s at 52 °C, and 40 s at 72 °C with a final extension of 7 min at 72 °C. PCR products were checked by electrophoresis on a 1.5% agarose gel. After electrophoresis, the PCR products were cut out, cleaned and purified by the use of BioFlux BioS-pin Gel Extraction kit (Bioertechnology Co., Ltd), and sequenced using the same primers by an automated DNA sequencer (Macrogen, Korea). Then, each sequence was checked in comparison to the GenBank database of the NCBI website. Finally, the sequences were deposited in GenBank of the NCBI website.

Bioassay

Insects and fungi isolates

Fresh leaves of grapevine plants infested with the aphid, *A. illinoisensis* were harvested on the same experimental day from the vineyard at Taif. Both fungal isolates were prepared as suspensions in six concentrations of 1×10^4, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 spores/ml. Then, 0.02% Tween 80 was added to each suspension for dispersing the conidia uniformly in the suspension (Selvaraj et al. 2012). The suspensions were vortexed for 5 min to obtain homogeneity in the suspension.

Experimental method

Five replicates (each contained 40 aphid individuals) with totally 200 aphid individuals were used for each concentration. For the bioassay, 1 μl of every single concentration was dropped straight on the aphid body (Eidy et al. 2016). Every Petri dish was filled by a thick layer (3–4 mm) of agar (0.1%). The individuals of adult aphid (1 day old) were carefully relocated by a fine camel hair-brush from the leaf of grapevine to Petri dishes. In the control, aphid adults were treated with 1 μ of distilled water with Tween 80 (0.02%) for each one. The plant leaves were changed daily with other fresh leaves for feeding aphids. The experiment was carried out at (25 ± 1 °C, 65–70% RH and a photoperiod of 16 L: 8D). The aphid mortality was checked daily till the 3rd day.

Statistical analysis

Abbott's formula (Abbott 1925) was used with the purpose of correcting the mortality data in the treatments with that in the control. The LC_{50} and LC_{90} values for both isolates were measured employing Probit analysis with the SPSS software program (SPSS 2015). The LC_{50} and LC_{90} values, 95% confidence intervals (lower bound and upper bound), slope, intercept, and chi square of both isolates were subjected to *t* test with a $P = 0.05$.

Results and discussion

Seventy-five leaf discs from each location were examined for detection of the endophytic *B. bassiana*. Some leaves had fungal growth (Fig. 1). All obtained mycelia having the same characteristics of *B. bassiana* were analyzed for ITS amplification with *B. bassiana* specific primers. Therefore, the endophytic *B. bassiana* was detected in 3 plant samples of location-1 and 2 plants of location-2, with a PCR product of 593 bp (Fig. 2). The 5 products were sequenced and the sequences showed that the 3 specimens from location-1 were identical. Moreover, the sequences of the 2 specimens from location-2 were also identical; meanwhile, they were differed than the specimens in location-1. Consequently, the 3 specimens from location-1 were considered as unique isolate and were named as Bb-Taif1, while the 2 specimens from location-2 were considered as the second isolate and were named as Bb-Taif2. The alignment of ITS sequences for both isolates resulted in identity of 99%. In this way, less geographical distances were related with less genetic variability where the isolation by distance of *B. bassiana* assumes a significant role in its genetic variation (Rehner et al. 2006). The differences between both isolate were in 5 nucleotides as follows: 2 nucleotides in the ITS1 and 3 in the ITS2 (Fig. 3). These 2 sequences were submitted in the NCBI GenBank database with the accession numbers of MN900613 for isolate Bb-Taif1 and MN900614 for isolate Bb-Taif2. In this sense, isolate Bb-Taif1 was identical in the sequence of ITS with previous isolate (accession number: LC338054), which isolated from the soil at the same location (Sayed et al. 2018). In our opinion, this finding means that the same isolate in the soil was colonized in the grapevine plants. This suggestion is in accordance with other investigations, while soil inoculation was used for endophytic

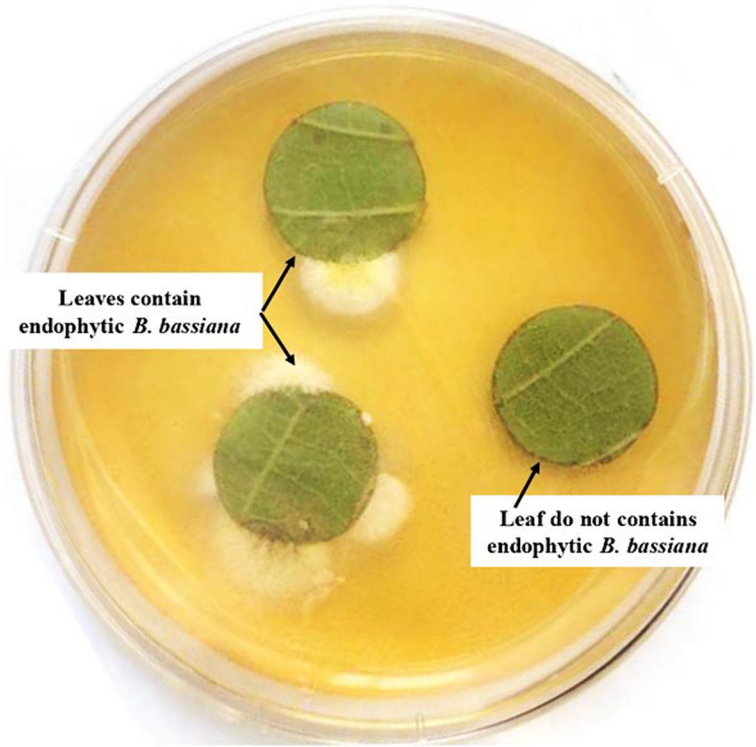

Fig. 1 Detection of endophytic entomopathogenic fungus (*Beauveria bassiana*) from leaves of *Vitis venifera*

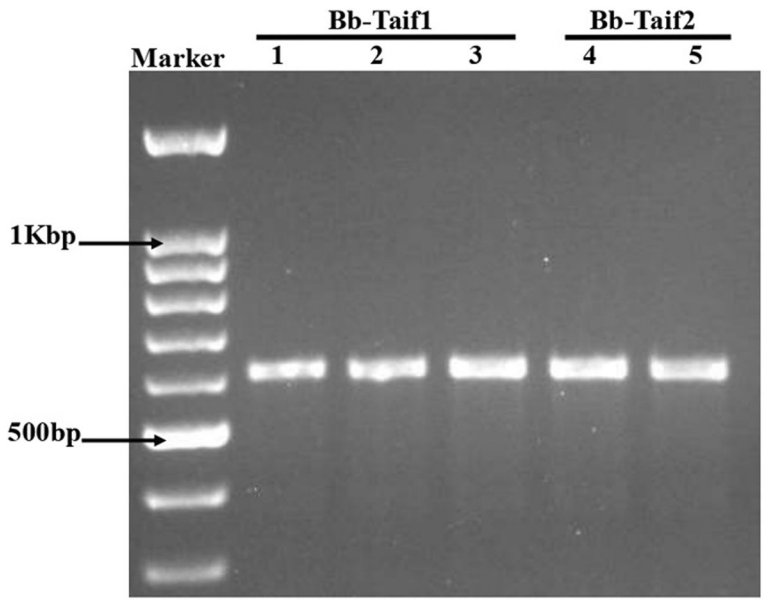

Fig. 2 PCR products of ITS genes in five endophytic specimens of *Beauveria bassiana* isolated from grape at Taif, KSA (3 specimens for Bb-Taif1 isolate and 2 specimens for Bb-Taif2)

Fig. 3 Sequences of ITS region of 2 isolates of endophytic fungus, *Beauveria bassiana* with alignment of both. The single neoclutide polymorphism (SNP) with red and highlighted color while the sequence indicates genes as 18S-partial sequence (1–53), ITS1 (54–214), 5.8S (215–371), ITS2 (372–534), and 28S-partial sequence (535–593)

establishment of *B. bassiana* in cassava plants (Greenfield et al. 2016). Also, grapevine inoculation trials via soil inoculation or root dipping resulted in endophytic colonization of *B. bassiana* but not at all plants (Rondot and Reineke 2018).

The values of LC_{50} and LC_{90} for the 2 tested isolates of endophytic *B. bassiana* against *A. illinoisensis* after 3 days of treatment are presented in Table 1. The LC_{50} value of isolate Bb-Taif1 (6.041×10^4) was significantly lower than of isolate Bb-Taif2 (3.199×10^5 spores/ml) ($t = 2.767$, $P = 0.024$). Also, there was a significant difference between the lowest bound of 95% confidence interval of LC_{50} values ($t = 2.278$, $P = 0.052$), while this difference was significant in the upper bound ($t = 3.476$, $P = 0.008$). The LC_{50} value of isolate Bb-Taif1 is compatible with Sayed et al. (2019) (6.46×10^4 spores/ml) for an isolate, which was isolated from the soil at the same location. This finding, coupled with the molecular conformity, reinforce the previous

Table 1 Values of LC_{50} and LC_{90} with 95% confidence intervals of the two tested endophytic isolates of *Beauveria bassiana* on grapevine aphid, *Aphis illinoisensis*

Isolates	LC_{50} (spores/ml)			LC_{90} (spores/ml)			Intercept	Slope ± SE	X^2
	Values	95% confidence intervals		Values	95% confidence intervals				
		Lower bound	Upper bound		Lower bound	Upper bound			
Bb-Taif1	**6.041×10^4**	**2.253×10^4**	**1.524×10^5**	**6.130×10^6**	**4.652×10^7**	**1.903×10^6**	**− 3.201**	**0.667 ± 0.020**	**9.5204**
Bb-Taif2	**3.199×10^5**	**1.708×10^5**	**6.044×10^5**	**7.474×10^7**	**4.367×10^8**	**2.783×10^7**	**− 3.081**	**0.562 ± 0.025**	**6.1148**
Sig.	*	**NS**	**	*	*	*	**NS**	*	**NS**

* (significant), ** (high significant), and *NS* (insignificant) with a $P = 0.05$

opinion that the isolate from the soil is the same endophytic isolate in this study. On the other hand, the LC_{50} value of isolate 1 is in accordance with previous finding of LC_{50} value for different isolates of *B. bassiana* against different aphid species such as Selvaraj et al. (2012) (1.5×10^4 spores/ml to the aphid, *Hyadaphis coriandri*), Eidy et al. (2016) (2.66×10^5 spores/ml against *Macrosiphum rosae*), Saranya et al. (2010) (4.5×10^4 spores/ml against *Aphis craccivora* Koch.), and Nirmala et al. (2006) (6.57×10^5 spores/ml against *Aphis gossypii* Glov.). In general, the virulence of the same isolate of *B. bassiana* varied against different aphid species such as the findings of Akmal et al. (2013) who found that the LC_{50} values were 6.28×10^5 and 6.76×10^6 spores/ml against *Brevicoryne brassicae* (Linn.) and *Schizaphis graminum* L., respectively.

On the same context of LC_{50}, in the present study, the LC_{90} value of isolate Bb-Taif1 (6.13×10^6 spores/ml) was significantly lower than of the isolate Bb-Taif2 (7.474×10^7 spores/ml) ($t = 2.739$, $P = 0.025$). Also, both of the lowest and highest bound of 95% confidence interval of LC_{90} values were differed significantly ($t = 2.332$, $P = 0.048$ and $t = 3.075$, $P = 0.015$, respectively). Meanwhile, the intercepts and χ^2 were not differed significantly ($t = 0.726$, $P = 0.488$ and $t = 1.072$, $P = 0.315$, respectively), while the difference in slope was significant ($t = 3.263$, $P = 0.011$).

Generally, the virulence of the 2 tested endophytic isolates indicated that the isolate Bb-Taif1 was higher in its virulence than the Bb-Taif2 isolate. Moreover, the findings of molecular characteristic and the virulence in this study and the findings of Sayed et al. (2018, 2019) showed that the isolate (Bb-Taif1) is endemic in the soil and establishment in the grapevine tissues as an endophytic fungus.

Conclusion

Using the endophytic indigenous isolate of *B. bassiana* for controlling aphids in vineyards achieved a high mortality rate in aphids' populations. Also, the isolate (Bb-Taif1) could be used as conidia spraying, soil inoculation, and/or root immersion for grapevine plants. Further investigations are needed on this isolate for its efficacy on other piercing sucking or chewing insect pests.

Abbreviations

ITS1: Internal transcribed spacer-1; ITS2: Internal transcribed spacer-2; PCR: Polymerase chain reaction; PDA: Potato Dextrose Agar; LC_{50}: The median lethal concentration for a population; LC_{90}: Lethal concentration for 90% of a population

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Authors' contributions

All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed in this study are available in this published manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

This study does not contain any individual person's data.

Competing interests

The authors declare that they have no competing interests.

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