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Pathogenicity of three entomopathogenic fungi, to the aphid species, *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae), and their Alkaline protease activities

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Abstract

The aim of the present study is to investigate the effect of three entomopathogenic fungi (EPF) (*Beauveria bassiana*, *Cladosporium cladosporioides*, and *Verticillium alfalfae*) on the aphid species, *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae). The selected EPF were isolated from the agricultural soil of the National Institute of Plant Protection (INPV) in Constantine, Algeria, and were tested against the aphid insects that were collected from the same area. The aphid species *M. dirhodum* were exposed to each fungal spore suspensions 10^7 conidia/ml for 10 s. Percent mortality was recorded at 1, 3, 5, and 7 days post treatment. Percentage mortalities, 7 days post treatment, were 95.83, 63.98, and 51.83% by *B. bassiana*, *C. cladosporioides*, and *V. alfalfae*, respectively. The higher protease activities were observed for isolate *V. alfalfae* with 95 U/ml, followed by *B. bassiana* with 38.26 U/ml, and finally *C. cladosporioides* with 35, 65 U/ml. The results presented in this study revealed that there was no relation between high alkaline protease activities and high virulence isolates.

Keywords: Entomopathogenic fungi, *Metopolophium dirhodum*, Mortality rate, Alkaline protease activity

Background

The aphids are the most harmful insects that affect wheat plants. *Diuraphis noxia* (Mordvilko), *Sitobion avenae* (F.), *Schizaphis graminum* (Rondani), *Rhopalosiphum padi* (L.), *Metopolophium dirhodum* (Walker), and *Sipha maydis* (Passerini) are the most inimical insect pests that depreciate substantially cereal cultures (Blackman and Eastop 2000).

M. dirhodum is the most important species that could destroy grains all over the world (Dixon 1987). It chooses its host during the cold season and then emigrates toward drought cereals in summer and is able to transmit a *Luteovirus* and provides a wide resistance against exterminators (Carter et al. 1980). The biocontrol methods, using entomopathogenic fungi (EPF), are among the most effective ones (Wells et al. 2000).

The efficiency of these fungi lies in the fact that they can fight against exterminators by invading insect crusts. They usually infect insects by active penetration through the cuticle and are able to control insects regarding their feeding environment.

The insect cuticle is the first barrier that fungi encounter. Accordingly, a wide variety of extracellular enzymes are synthesized for the enzymatic processes involved in the degradation of protein, chitin and lipids, which are the basic cuticle components (St. Leger et al. 1986). Different studies suggested that proteases and chitinases are major determinants of fungal virulence in the complex and multifactorial phenomenon insect host/pathogen relationship (Fang et al. 2007).

Protease Pr1 was also found as a virulence indicator in EPF (Castellanos-Moguel et al. 2007). High pathogenicity on *Heliothis armigera* and cotton aphid has been observed by locally isolated EPF grown on Sabouraud dextrose medium by laboratory bioassay methods (Shahid et al. 2012). These experiments were designed

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to check similarities at the level of virulence-determining factors. VCP1 (VCP1 as abbreviation means "*Verticillium chlamydosporium*") serine-like protease virulence factors, which are pathogenic toward both *Heliothis* and aphids, were grown in liquid medium at a large scale (Shahid et al. 2003).

The activities of protease have been determined in *M. anisopliae* var. *anisopliae*, *B. bassiana*, and *Verticillium lecanii* when grown on the cuticle of the locust *Schistocerca gregaria* (St. Leger et al. 1986).

Thereby, the main objective of the present study was to evaluate the most effective fungi against *M. dirhodum* and the production of an alkaline protease using Locust meal of *Ocneridia voleximii* (Pamphaginae, Orthoptera) as a basal substrate, which suggested to select the most widespread strains for biological control of aphids.

Materials and methods

Insects' rearing

Metopolophium dirhodum was collected from a wheat variety (Cirta HD.122) (*Triticum aestivum* L.). The aphid was cultured on wheat for several generations, before the commencement of the experiment, at the National Institute of Plant Protection' suburb (INPV) of Constantine-Algeria.

Fungal isolates

The virulence of three fungal isolates (*Beauveria bassiana*, *Cladosporium cladosporioides*, and *Verticillium alfalfae*) was tested. The fungi used in this study were isolated from an agricultural soil of the INPV. Isolation of EPF was determined according to the method described by (Vinayaga Moorthi et al. 2015), with minor modification. One gram (1 g) of the soil was diluted in 9 ml of distilled water, and then, 100 µl of the dilutions (10^{-3} , 10^{-4} , 10^{-5}) was planted in Potatoes Dextrose Agar (PDA: 200 g potatoes, 20 g D. Glucose, and 20 g Agar), supplemented with Chloramphenicol (10 mg/1 L). The dishes were incubated for 2 weeks at 28 °C. The macroscopic and microscopic of resultant fungal growths were compared in terms of taking into account the standard description of mycelium and spore (De Hoog 1972; Samson et al. 1988).

Pathogenicity bioassay

The aphids were immersed for 10 s in each fungal suspension, which was prepared at the concentration (1.10^7 conidia/ml) of sterile distilled water and supplemented with a drop of Tween 80 (0.05%). Dishes were kept at room temperature of 25 °C and 60% R.H. The mortality rates of the aphids were evaluated on 1st, 3rd, 5th, and 7th day after inoculation. All the treatments were repeated six times. Obtained data were analyzed. Tukey test was applied after one-way ANOVA for

numbers of live individuals, using SPSS[®] for data analysis (version 17.00 Software. 2006 SPSS.Inc.Chicago.il, USA). The percentage effects of the three fungal isolates were calculated and corrected by Abbott's (1925) formula:

$$\text{Abbott's corrected mortality}\% = \frac{(\text{mortality in control} - \text{mortality in treatment})}{\text{mortality in control}} \times 100$$

Enzymes' essays

Screening for protease enzyme production

The three EPF (*B. bassiana*, *C. cladosporioides*, and *V. alfalfae*) were tested for their ability to produce the protease enzyme. Caseins hydrolysis was added to 20% of agar and then 0.2% of skimmed milk (called Candia) was also added to the agar plates (Larsen et al. 1998). The plates were then incubated at 28 °C for 3 days. The enzyme activity was indicated by the formation of a clear zone around the colonies.

Fungi and inoculum's preparation

The tested fungal isolates were propagated on PDA medium in plates at 28 °C. After 2 weeks, the plates were scraped with 10 ml of sterile water that contained 0.05% Tween 80. The spore suspension was adjusted to the desired concentration (10^7 conidia/ml) after counting with Malassez cell.

Fermentation condition

The culture medium used for production of alkaline protease was carried out by using Locust meal of *Ocneridia voleximii* (Pamphaginae: Orthoptera) which is considered as a basic substrate provided by the laboratory of biosystems and ecology of Arthropod at Mentouri Brothers University of Constantine, Algeria. Locust material was dried in shade at room temperature and then ground by using a blender. Two grams of powdered locust was soaked in 50 ml of distilled water. A fungal suspension (1 ml) containing 10^7 of conidia/ml was inoculated into Erlenmeyer flasks containing 50 ml of culture medium with an adjusted pH (pH=4.5). Flasks were kept at 28 °C under agitation at 120 g for 3 days. The mycelia mass was obtained by filtering the content of each flask and then drying it at 105 °C until it reached a constant weight. The filtrate was used as a crude enzyme extract for the enzyme's assay. The experiment was triplicated, and the mean values were calculated.

Alkaline protease activity

Based on Mechakra et al. 1999 method, a proteolytic activity assay was done using casein as the substrate. The enzyme activity was determined by incubating 1 ml of

crude enzyme extract (EE) with 2.5 ml of casein sodium citrate (0.02 M) and 1.5 ml of Tris-HCl (0.2 M/0.1 M) with pH = 8. The mixture was incubated for 30 min at 40 °C, and the reaction was stopped by adding 5 ml of Trichloro-acetic (TCA). Thereafter, it left for 30 min in ice. The precipitated casein was then filtered off. 0.5 ml of the filtrate was taken in a test tube with 2.5 ml of NaCO₃ in 2% NaOH (0.1 N), and then, 0.25 ml of the follin Ciocalten reagent (1 ml diluted with 3-ml distilled water) was added. A proteolytic activity was determined by the absorbance reading at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate. Tyrosine was used as standard. One unit of protease activity was defined as the amount of enzyme that produced 1 mM of Tyrosine per minute above conditions. Tyrosine was taken as the standard for all the enzyme assay calculations.

Results and discussion

Pathogenicity bioassay

As illustrated in Fig. 1, 7 days post treatment, the rate of mortality of *M. dirhodum* varied relatively between 52 and 96%. The results revealed that *B. bassiana* had the most effective toxicity (95.83%), followed by *C. cladosporioides* (63.98%) and then *V. alfalfae* (51.83%).

The scale values of the population density of *M. dirhodum* are given in Table 1. Aphid population densities at the treatments with the fungal isolates were compared with control at 1, 3, 5, and 7 days post treatment. Before treatment, the population densities were not significantly different at any treatment. The lowest population densities (1.00 and 0.017 individuals) were recorded on *B. bassiana* at 5 and 7 days post treatment, respectively, while the highest ones (2.67 and 1.67 individuals) were observed on *V. alfalfae* on the same dates. The respective population densities on *C. cladosporioides* were (2.33 and 1.33 individuals) at 5 and 7 days post treatment.

All isolates produced different mortality values among treatment; the highest mortality value was obtained on 7 days post treatment ($F_{7DAA} = 32.698$, $df = 3$, $p \leq 0.00$).

The results' statistics in 3rd and 5th were significant [$(F_{3DAA} = 8.333$, $df = 3$, $p \leq 0.001$); ($F_{5DAA} = 14.556$, $df = 3$, $p \leq 0.00$)] .

More than 750 fungal species were reported as potent agents against insect propagation (Gillespie et al. 1998). Saranya et al. (2010) stated that *C. cladosporioides* caused 77.50% mortality in *Aphis craccivora* (Koch) at a concentration of 10⁸ conidia/ml. *B. bassiana* killed 96% of the aphids within 7 days after application. These results corroborate those of Murerwa et al. (2014) who reported that *B. bassiana* developed a considerable activity against *M. dirhodum*, and the mean mortalities ranged from 84 to 90% 10 days after application. Bensaci et al. (2015) revealed that the maximal mortality rate of *C. oxysporum* against *Aphis fabae* was 67.90% at the concentration of 10⁸ conidia/m.

On the contrary, Abdel-Baky and Abdel-Salam 2003 showed that the maximal mortality rates of *Cladosporium* spp. on *Aphis gossypii* and *Aphis craccivora* were 37.5 and 38%, respectively, 3 days after application with a concentration of 10⁶ conidia/ml.

In the present study, the results that describe the effect of *V. alfalfae* on aphids were approximately similar to those reported by Chavan et al. (2008). Their results showed that the efficacy of *V. locanii* was high in a sense that they had an immense mortality rate ranged between 68.23 and 89.54% on aphids.

Alkaline protease activity

The screening of fungi in order to determine their protease activity was carried out by hydrolysis on Agar plates that contain 0.2% Skim milk in 20% agar. After incubation at 28 °C for 7 days, the enzyme activities were detected by the appearance of a zone around the fungal colonies. *V. alfalfae* showed the highest zone around the colony with $\phi = 5.1$ cm, followed by *C. cladosporioides* with $\phi = 2$ cm, and then *B. bassiana* with $\phi = 1.3$ cm (Fig. 2).

Alkaline protease activity in the culture of locust meal of *O. volestii* was detected at 28 °C for 3 days. The higher protease activities were observed for *V. alfalfae*

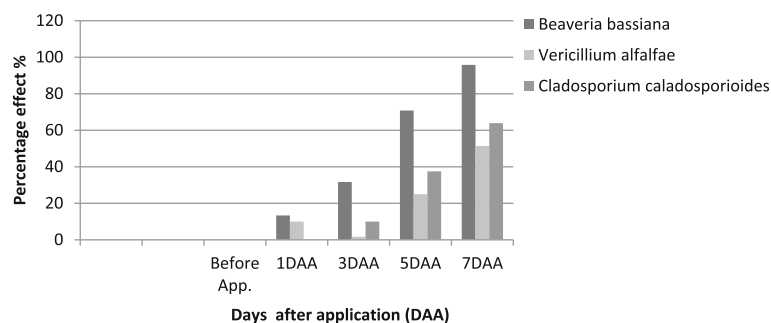


Fig. 1 Percentage mortality of three fungi on *Metopolophium dirhodum*

Table 1 The scale values of the population density of *Metopolophium dirhodum*

Fungal isolates	Days post treatment				
	Pre-treatment	1st	3rd	5th	7th
<i>Beauveria bassiana</i>	5.00 ± 0.00 ^a	4.33 ± 0.21 ^b	3.17 ± 0.17 ^c	1.00 ± 0.37 ^c	0.017 ± 0.17 ^d
<i>Cladosporium cladosporioides</i>	5.00 ± 0.00 ^a	5.00 ± 0.00 ^b	4.17 ± 0.17 ^{abc}	2.33 ± 0.33 ^b	1.33 ± 0.33 ^c
<i>Verticillium alfalfae</i>	5.00 ± 0.00 ^a	4.5 ± 0.22 ^b	4.5 ± 0.34 ^{ab}	2.67 ± 0.21 ^{ab}	1.67 ± 0.21 ^{bc}
Control	5.00 ± 0.00 ^a	5.00 ± 0.00 ^a	4.67 ± 0.21 ^a	3.67 ± 0.21 ^a	3.50 ± 0.22 ^a
<i>P</i> value	NS	0.000	0.000	0.000	0.000
S.E.M	0.00	0.062	0.23	0.21	0.26

^{a,b,c} means, with different superscripts within a same line, are significantly different according to Tukey's HSD multiple range test ($P < 0.05$). *P* probability, S.E.M standard error mean. NS not significant

with 95 U/ml, followed by *B. bassiana* with 38.26 U/ml, and then *C. cladosporioides* with 35, 65 U/ml.

The pH of the medium showed a large change in the case of isolates *V. alfalfae* (7.89), *B. bassiana* and *C. cladosporioides* (6.92) knowing that the initial pH was adjusted to 4.5.

This increase is explained by the release of alkaline molecules such as the ammonia that results from the degradation of proteins and leads to the decreases of the production of organic acids. This indicates also that the strains had certainly used the locust meal of *O. vloximii*, differently, and as the only source of carbon in the protein production. In effect, the variations of pH are considered as indicators of change in the metabolic process, including enzymes (Sandhya et al. 2005).

Extracellular proteases were even found in the insect haemolymph (Shimizuy et al. 1993). An immense variation in the protease activity was observed among the *B. bassiana*, *C. cladosporioides*, and *V. alfalfae*. A level of protease was observed in *V. lecanii*, and the highest levels were produced, solely, in cuticle substrate. Insect cuticle stimulated the protease production by both *V. locanii* and *V. alboatum* and with the adaptation of *Verticillium* spp. The protease production occurred in the insect cuticle that does not require substantial changes in the regulatory conditions of protease expression genes. These results are similar to those reported by (Barranco-Florida et al. 2002) when the proteolytic was lower submerged fermentation (cuticle of *Sphenarium purpurascens*, 20 g/l; (NH₄)₂ SO₄, 3 g/l; MgSO₄, 0.6 g/l; NaCl, 0.5 g/l; KH₂PO₄, 10 g/l). They were 66.35

and 92% lower for proteolytic in the strains *V. lecanii* ATCC26854, ATCC 46578 and wild strain (WS).

In the present study, the protease activity by *B. bassiana* (38.26 U/ml) was approximately similar to those reported by Ito et al. (2007), demonstrating high protease activity on the 5th day of culture in *B. bassiana*. The time for maximum protease production does not necessarily depend upon the media constituents. Kucera (1971) observed high amounts of protease release 3 days after inoculation with different nitrogen sources. Proteolytic activity of *B. bassiana* is influenced by the culture age. This is probably due to the nutrient limitation or the autolysis of the culture (Braga et al. 1999).

Fungal pathogenesis of insect is related to many different factors such as hosting, parasitizing, and the environment as well. In the initial contact between fungus and insect, after the adhesion process, the penetration of the fungal pathogen into cuticle depends relevantly on the action of the enzymatic activities (St. Leger et al. 1987).

Jakovljević (2016) found that the alkaline protease activities of *C. cladosporioides* were enhanced by the addition of pollutant for 56.88% in Czapek-Dox liquid nutrient medium with the addition of 0.5%.

Conclusion

Under the present experimental conditions, the results revealed that there was no relationship between a high alkaline protease activity and high virulence isolates. *B. bassiana* had the most effective toxicity (95.83%) but the high alkaline protease activity was observed in *V. alfalfae* (95 U/ml). Many factors influence directly and



Fig. 2 Screening of fungi protease enzyme production, **a** *Verticillium alfalfae*, **b** *Beauveria bassiana*, **c** *Cladosporium cladosporioides*

indirectly protease activity and the virulence isolates in this process. More research is needed about this complex mechanism.

Obtained results showed that the fungal isolates used might be utilized as biocontrol agents against the aphids. Among them, *B. bassiana* was the most promising one. Further studies should include evaluating the efficacy of the isolate in the field. However, this present work indicated the potentiality of *V. alfalfae* as a new resource of alkaline protease, which may suggest that protease activities could be used in the selection of candidates for aphids biological control.

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Authors' contributions

OA, MMS and AO collaborated in the creation of the manuscript. ALB and participated in the pathogenicity test. In addition to that, OA, MM, AO, FK and AB elaborated in the identification of the fungi and enzyme's test. Finally, BN is the one that produced locusts (*Ocniridia voleximii*). All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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