ORIGINAL RESEARCH PAPER

Effect of entomopathogenic *Aspergillus* strains against the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae)

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Abstract Aphids (Homoptera: Aphididae) are sap-sucking insect pests that feed on several plants of agronomical importance. Entomopathogenic fungi are valuable tools for potential aphid control. As part of a selection process, laboratory bioassays were carried with five different concentrations of *Aspergillus clavatus* (Desmazières), *Aspergillus flavus* (Link) and *Metarhizium anisopliae* ((Metschnikoff) Sorokin) spores against the pea aphid, *Acyrthosiphon pisum* (Harris). *Aspergillus* isolates induced higher mortalities than *M. anisopliae*, which is a well-known entomopathogen in the literature. Lethal concentrations (LC_{50} and LC_{90}) were 1.23×10^3 and 1.34×10^7 spores/ml for *A. flavus*, 4.95×10^2 and 5.65×10^7 spores/ml for *A. clavatus*, and 3.67×10^3 and 9.71×10^7 spores/ml for *M. anisopliae* 5 days after treatment. Mycelia development and sporulation on adult cadavers were observed 48 h after incubation. The intrinsic growth rate of *A. pisum* decreased with increased spore concentration for all fungal strains, suggesting an increase in pathogen fitness related to a consumption of host resources. In conclusion, *Aspergillus* species could be useful in aphid control as pest control agents despite their saprophytic lifestyle. This is also to our knowledge the first report of *A. clavatus* and *A. flavus* strains pathogenic to aphids.

Keywords Aphid · *Aspergillus* · *Metarhizium* · Entomopathogenic fungi · Biological control

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Introduction

The *Acyrthosiphon pisum* (Harris) aphid (Homoptera: Aphididae), also called pea aphid, is a pest of many cultivated and wild plants such as the worldwide crop *Vicia faba* (L.), limiting their growth. These aphids have also been reported to be a vector of some viral diseases (Brault et al. 2010; Emden and Harrington 2007). Different strategies have been employed to control these pests. The most widely used methods are physical, chemical and more recently an integrated approach that includes biological control using many kinds of organisms as parasitoids and pathogens. Entomopathogenic fungi are well known to be effective against insects. Their pathogenicity depends on the fungal species and strain virulence. In the latter case, substrate and culture methods are of primary

importance for spore effectiveness by increasing or decreasing the secretion of insecticidal compounds recognized as virulence factors (Shah et al. 2005). In this context, agricultural by-products could be used as inexpensive media with high spore yield production (Sahayaraj and Namasivayam 2008). Various entomopathogenic fungi such as *Lecanicillium lecanii* (Zare and Gams) (Jung et al. 2006), *Beauveria bassiana* ((Balsamo) Vuillemin) (Sivasundaram et al. 2007) and *Metarhizium anisopliae* ((Metschnikoff) Sorokin) (Dong et al. 2007) have been effectively used to control aphids, lepidopteran larvae and other pests. Vu et al. (2007) also demonstrated the pathogenicity of many entomopathogenic fungi such as *M. anisopliae* against aphids. Many other studies have shown the pathogenicity of fungi against aphids (Hall 1979, 1980; Khan et al. 1990), but by direct application on the cuticle or by leaf treatment. However, because of the high growth rates of aphid populations, it is unlikely that they can be fully controlled in all cases. Due to emerging insecticide resistances, the research into biological control of aphids still continues. In this present work, we showed the effectiveness of three fungal isolates, *M. anisopliae*, *Aspergillus flavus* (Link) and *A. clavatus* (Desmazières), regarding the adult mortality and population growth rate by directly treating *V. faba* plants infected with *A. pisum* aphids in laboratory conditions.

Materials and methods

Fungal cultures

All the fungal strains were identified by the Mycothèque de l'Université catholique de Louvain (MUCL, Belgium). *M. anisopliae* (Seye et al. 2012, 2013) and *A. clavatus* (Seye and Ndiaye 2008; Seye et al. 2009; accession no. MUCL 55275) were recently isolated from *Oedaleus senegalensis* (Krauss) (Orthoptera: Acrididae). *A. flavus* (accession no. MUCL 55276) was isolated from *Agriotes lineatus* (L.) larvae (Coleoptera: Elateridae). All these isolates were cultured in 250-ml Erlenmeyer flasks on a solid-state substrate constituted by 5 g wheat bran supplemented with 20 ml of a nutrient solution (1 % peptone, 1 % yeast extract, 0.005 % chloramphenicol) and previously sterilized at 121 °C for 20 min. Seven days after, fungal masses produced on the media were washed with 150 ml distilled water containing 0.05 % Tween 80 on a rotary shaker (150 rpm) for 2 h. These solutions were then filtered and centrifuged (8000 rpm, 15 min) to remove the conidia. For each strain, the conidia were diluted in sterilized water containing 0.05 % Tween 80, and final suspensions were adjusted to 10^3, 10^4, 10^5, 10^6 and 10^7 spores per ml using a hemocytometer (Thoma®).

Aphid rearing

A. pisum aphids were isolated on *Phaeolus vulgaris* L. (France, 2011) and reared in laboratory conditions on ordinary broad bean (*V. faba*) plants growing in perlite/vermiculite mix pots. Rearing conditions were a 16L:8D photoperiod, 75 % relative humidity and 25 ± 2 °C temperature.

Bioassays

For each conidial suspension, 20 adult female aphids were dropped on a young plant in a pot (10 cm diameter) surrounded by a transparent plastic sleeve closed at the ends with muslin and directly treated. Each fungal strain was applied (1.6 ml of conidial suspension) with a dedicated sprayer (Di Martino spa—Conico 1000) to 20 cm with the top of the plant. This volume was sufficient to cover the entire system without impacting aphid viability. The control aphids were treated only with distilled water containing 0.05 % Tween 80. Bioassay conditions were a 16L:8D photoperiod, 75 % relative humidity and 25 ± 2 °C temperature. Aphid mortality and nymphs produced were recorded daily for 5 days after inoculation. All newborn nymphs and dead adults were removed. The cadavers were transferred to plates with moist filter paper in order to allow fungal filament emergence and placed in room conditions as previously described. Each treatment was made in four replicates of 20 adult female aphids with a new conidial suspension in different time periods.

Statistical analysis

The lethal concentrations (LC_{50} and LC_{90}) were calculated by regression analysis. Aphid mortalities were corrected using Abbott's formula (Abbott 1925), and corrected mortality proportions were linearized using logit transformation (Dagnelie 1970): $\log \text{it}(P) = \ln(P/(1 - P))$. A single linear regression was used for modeling the relationship between logit-transformed mortality and logarithm-transformed values of fungal concentrations as explanatory variable: $\log \text{it}(P) = \text{slope} \times \ln(\text{concentration}) + \text{intercept}$. The relationship between aphid mortality and spore concentrations was assessed considering Snedecor-F distribution and p values. Reliability of the regression coefficients was assessed using Student's t test.

The lethal times (LT_{50}) were calculated by Kaplan-Meier analysis, and the log-rank test (providing the chi-square and p value) was carried out to check for significant differences between treated and control aphids.

The number of nymphs produced daily per female aphid was expressed as intrinsic growth rate (Wyatt and White 1977) and subjected to the ANOVA-1 test to check for

significant differences between treated and untreated aphids for each time interval.

All analyses were performed with Statistica 9 software. In all the cases, results were considered statistically significant when the p value of the analysis was lower than 5 % ($p < 0.05$).

Results and discussion

Aphid mortality was monitored daily up to 5 days (four replicates). Control mortalities ranged from 3.75 (day 1) to 17.50 % (day 5) for *A. flavus*, from 2.5 to 12.5 % for *A. clavatus* and from 3.75 to 10.00 % for *M. anisopliae*. Corrected percent mortality increased with time interval (day 1 to day 5) and spore concentration (10^3 to 10^7 spores/ml). At day 5, mortality of aphids ranged between 49.90–90.90 % for *A. flavus*, 51.47–84.06 % for *A. clavatus* and 30.37–78.05 % for *M. anisopliae* depending on the spore concentration (Fig. 1). Corresponding lethal concentrations (LC_{50} and LC_{90}) were, respectively, 1.23×10^3 and 1.34×10^7 ($F_{(1,17)} = 59.17$; $p < 0.001$), 4.95×10^2 and 5.65×10^7 ($F_{(1;17)} = 20.44$; $p < 0.001$) and 3.67×10^3 and 9.71×10^7 ($F_{(1,18)} = 26.92$; $p < 0.001$)

spores/ml (Table 1). At the highest concentration of 10^7 spores/ml, lethal times (LT_{50}) were reached, respectively, with *A. flavus* at day 2.50 ($\chi^2_{(0.95,1)} = 99.68$; $p < 0.001$), with *A. clavatus* at day 2.85 ($\chi^2_{(0.95,1)} = 92.70$; $p < 0.001$) and with *M. anisopliae* at day 3.26 ($\chi^2_{(0.95,1)} = 80.34$; $p < 0.001$). It was demonstrated that there were rapid declines in aphid populations for 5 days. Regarding the lethal times and concentrations, fungal strains would not have the same pathogenicity. *Aspergillus* isolates induced higher mortalities than *M. anisopliae*, which is a well-known entomopathogen in the literature. These variations might be due to the differences in the virulence of the fungal isolates, the host species or the method used for fungal application. For example, differences in the virulence of four *M. anisopliae* isolates was shown against *Aphis craccivora* (Koch) with LC_{50} ranging from 3.1×10^5 to 7.4×10^6 spores/ml (Ekesi et al. 2000). Similarly, Loureiro and Moino (2006) showed susceptibility differences in *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover) treated with 10^6 spores/ml of the same *M. anisopliae* isolate with LT_{50} of 2.91 and 3.9 days after treatment. Regarding other studies, our results are similar to those of Saranya et al. (2010) against *A. craccivora*

Fig. 1 *Acyrthosiphon pisum* adult female mortality after 5 days of exposure to *Aspergillus flavus*, *Aspergillus clavatus*, and *Metarhizium anisopliae* spores depending on tested concentrations. Values are mean corrected percent mortality of four replicates with standard error (SE)
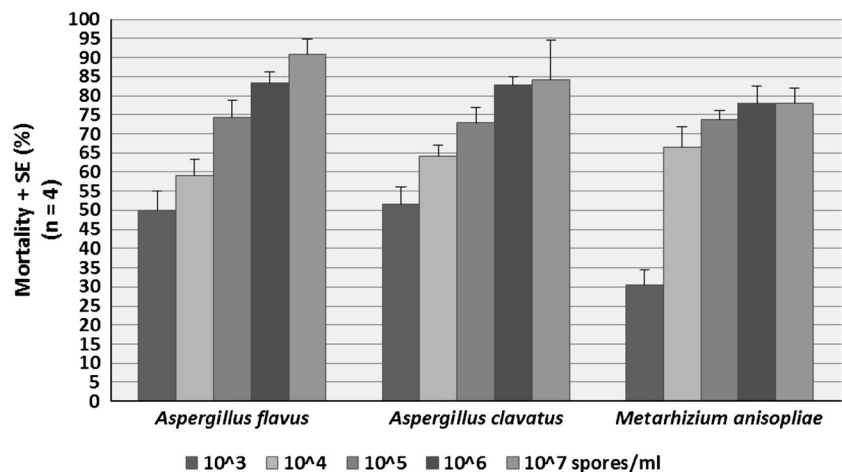
Table 1 Lethal concentrations (LC_{50} and LC_{90} expressed as spores/ml) of *Aspergillus flavus*, *Aspergillus clavatus*, and *Metarhizium anisopliae* against *Acyrthosiphon pisum* adult females after 5 days of spore exposure

Fungal strain	LC_{50}	LC_{90}	Coefficient	Standard error	T test (df)	p value
Aspergillus flavus	1.23×10^3	1.34×10^7				
Slope			−1.68	0.36	−4.67 (17)	<0.001
Intercept			0.24	0.03	7.69 (17)	<0.001
Aspergillus clavatus	4.95×10^2	5.65×10^7				
Slope			−1.77	0.50	−3.56 (18)	0.002
Intercept			0.22	0.04	5.19 (18)	<0.001
Metarhizium anisopliae	3.67×10^3	9.71×10^7				
Slope			−1.17	0.49	−2.40 (17)	0.028
Intercept			0.19	0.04	4.52 (17)	<0.001

adults for the same treatment time. This was not the case for Yokomi and Gottwald (1988) who reported 100 % mortality of three aphid species [*M. persicae*, *A. gossypii* and *Aphis citricola* (Van Der Goot)] with 10^6–10^7 concentrations of five isolates of *L. lecanii* and *Hirsutella thompsonii* (Fisher) after 4-day treatment.

Postmortem observations revealed mycelia development (corresponding to the inoculated strain) around the dead treated aphids 48 h after incubation in petri dishes for each fungal isolate (Fig. 2). No mycelia appeared from dead control aphids. This suggested that conidia adhered to aphid's cuticle when sprayed and led to their death. Even though *Aspergillus* species such as *A. clavatus* and *A. flavus* are saprophytic fungi, the isolates used in this study might have completed an infectious cycle on aphids as previously reported for other insects. Previously, microscopic observations revealed that *A. clavatus* adhered to and penetrated the cuticle of mosquito larvae (Diptera: Culicidae). Surviving *Culex quinquefasciatus* (Say) larvae that reached the pupal stage produced infected adults (Seye et al. 2009). Similarly, surface-inoculated *A. flavus* completed its life cycle on *Bombyx mori* (L.) larvae in 6 to 7 days including hemocoele invasion (Kumar et al. 2004). Moreover, mycelia development and sporulation observed on dead adults could promote fungal persistence in the environment. Aphid reproduction is very fast, and densities could increase rapidly. The autodissemination of spores between adults and their nymphs could be raised as an objective study since the high densities of aphids could increase the contact possibilities among them and consequently horizontal transmission.

Untreated adults produced more nymphs than treated adults. The production of *A. pisum* nymphs decreased with increased conidial concentration for all fungal strains (Fig. 3). The ANOVA-1 test showed that the intrinsic growth rates varied significantly from untreated adults on day 5 ($p = 0.016$) for *A. flavus*, on days 4 ($p = 0.003$) and 5 ($p < 0.001$) for *A. clavatus* and on days 4 ($p = 0.005$) and 5 ($p = 0.011$) for *M. anisopliae*. Many studies showed that entomopathogenic fungi could affect the fecundity of insect species (Castillo et al. 2000; Mulock and Chandler 2001; Quesada-Moraga et al. 2004; Scholte et al. 2006). Our results are consistent with Baverstock et al. (2006), which also revealed that *Pandora neoaphidis* [(Remaudière and Hennebert) Humber] and *B. bassiana* infection appears to have direct effects on adult *A. pisum* reproduction, but no indirect effects on the fitness of their progeny. By contrast, Wang and Knudsen (1993) did not find similar effects with *B. bassiana* against the Russian wheat aphid [*Diuraphis noxia* (Kurdyumov)]. Our results are consistent with the hypothesis that a reduction in aphid fecundity may be related to an increase in pathogen fitness as aphid resources are used for mycelia development and conidia production (Baverstock et al. 2006).

Fig. 2 Non-treated *Acyrthosiphon pisum* (**a**) and treated with *Aspergillus clavatus* (**b**), *Metarhizium anisopliae* (**c**) and *Aspergillus flavus* (**d**) 48 h after incubation on wet paper. Filaments (*arrows*) appeared around the aphid cuticle. Compared to the control (**a**), the treated adults were attacked by fungi. The fungal development was more effective with *A. clavatus* (**b**) than *M. anisopliae* (**c**) and less with *A. flavus* (**d**). *h* head, *th* thorax, *ab* abdomen
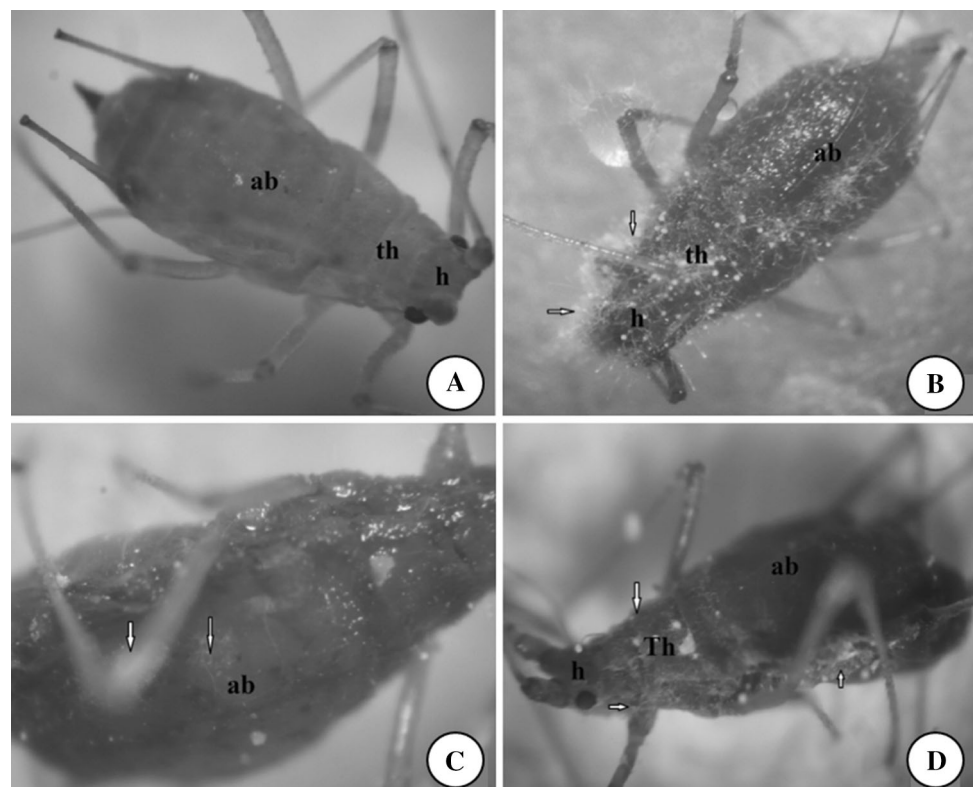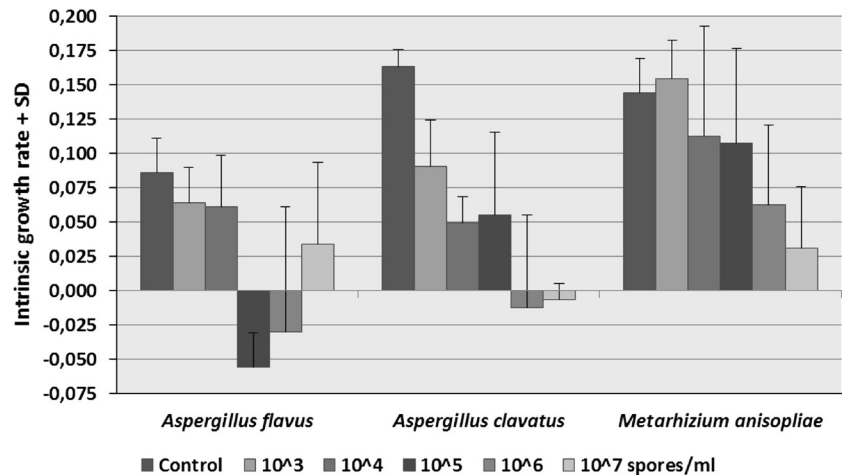
Fig. 3 Intrinsic growth rate [with standard deviation (SD)] in *Acyrthosiphon pisum* adult female populations 5 days after application of different spore concentrations of *Aspergillus flavus*, *Aspergillus clavatus* and *Metarhizium anisopliae*

Aspergillus species display a wide diversity of lifestyles including in clinical, industrial and agricultural environments; some of them may be opportunistic pathogens of a wide range of organisms including agricultural pests (Gibbons and Rokas 2012). To our knowledge, *A. clavatus* and *A. flavus* were reported here for the first time to be pathogenic against aphids. Toxicity tests and postmortem observations suggested that *Aspergillus* species could be useful in aphid control as pest control agents. However, these saprophytic fungi not target only insects, but can also affect immunodepressed humans, mammals and birds (Tell 2005). Non-aflatoxin-producing and non-toxigenic *A. flavus* strains are currently studied in biological control to reduce preharvest contamination of crops with aflatoxin (Ehrlich 2014). Their effects on non-targeted organisms including human health should be investigated.

In conclusion, the adult *A. pisum* aphid was susceptible to the entomopathogenic fungi *A. clavatus*, *A. flavus* and *M. anisopliae*. Adult mortality increased with time interval and concentration. The reproductive potential of these aphids also decreased with increased concentration. *A. clavatus* and *A. flavus* were here reported for the first time to be pathogenic against aphids. These results suggest that these fungi may be candidates for aphid control. However, toxin extraction, identification and investigation on non-targeted organisms should be performed before use in biological control.

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