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Evaluation of Native Entomopathogenic Fungi Isolates for Microbial Control of the Mediterranean Fruit Fly (*Ceratitis Capitata* (Diptera: Tephritidae)) Pupae and Adults

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Abstract

Two isolates of the entomopathogenic fungi (EPF) *Beauveria bassiana* (Hypocreales: Cordycipitaceae), an isolate of *Aspergillus flavus* (Eurotiales: Trichocomaceae), of *Verticillium* sp. (Glomerellales: Plectosphaerellaceae) and of *Aschersonia* sp. (Hypocreales: Clavicipitaceae) were evaluated for their pathogenicity against the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae) pupae and adults under laboratory conditions, using four different concentrations. Pathogenicity towards pupae and adults was tested via spraying and body contact bioassays respectively. Average mortality of treated individuals ranged from 60 to 100% for pupae and from 39 to 100% for adults depending on the fungal isolates and concentrations. The highest mortality levels were observed for individuals treated by *Verticillium* sp., *A. flavus* and *Aschersonia* sp., while the *B. bassiana* isolates were the less effective against both adult and pupae. Estimated lethal concentrations 90 ranged from $3x10^5$ to 3.8×10^7 conidia/ml for adults and from 6.58×10^3 to 2.5×10^5 conidia/ml for pupae with lower values calculated for *A. flavus*, *Verticillium* sp. and *Aschersonia* sp. Mean lethal times 90 were less than 79h for *C. capitata* adults and ranged from 94 to 273 hours for pupae according to the fungal isolates. Microscopic observation of treated pupae showed a mycelium growth on the bodies of death individuals, while visible signs of mycosis are less observed for adults. Results of these experiments demonstrated that native EPF isolates could be a promising biocontrol agent to manage safely *C. capitata* and might be used against pupae by soil application or against adults by cover or bait sprays.

Keywords: *Aschersonia* sp.; *Aspergillus flavus* (Eurotiales: Trichocomaceae); *Beauveria bassiana* (Hypocreales: Cordycipitaceae); Entomopathogenic fungi; *Ceratitis capitata* (Diptera: Tephritidae); *Verticillium* sp. (Glomerellales: Plectosphaerellaceae).

1. Introduction

Attacking over 300 host plant species, the Mediterranean fruit fly (*Ceratitis capitata Wiedemann*) (Diptera: Tephritidae) or medfly is one of the most destructive pests of fruit production worldwide [1, 2]. In Morocco, the

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medfly survives in the *Argania spinosa* forests and invades continuously agricultural areas [3, 4]. Medfly damages on fruit production are caused by female laying eggs in ripening or ripe fruits and larval development inside. Because of the larvae formation inside the fruit, the control is exceedingly difficult and expensive. Consequently, the medfly is responsible of extensive economic losses and fruit qualitative degradation [5, 6]. Besides causing large damages to fruit production, producer countries lose their international markets due to the strict quarantine regulations established by importing countries. Accordingly, the success of fruit industries in the Mediterranean region and Morocco depends heavily on the medfly management [6, 7].

Currently, the control of *C. capitata* is mostly based on chemicals either by over spray method or using attractive baits mixed with insecticides [5, 6]. The application of these insecticides in oftentimes is in the beginning of fruit maturation period when they are susceptible to the oviposition by the *C. capitata* female [6, 8]. This application of chemical compounds is extremely important to maintain the fruit production and to reduce medfly damages. However, these chemicals when misused can induce ecological and toxicological bad effects [9-11] which requires the development of alternative management strategies. In addition, a regular application of insecticides could induce a development of resistance in insect populations. [10, 12] demonstrated that field populations of *C.capitata* from citrus and other fruit crops in Spain showed lower susceptibility to Malathion compared with laboratory populations. This resistance is correlated with the frequency of Malathion treatment in the field. More importantly, this resistant strain showed cross-resistance to other organophosphates. Furthermore, the use of Malathion and many organophosphorates has been limited by several importing countries in particular by the EU (According to the European Guideline 91/414).

Moreover, the medfly current management strategy is only relied to adults while pupae and larvae are not controlled. Third instar medfly larvae are usually pupariating in soil; however the control measures against pre-pupating larvae and pupae in soil are poorly applied [13]. To reduce chemicals, entomopathogenic fungi (EPF) could be applied as microbial insecticides in integrated pest management programs or as alternatives [14, 15]. Control strategy of *C. capitata* via entomopathogenic fungi includes disseminating the fungi among medfly populations by attracting adults to bait stations where they are infected with the pathogen and transmit the disease to other individuals [16] as well as targeting soil pupariating larvae and pupae by soil inoculation [13, 17]. The possibility of medfly management using entomopathogenic fungi was proved by many researches which have shown the susceptibility of medfly larvae, puparia and adults to fungal infection by isolates of *Beauveria bassiana* Ekesi, *et al.* [18], Quesada-Moraga, *et al.* [19], Imoulan and Elmeziane [20], *Beauveria Brongniarti* [21], *Metarhizium anisopliae* [17, 18, 22, 23], *Paecilomyces lilacinus* [24, 25], *Paecilomyces fumosorosea* [23] and *Verticillium lecanii* [26] . With the exception of Imoulan, *et al.* [24], Imoulan and Elmeziane [20] studies studies, the entomopathogenic isolates tested against *C. capitata* have never been isolated from medfly infected individuals or from soils naturally containing larvae and pupae of this insect. The introduction of non-indigenous entomopathogenic strains can probably reduce the effectiveness of these biocontrol agents and could pose ecological risks. Therefore, the selection of indigenous fungi strains increases the possibility of an effective safe control [27, 28]. Besides, if applied as biopesticides, these strains can overcome environmental stress and adapt better to environmental conditions [24, 29]. Indeed, several studies have reported that the tolerance of entomopathogenic fungi to climatic conditions is strongly related to its natural habitat [24, 30, 31].

Accordingly, the present study aims to evaluate the pathogenicity of three native isolates of the entomopathogenic fungi A. *flavus* Link (Eurotiales: Trichocomaceae), *Verticillium* sp. (Glomerellales: Plectosphaerellaceae) and *Aschersonia* sp. (Hypocreales: Clavicipitaceae) and two isolates of B. *bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) against *C. capitata* pupae and adults. These isolates are locally isolated form Argan forest soil using *C. capitata* pupae as bait. Some of those EPF species have been tested for the first against this pest.

2. Material and Methods

2.1. Insect Colony

In this study, the experiments were conducted on reared *C. capitata* pupae and adults.

C. capitata larvae were recovered from field-infested Argan (*Argania spinosa*) fruits then reared in the laboratory of Microbial Biotechnologies and Plant Protection (LBMPV) of the faculty of science, Ibn Zohr university, Morocco. The breeding was carried out in an insectary under controlled conditions of temperature and photoperiod. The temperature was maintained at 25 ± 2°C and the photoperiod at 14 h / 10 h (Light/Darkness). Medfly adults were provided with water and a sugar-yeast nutrient medium (¾ sucrose + ¼ yeast extract (w/w)). The larval medium consists of 940g of wheat bran (94%), 50g of yeast extract (5%), 5g of Nipagine and 5g of glucose in 1000ml of distilled water [4]. The *C. capitata* pupae and adults are used thereafter for the pathogenicity tests.

2.2. Fungal Origin

The entomopathogenic isolates used during this study were obtained from an entomopathogenic collection of the laboratory of microbial biotechnologies and plant protection (LBMPV). This collection was created during previous experiments conducted to isolate indigenous medfly entomopathogenic fungi from Argan forest soil using this insect's pupae as a bait. In our knowledge, this is the only entomopathogenic fungi collection isolated from the *C. capitata* natural habitat by using this insect's pupae as bait.

Five isolates of entomopathogenic fungi belonging to four genera were screened against *C. capitata* pupae and adults under laboratory conditions. The selected fungi are: two isolates of Beauveria bassiana OS1 and NS10, an isolate of Aspergillus flavus NS14, *Verticillium* sp. Pt34 and *Aschersonia* sp. Pt14.

All the fungal isolates have been identified on the basis of macroscopic and microscopic criteria, using the specialised keys of Pitt, *et al.* [32] and Samson, *et al.* [33]. While, *Beauveria bassiana* OS1, *Beauveria bassiana* NS10 and *Aspergillus flavus* NS14 have been molecularly characterized in the "Molecular Biology and Functional Genomics Platform" at the National Center for Scientific and Technical Research (CNRST) in Rabat, Morocco. Fungal isolates samples were cultivated on potato dextrose agar medium (PDA) for 7 days at 25 °C. The DNA was extracted from 50-200 mg of mycelium (fresh weight) using the Isolate II Plant DNA (BIOLINE®) according to the manufacturer's instructions.

Polymerase chain reactions (PCR) with specific primers on partial sequences were used to confirm the morphological identification of each fungal isolate (*B. bassiana* OS1, *B. bassiana* NS10 and *A. flavus* NS14). PCR reactions were performed using the internal transcribed spacer region (ITS-1) forward (TCCGTAGGTGAACCTGCGG) and ITS-4 reverse (TCCTCCGCTTATTGATATGC) primers for *Aspergillus* sp. [34]. For *Beauveria* sp. isolates; a 1500 bp fragment spanning the 3' 2/3 of the Bloc gene was amplified with primers B5.1fw (5'-CGACCCGGCCAACTACTTTGA-3') and B3.1rw (5'-GRCTTCCAGTACCACTACGCC-3') [35, 36]. PCR was performed in a 25 µl volume reactions with the following concentrations: containing 150 ng of each fungal isolate DNA, Taq polymerase buffer (5x), 0.2 mM of each dNTP, 1 µl of each primer and 0.2 µl of Taq polymerase 5U/µl (MyTaq DNA polymerase kit, Bioline®) [37]. The reaction mixture was supplemented with 2 mM of MgCl2 concentration. The PCR thermal was performed in a Thermal Cycler (Verity, ABI 3130xl), using a regime starts with an initial denaturation cycle at 95ºC for 1 minute; followed by 35 thermal cycles of denaturation for 15 seconds at 95 °C, annealing for 20 seconds at 60 ºC followed by extension for 15 seconds at 72ºC; and ends with final extension cycle at 72ºC, for 3 min. The reaction was held at 4 °C. A volume of 5µl of the amplified products were purified using purification Kit (ExoSAP-IT) to remove primers and nucleotides (dNTPS) not used during the PCR reaction.

PCR products were prepared for sequencing in the forward and reverse directions using 2 µl of the primers, 1µl of the BigDye Terminator v3.1 cycle sequencing kit's, 3µl of sequencing buffer (5x), 1.5 µl of DNA matrix and 2.5µl of Milli-Q water. Thereafter, the products were sequenced using an Applied Biosystem ABI 3130xl sequencer (Genetic Analyzer, 16 capillaries, Applied Biosystem). BLAST similarity searches were performed in the non-redundant nucleotide database of GenBank [34, 38] to identify/verify species or genus affiliation of collected isolates.

2.3. Fungal Suspension Preparation

The fungi were routinely grown on PDA medium incubated in 25 °C. After two weeks of incubation, spore suspensions intended for the pathogenicity tests were prepared under aseptic conditions. Conidia were harvested directly by scraping the fungal surface and by adding 10 ml of sterile distilled water containing 0.1 % Tween 80 to the petri dishes [22]. The obtained suspension was poured into sterile glass tubes then shaken for 8 to 10 minutes using a vortex mixer to detach the conidia. Thereafter, the sporale suspension was filtered through sterile gauze to produce a homogenous suspension.

The concentration of the suspension was determined using a haemocytometer, then diluted with sterile distilled water to obtain the conidial concentrations for pathogenicity tests. In order to determine the lethal concentrations 50 and 90 for each isolate, four concentrations of spores were tested as follow: 107, 106, 105 and 104 conidia /ml.

2.4. Conidia Viability Studies

The conidial viability of each isolate was determined by germination studies before the bioassay was performed. For each fungal isolate, a 50 µl of a 1 x 105 conidia / ml suspension was spread inoculated on agar water medium plates then the dishes were incubated at 25 ± 2 ° C in the dark. Three Petri dishes were used for each isolate. The germination rate was determined for each Petri dish after 18 h of incubation, out of a total of 300 conidia, at 400x magnification. Conidia were considered to have germinated when the germ tube was longer than the conidium's diameter. Table 1 shows the percentage of conidial germination of the tested fungi.

2.5. Laboratory Bioassay on Pupae

2.5.1. Disinfection of Pupae

Three-to four-day-old pupae were surface sterilized by soaking in 0.3% sodium hypochlorite solution for 1 minute then rinsed five times with sterile distilled water as described by Ebling [39]. Disinfected and rinsed pupae are placed on sterile Whatman paper to dry before pathogenicity testing procedures.

2.5.2. Infection of Pupae by EPF

For the pathogenicity bioassay against pupae, twenty disinfected pupae were placed on sterile Whatman paper in sterile Petri dishes. Subsequently, these pupae were treated by spraying about 2 ml of the spore suspension. While, the control pupae were treated by sterile distilled water containing 0.1% of tween 80. Three repetitions were performed for each concentration (107, 106, 105 and 104 conidia/ml). The Petri dishes containing the treated insects are incubated in the dark at 25 ± 2 °C.

In order to determine the lethal times 50 and 90, the mortality rate of pupae was noted after each 24 hours after inoculation. Observation of the treated individuals was carried out daily under a binocular magnifier at 40x magnification. To prevent horizontal transmission of the pathogen (EPF) between treated pupae, regular elimination of infected individuals was carried out. Lethal concentrations for each isolate were calculated after the emergence of adults, at the end of the test.

A re-isolation of the entomopathogenic fungi from the insects was done to verify that the tested fungi are the responsible for the observed mortality (Koch's postulates). Due to the natural mortality in control dishes, the corrected percentage of pupae mortality in each Petri dish was calculated according to Abbott [40] formula.

2.6. Laboratory Bioassay on Adults

The pathogenicity of the five entomopathogenic isolates toward medfly adults was tested by bodily contact bioassay. In this experiment, two- to three-day-old adults (males and females) have been contacted with entomopathogenic fungi spores according to the protocol of Beris, *et al.* [23].

During this experiments sterile Petri dishes of 9 cm in diameter, 1.5 cm in height and an area of 63.62 cm2 were used. A volume of 3 ml of the spore suspension (1 x 107, 1 x 106, 1 x 105 and 1x104 spores/ml corresponding to 4.71 x 105; 4.71 x 104; 4.71 x 103 and 4.71 x 102 spores/cm2, respectively) was poured into the plate and the lid of each Petri dish. The spore suspension was spread equally on the entire surface of the dish. For the control treatment, 5 ml of sterile distilled water containing 0.1% Tween 80 was used. Subsequently, the excess of the fungal solution in the Petri dishes was eliminated before adding the insects. For each treatment, three dishes were each filled with ten *C. capitata* adults. These adults have been anesthetized previously with ice to facilitate their handling.

After 2 hours of bodily contact with the fungal suspension, the treated adults have been anesthetized with ice then transferred to sterile Petri dishes containing water and nutrients (sugar and yeast extract). The dishes were incubated at 25 ± 2 ° C with controlled photoperiod (14/10 (L / D)). Adult mortality was measured every 24 hours after treatment until the death of all individuals. Lethal concentrations for each isolate were calculated after 96 h of treatments.

2.7. Statistical Analysis

Data analysis of the mortality rates of pupae and adults was conducted using the statistical software Statistica V6 [41]. The comparisons of the corrected mortality percentages related to each isolate and each concentration were carried out with the two-way analysis of variance test (ANOVA). Corrected mortality results of pupae and adults were previously log10 (x + 1) transformed to comply with ANOVA assumptions. The classification of homogeneous groups and the confirmation of the existence of significant differences between the means were carried out with the Fisher-LSD test at a confidence interval of 95%, $p < 0.05$. Mean lethal concentrations (LC50 and LC90) and lethal times (LT50 and LT90) were estimated using Probit analysis after log10 transformation of mortality percentages using SPSS V 20.0 software [42].

3. Results

3.1. Bioassay on Pupae

3.1.1. Mortality Rates

Overall, pupae mortality in treatments with fungal suspensions is much higher than mortality observed in the control (natural mortality) which is less than 10%. Analysis of variance of the corrected mortality rates showed a high significant difference between isolates in different concentrations ($F_{12,40} = 6.7$, $p<0.001$). Pupae mortality ranged from 60 to 100 % depending on the fungal isolates and applied concentration (Fig.1 (A)). The highest mortality rates are observed in pupae treated by *Aspergillus flavus* NS14, *Aschersonia* sp. Pt 14 and *Verticillium* sp. Pt34, while *Beauveria bassiana* isolate NS10 was related to the lowest mortality. The classification of applied concentrations using Fisher-LSD test showed that 1 x 107 and 1 x 106 conidia/ml are the most effective concentrations. However, no statistical difference was found between these two concentrations (group a). The concentration of 1 x 104 conidia/ml has showed the lowest mortality results specially for the two Beauveria bassiana isolates (NS10 and OS1). Concerning the concentration 1 x 107 conidia/ml, the mortality ranged from 97.67 ± 4.02 % for *Beauveria bassiana* NS10 and 100 ± 0 % for the other isolates. For the concentration of 106 conidia/ml, mortality rates are about 100% for *Verticillium* sp. Pt34 followed by *Beauveria bassiana* NS1 and Aspergillus flavus NS14 with 97.67%, while *Beauveria bassiana* OS1 and Aschersonia sp. Pt14 showed the lowest mortality levels (about 95%) for this concentration. At a concentration of 1 x 105 conidia/ml, the *Verticillium* sp. Pt34 isolate keeps its high pathogenicity level with 100% followed by Beauveria bassiana OS1, *Aschersonia* Pt14 and *Aspergillus flavus* NS14 with mortality rates up to 93%.

For all concentrations the most pathogenic isolate was *Verticillium* sp. Pt34 while the *Beauveria bassiana* NS10 was the less effective isolate. However, all the tested isolates have been showed a high significant pathogenicity toward medfly pupae. Microscopic observation of treated pupae showed that all the death individuals have a mycelium growth on their bodies. The re-isolation of this mycelium confirms that the tested fungi are the responsible for the observed mortality.

3.1.2. Lethal Concentration

Obtained mortality for each isolate-concentration was used to estimate the lethal concentrations 50 and 90 using the Probit model (Table 2). The obtained results showed that isolates with hight pathogenicity levels are mostly related to small lethal concentrations (LC 50 and LC 90).

Regarding the LC50, the lowest concentrations were obtained by *Aspergillus flavus* NS14 and *Aschersonia* sp. Pt14 with LC50 of less than 2 conidia/ ml; while the highest values were obtained for *Beauveria bassiana* NS10 and Verticillium sp. Pt34 with LC50 up to 7 x 103 conidia/ml. In general, LC50 values do not really reflect the actual effectiveness of the fungal isolates as observed for *Verticillium* sp. (Pt34). On the other hand, LC90 results are more

reliable for determining the most virulent isolate. The lowest LC90 is obtained by *Aspergillus flavus* NS14 (6.58x103 conidia/ml), followed by *Verticillium* sp. Pt34, *Aschersonia* sp. Pt14 with values less 1.2x104 conidia/ml. While the highest value was observed for the *Beauveria bassiana* NS10 isolates with LC90 up to 2.5 x 105 conidia/ml. This isolate has showed low mortality levels compared to others.

3.1.3. Lethal Time

The lethal time was estimated at the concentration of 1 x 107 conidia/ml which the most effective for all isolates (Table 3). For the lethal time 50, the shortest durations were recorded by *Beauveria bassiana* OS1, *Verticillium* sp. Pt34 and *Aschersonia* sp. Pt14 with values less than 50 hours. Likewise, these isolates have shown the lowest LT90 values of about 100 h. Whereas, *Aspergillus flavus* NS14 and *Beauveria bassiana* NS10 are the isolates with the highest lethal time values.

3.2. Bioassay on Adults

3.2.1. Mortality Rates

Average mortality rates after 96 h of treatment of *C. capitata* adult using fungal suspensions showed a significant effect of the fungal isolate and concentration ($F_{12,40}=3.12$, $p=0.0034$). Corrected mortality percentages ranged from 39.13 to 100% depending on fungus and concentration (Fig. 1 (B)). The lowest results were observed at the concentration of 104 conidia/ml for all the isolates, while the highest mortalities were observed at 1 x 107 and 1 x 106 conidia/ml. As observed for pupae, the *Beauveria Bassiana* isolates NS10 and OS1 isolate were the less effective fungi against adults specially at low doses. Nevertheless, these isolates have reached mortality levels over 91 % using a conidial suspension of 1 x 107 conidia/ml. On the other hand, the highest results were observed for adults treated by *Verticillium* sp. Pt34 with mortality rates that exceed 95.6% for all conidial concentrations above 1 x 105 conidia/ml. *Aspergillus flavus* NS14 and Aschersonia sp. Pt14 have been also showed high effectiveness against *C. capitata* adult with mortality ranged from 82.6% to 100% for all the applied concentrations.

3.2.2. Lethal Concentration

The lethal concentration values were estimated after 96 h of treatment. Obtained results showed that the tested fungal isolates are highly effective against *C. capitata* adults at low concentrations (Table 4). The lowest results were observed for *Aspergillus flavus* NS14 with LC90 of about 3 x 105 conidia/ml, followed by *Aschersonia* sp. Pt14 and *Verticillium* sp. Pt34 with LC90 less than 8 x 105 conidia/ml. Considered the less effective fungi, *Beauveria bassiana* isolates have a high LC90 values up to 5.1 x106 conidia/ml for OS1 and 3.8 x 107 conidia/ml for NS10.

3.2.3. Lethal Time

Monitoring of *C. capitata* adult mortality after treatment with fungal suspensions of 1 x 107 conidia/ml of the five studied isolates, revealed that these fungi are highly effective in short time (Table 5). Overall, the tested fungi require less than 30 h to produce mortality of 50% of adults. However, the shortest time to reach 50% mortality is about 27 hours recorded by the *Aspergillus flavus* NS14 and *Beauveria bassiana* OS1. Regarding LT90 values, the mean lethal time 90 were higher for *Beauveria bassiana* compared to other isolates. The lowest LT90 values were recorded for *Aspergillus flavus* NS14 and *Verticillium* sp. Pt34 with values less than 55h. Concerning the control which represents the natural mortality, the mortality of 50% of adults requires about 163 h while the mortality of 90% requires more than 372 h. Generally, the mean lethal times of adults are reduced compared to pupae.

4. Discussion

The pathogenicity of five locally isolated entomopathogenic fungi B. bassiana, A. *flavus, Aschersonia* sp. and *Verticillium* sp. have been tested *in-vitro* against pupae and adults of the Mediterranean fruit fly (Ceratitis capitata). Some of those EPF species were tested for the first against this pest. The five fungal isolates have been shown remarkable pathogenicity ranged from 39 to 100 % depending on the fungal isolates, applied concentration and insect stage. Estimated lethal concentration 90 (LC90) values ranged from 3 x 105 to 3.8 x 107 conidia/ml for adults and from 6.58 x 103 to 2.5 x 105 conidia/ml for pupae. Whereas, LT90 values were less than 79 h for adults and ranged from 94 to 273 hours for pupae.

Microscopic observation of treated pupae showed that all the death individuals have a mycelium growth on their bodies, while visible signs of mycosis are less observed for adults. Besides direct mortality of treated pupae, monitoring of adults emerged from these pupae shows a high and quick mortality of these adults. This finding is confirmed by other authors who, after exposing of *C. capitata* and other insect pupae to EPF, reported a high mortality levels and fungal growth on emerged adults [13, 17, 23].

Most bioassays to control biologically the medfly using EPF are carried out on adults [13, 19, 21, 26], while only few researches have evaluated the insecticidal effect on both pupae and adults stages [17, 19, 23]. The entomopathogenic fungi *Beauveria, Metarhizium, Paecilomyces (=Isaria)* and *Verticillium* are the most studied genera for their pathogenicity against *C. capitata*. This pest individuals have been exposed to entomopathogenic fungi and their extracts of different strains of *B. bassiana, B. Brongniarti, M. anisopliae, P. fumosorosea, P. lilacinus* and *V. lecanii* isolated from soils or insects from Greece [21, 23], from Spain [19, 26, 43], from Kenya Dimbi, *et al.* [17], Ekesi, *et al.* [13] and from Morocco [20]. Mortality levels produced by these isolates ranged from 20 to over 90% depending on fungal isolate, concentration and application method. However, none of the previous

researches have studied indigenous isolates obtained from *C. capitata* infected individuals or from its natural habitat. The application of native strains as biological control agents is desirable to reduce ecological risks. In addition, these strains are more suited to the climatic conditions of the region [27, 44]. The use of indigenous isolates could explain the high mortality rates observed during our tests which is about 100% for the majority of isolates.

In general, susceptibility of adults to the fungal infection was much higher than pupae under the treatment protocols used during this study. The same, previous studies have been mentioned that *C. capitata* pupae are more resistant to EPF infection compared to adults [19, 23]. Our EPF isolates have produced high pupae mortality levels reaching 100% for some strains with mycelial growth on death individuals which facilitates the spread of the pathogen through the insect population. However, the comparison with other researches must be made with caution because of the differences in fungal isolates, infection protocols, doses, as well as insect population histories. These conditions can affect the effectiveness and the development of the pathogen [23, 27, 36]. Further, the difference between fungal isolates can be explained by the genetic variation between these isolates, which influences enzymatic secretion during the contact phase, the formation of the appressorium and the fungal penetration [45-47]. During this study, *Verticillium* sp. Pt34 was the most effective isolate against *C. capitata* pupae and adults producing about 100% mortality of adults and pupae at the lowest conidial concentration (1 x 104 conidia/ml). Previous researches have reported that Verticillium genus contains species with entomopathogenic abilities such as *V. lecanii, V. dahliae and V. hemipterigenum* [26, 48, 49]. Furthermore, this genus is well-known by the production of insecticidal secondary metabolites in particular the dipicolinic acid and phenylalanine anhydride [50]. To our knowledge, only the research of Castillo, *et al.* [26] has been reported to pathogenicity of *Verticillium* to the *C. capitata* by studying the effect of *V. lecanii* conidial suspension and their extracts on the longevity and fecundity of this pest adults. Nevertheless, the obtained results by Castillo, *et al.* [26] were not encouraging with mortalities less than 10% and a reduction of fertility of about 25%. Compared to previous researches, our *Verticillium* sp. isolate Pt34 have shown a great virulence to medfly adults and pupae which makes of it a good candidate for a biopesticide preparation. On the other hand, our *Beauveria Bassiana* isolates seems to be the less effective against *C. capitata* adults and pupae. However, obtained results are similar to the mortalities recorded by some researchers [19, 20] and higher than others [17, 23]. Although, lethal concentration (LC) and lethal time (LT) values recorded by our *B. Bassiana* isolates are very low compared to previous studies with LC50 less than 8 x 103 conidia/ml for both pupae and adults and an LT50 ranged from 30 h for adults to 80 h for pupae. The closest results to our B. Bassiana isolates virulence were recorded by Quesada-Moraga, *et al.* [19] research. Quesada-Moraga, *et al.* [19] exposed *C. capitata* adults to ten isolates of *B. bassiana* by spraying, and demonstrated the sensitivity of these adults to these isolates with a lethal concentrations (LC50) of the four most virulent isolates ranged from 4.9 x 105 to 2 x106 CFU/ml and an LT50 varying from 4.6 to 5.3 days. Besides their high mortality levels, B. bassiana are known for their ability to reduce the fertility and fecundity of adults [19].

During this study, fungal isolates belonging to *Aspergillus* and *Aschersonia* genera have been tested, for the first time, for their pathogenicity against *C. capitata*. These isolates have shown a great effectiveness against this pest with mortalities up to 100% at short lethal time and low lethal concentration. These isolates have never been recorded as *C. capitata* EPF, however their entomopathogenic ability was confirmed by several studies [11, 27, 33, 51]. *Aspergillus flavus* NS14 showed a very high virulence towards *C. capitata* adults and pupae with mortalities ranging from 82% for 1x 104 conidia/ ml to 100% for 1 x 107 conidia / ml. However, the majority of death insects does not show early mycelium growth on their bodies which explain the high lethal times recorded for this isolate. Accordingly, several *Aspergillus flavus* isolates have been reported for their enzymatic secretion and entomopathogenic ability [52, 53]. Drummond and Pinnock [54], Gupta and Gopal [52] and Zeng, *et al.* [53] reported the production of Aflatoxin B1 and G1 by strains of *A. flavus* isolated from the *Saccharicoccus sacchari* larvae and from several coconut pests. These Aflatoxins are known for their insecticidal, larvicidal and chemo-sterilizing properties against several species of insects and mites [52, 54]. . In addition, many extracellular chitinase isoenzymes have been detected in culture filtrates of *Aspergillus flavus* after electrophoresis on polyacrylamide gels [55]. Furthermore, Lage De-Moraes, *et al.* [56] demonstrated the possibility of using conidial suspensions of *A. flavus* to control larvae of *Culex quinquefasciatus* and *Anopheles stephensi*, two diseases-vector mosquitoes, with over 80% mortality. *Aschersonia* species such as *A. aleyrodis* and *A. goldiana* have been also reported for their pathogenicity against several pests [33, 44, 57]. Moreover, this genus is known by its secondary metabolites and cytotoxicity against insect cells but not mammalian cells [58-60]. Destruxins isolated from organic extracts from cultures of *Aschersonia aleyrodis* and other *Aschersonia* species are worldwide used as biopesticides [58, 61].

Overall, the EPF isolates used during this study have shown a high virulence against *C. capitata* pupae and adults with low lethal concentrations and short lethal times. This effectiveness could be explained by their origin (Argan forest soil, the natural environment of the medfly) and by the isolation method which uses *C. capitata* pupae as bait. These two parameters probably increased the chance to isolate specific pathogens to the Mediterranean fruit fly. Those results indicate that the use of these indigenous isolates remains a promoting solution the control safely medfly pupae and adult reducing the environmental and ecological risks. Furthermore, all the fungal genera tested during this study are considered by several studies as endophytic fungi of plants [28, 33, 51]. These endophytic fungi have been reported for their potential to promote plant resistance to insects [51, 62]. In addition, some endophytic entomopathogenic fungi can promote plant resistance to plant-parasitic microorganisms and act as a growth stimulant [51, 62].

This study identifies *Verticillium* sp. Pt34, *Aspergillus flavus* NS14 and *Aschersonia* sp. Pt14. as excellent candidates for an effective application to control biologically *C. capitata* adults and pupae. These isolates produced high mortality levels at short time with low fungal concentrations and could be used against pupae by soil application or against adults by cover or bait sprays. Accordingly, EPF native isolates might be a promising

biocontrol agent to manage C. capitata. Nevertheless, their application in field requires more studies to understand the factors controlling their effectiveness, their survival in the natural environment and their migration and retention in soil. Moreover, the possibility of using these agents in integrated pest management strategies should be evaluated.

Conflict of Interest

The authors declare that they have no financial/commercial conflicts of interest.

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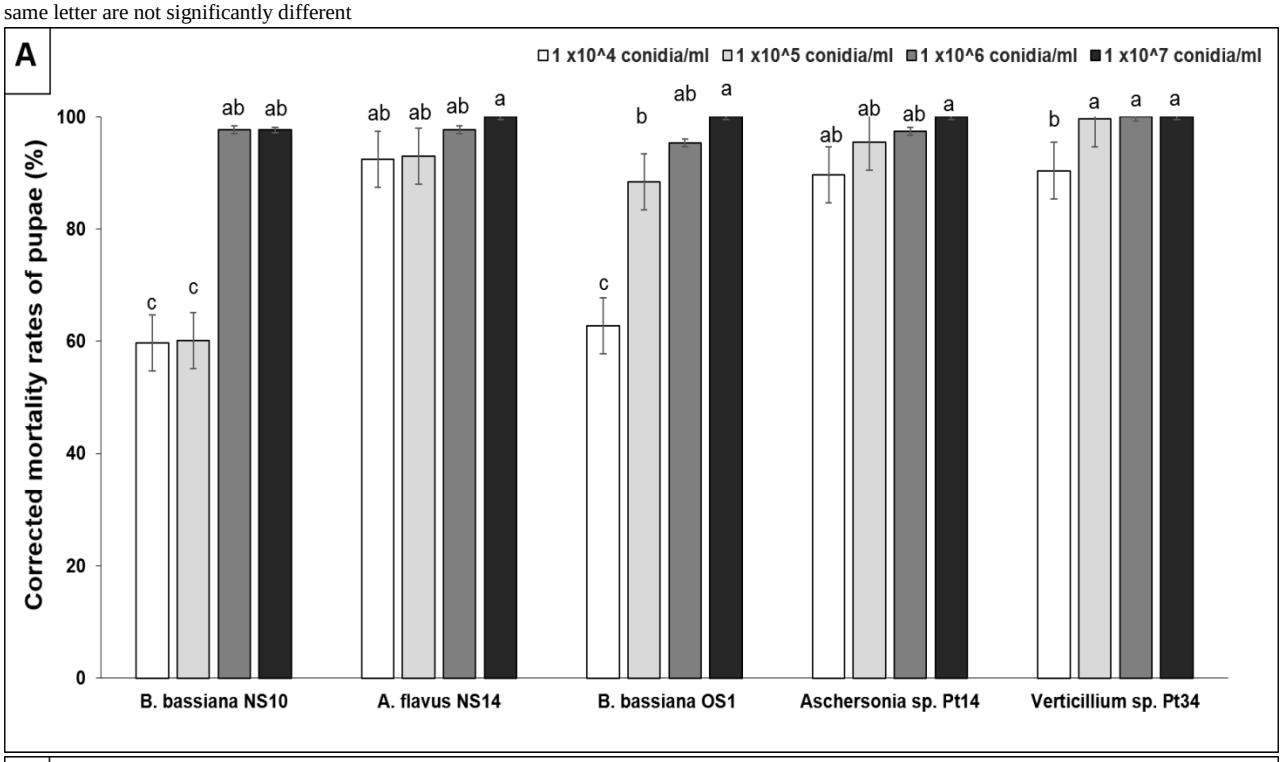
Fig-1. Corrected mortality rates caused by direct spray of four conidial concentrations (10^7, 10^6, 10^5 and 10^4 conidia /ml) of five EPF isolates on *C. capitata* pupae **(A)** and adults **(B)** - Mean (±SE) of corrected mortality, Means followed by different letters differ significantly (comparisons were performed using Fisher-LSD test, P< 0.05), Letters indicate difference between both concentrations and isolates. Treatments sharing the same letter are not significantly different



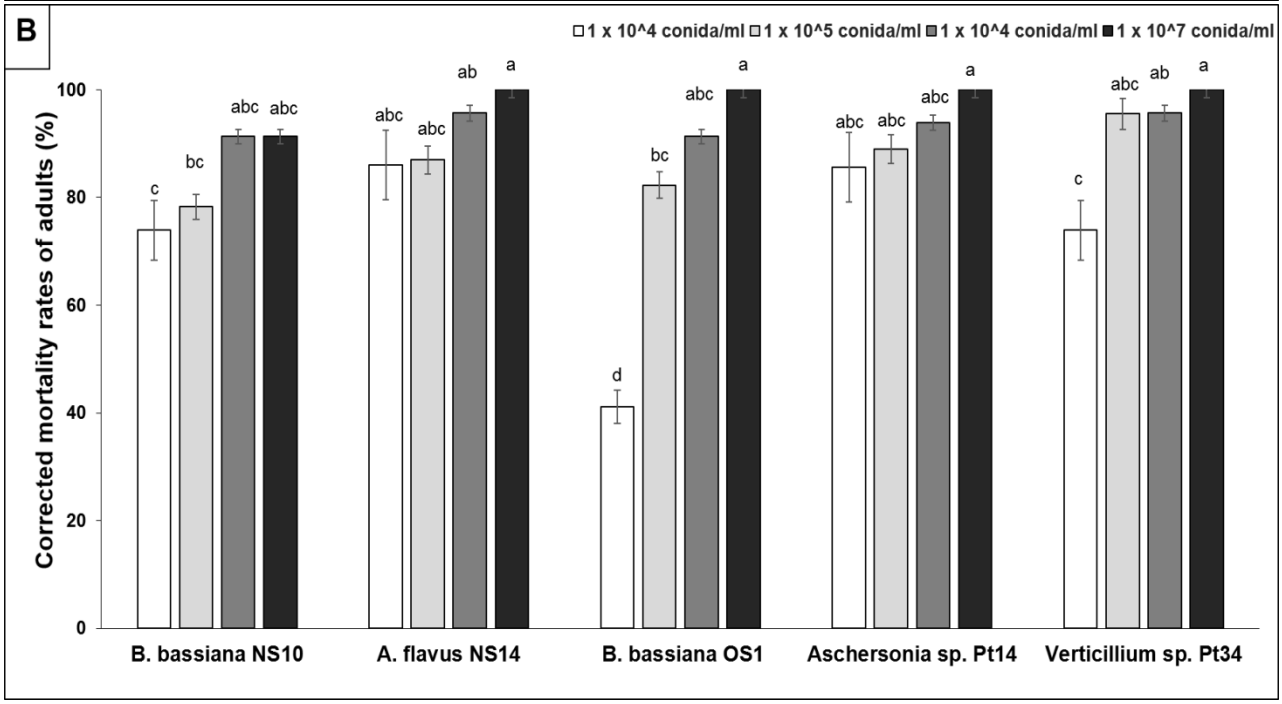


Table-1. Origin and percentage of Conidial Germination of the isolates tested against Ceratitis capitata adult and pupae. With Gr = Germination rates and Sd = Standard deviation. Means followed by different letters differ significantly (comparison was performed Newman-Keuls test, P< 0.05)

Isolates	Origin	Gr ± Sd (%) (p=0.0005) ***
Beauveria bassiana NS10	Argan forest soil	82.10^{a} ± 1.61
Aspergillus flavus NS14	Argan forest soil	94.26^{a} ± 1.34
Beauveria bassiana OS1	Argan forest soil	82.73^{c} ± 4.33
Aschersonia sp. Pt14	Argan forest soil	84.13^{bc} ± 1.37
Verticillium sp. Pt34	Argan forest soil	88.41^{b} ± 2.03

Table-2. Estimated lethal concentrations 50 and 90 of pupae for each fungal isolate using the Probit model. Mean ± Confidence interval (CI) at 95% are represented. LC50 or 90 are considered significantly different when the 95 % CI fail to overlap. Chi-square ($\chi 2$) testing goodness of fit of observed concentration-mortality to expected concentration-mortality responses; Since p-value is greater than 0.1, no significant difference was observed between expected and observed responses; df= degrees of freedom

Isolate	LC_{50} (conidia/ml), (CI 95%)	LC_{90} (conidia/ml), (CI 95%)	Slope ± SE	Intercept ± SE	Goodness of fit – χ^2 (df=2)
***B. bassiana* NS10**	7.104E+03 (4.31E+3-1.542E+4)	2.524E+05 (1.49E+5 - 5.29E+5)	0.888 ± 0.113	-3.517 ± 0.535	2.646 p=0.266
***A. flavus* NS14**	1.429 (1.57E-11-1.47E+2)	6.585E+03 (1.19E+1 - 3.38E+4)	0.350 ± 0.124	-0.054 ± 0.620	2.771 - p=0.250
***B. bassiana* OS1**	3.53E+02 (1.029E+2-7.57E+2)	1.706E+04 (9.63E+3 - 3.76E+4)	0.761 ± 0.111	-1.940 ± 0.421	1.537 - p=0.464
***Aschersonia* sp. Pt14**	1.634 (9.37E-1 - 1.47E+2)	1.165E+04 (1.56E+2 - 6.23E+4)	0.327 ± 0.110	-0.070 ± 0.556	2.832 - p=0.243
***Verticillium* sp. Pt34**	1.07E+03 (1.975-3.33E+3)	1.133E+04 (4.22E+3 - 2.20E+4)	1.254 ± 0.456	-3.802 ± 1.885	0.711 - p=0.701

Table-3. Estimated lethal times 50 and 90 of pupae for each fungal isolate using the Probit model. Mean ± Confidence interval at 95% are represented. LT50 or 90 are considered significantly different when the 95 % CI fail to overlap. Chi-square ($\chi 2$) testing goodness of fit of observed time-mortality to expected time-mortality responses; Since p-value is greater than 0.1, no significant difference was observed between expected and observed responses; df= degrees of freedom

Isolate	LT_{50} (h), (CI 95%)	LT_{90} (h), (CI 95%)	Slope ± SE	Intercept ± SE	Goodness of fit - χ^2 (df=6)
***B. bassiana* NS10**	80.529 (55.511- 104.644)	273.908 (204.315 - 437.488)	2.411 ± 0.397	-4.594 ± 0.850	3.828 - p=0.700
***A. flavus* NS14**	85.793 (62.562- 107.518)	210.538 (184.870 - 321.142)	3.360 ± 0.528	-6.657 ± 1.136	3.346 - p=0.764
***B. bassiana* OS1**	43.044 (30.195 - 55.908)	94.197 (71.936 - 141.120)	2.697 ± 0.463	-6.156 ± 1.233	0.527 - p=0.997
***Aschersonia* sp. Pt14**	49.601 (37.071 - 65.306)	96.932 (76.510 - 136.093)	4.680 ± 0.880	-8.016 ± 1.640	4.167 - p=0.654
***Verticillium* sp. Pt34**	48.993 (33.478 - 66.031)	120.664 (104.307 - 211.165)	2.833 ± 0.468	-4.813 ± 0.917	9.144 p=0.166

Table-4. Estimated lethal concentrations 50 and 90 of adults for each fungal isolate using the Probit model. Mean ± Confidence interval at 95% are represented. LT50 or 90 are considered significantly different when the 95 % CI fail to overlap. Chi-square ($\chi 2$) testing goodness of fit of observed concentration-mortality to expected concentration-mortality responses; Since p-value is greater than 0.1, no significant difference was observed between expected and observed responses; df= degrees of freedom

Isolate	LC_{50} (conidia/ml), (CI 95%)	LC_{90} (conidia/ml), (CI 95%)	Slope ± SE	Intercept ± SE	Goodness of fit – χ^2 (df=2)
***B. bassiana* NS10**	8.972E+03 (4.23E-9 - 1.04E+5)	3.803E+07 (1.99E+7 - 3.80E+19).	0.413 ± 0.208	-1.634 ± 1.112	2.660 - p=0.264
***A. flavus* NS14**	1.840E+03 (1.04E-23 - 2.13E+4)	3.030E+05 (3.09E+4 - 6.42E+011)	0.578 ± 0.293	-1.888 ± 1.455	0.364 - p=0.834
***B. bassiana* OS1**	7.55E+03 (1.64E-1 - 3.66E+3)	5.10E+06 (6.68E+3-3.69E+7)	0.794 ± 0.320	-2.286 ± 1.238	0.191 - p=0.909
***Aschersonia* sp. Pt14**	2.50E+03 (1.96E-3 - 2.79E+4)	5.64E+5 (6.61E+4 - 3.34E+12)	0.545 ± 0.265	-1.851 ± 1.343	0.717 - p=0.699
***Verticillium* sp. Pt34**	7.12E+03 (5.54E-1 - 2.91E+4)	7.82E+5 (4.208E+4 - 1.13E+11)	0.832 ± 0.339	-3.151 ± 1.630	0.308 - p=0.857

Table-5. Estimated lethal times 50 and 90 of adults for each fungal isolate using the Probit model. Mean ± Confidence interval at 95% are represented. LT50 or 90 are considered significantly different when the 95 % CI fail to overlap. Chi-square ($\chi 2$) testing goodness of fit of time-mortality response; df= degrees of freedom. Chi-square ($\chi 2$) testing goodness of fit of observed time-mortality to expected time-mortality responses; Since p-value is greater than 0.1, no significant difference was observed between expected and observed responses; df= degrees of freedom

Isolate	LT_{50} (h), (IC 95%)	LT_{90} (h), (IC 95%)	Slope ± SE	Intercept ± SE	Goodness of fit – χ^2 (df=2)
***B. bassiana* NS10**	28.883 (8.705 - 41.492)	77.894 (53.434 - 318.380)	2.974 ± 1.057	-4.345 ± 1.763	0.616 - p=0.735
***A. flavus* NS14**	27.243 (14.167 - 36.077)	51.764 (38.916 - 112.416)	4.597 ± 1.487	-6.598 ± 2.331	0.822 - p=0.663
***B. bassiana* OS1**	27.097 (12.463- 49.90)	65.515 (41.007 - 129.640)	4.114 ± 1.354	-5.895 ± 2.155	1.641 - p=0.440
***Aschersonia* sp. Pt14**	30.051 (16.901 - 39.691)	59.622 (44.674 - 106.540)	4.307 ± 1.313	-6.365 ± 2.110	2.190 - p=0.335
***Verticillium* sp. Pt34**	29.536 (17.859 - 38.456)	54.191 (41.318 - 107.659)	4.862 ± 1.468	-7.149 ± 2.318	0.936 - p=0.626
Control	163.048 (112.068 - 3410.66)	372.892 (185.934 -7747.011)	0.029 ± 0.014	-3.231 ± 1.172	0.208 - p=0.901