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BIOLOGICAL CONTROL WITH THE FUNGI *PENICILLIUM ROQUEFORTI* AGAINST *CERATITIS CAPITATA* L. (DIPTERA: TEPHRITIDAE)

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ABSTRACT

The fight against pests of fruit crops remains essentially chemical. Currently, we realized that it has harmful effects on the environment and on humans. A new perspective of biological control through the use of biological agents such as fungi gives promising results. In the laboratory, we tested the insecticidal activity of four doses of the conidial suspension of *Penicillium roqueforti* (class of Eurotiomycetes) on the third larval stage of *Ceratitis capitata*. The results reveal that the rate of larval mortality increases after 24 hours of inoculation up to 21.25% at the dose d4 (5.10^6 spores / ml). Similarly, the rate of larvae transformed in pupae has reached 77.5% with dose d3 (4.10^6spores / ml). The rate of malformed pupae, increases to 71.25% for the higher dose represented by d4 (5.10^6 spores / ml). The adult emergence rate of *C. capitata* decreases with increasing dose to be canceled dose d4. This rate decreases for adults formed from larvae placed in the soil contaminated with *P. roqueforti* to 12.5% at the dose d2 (3,2. 10^6 spores / ml). We conclude that this biological test showed that *P. roqueforti* presents a biopesticidal potential against larvae and pupae of *C.capitata*.

KEYWORDS: *Ceratitis Capitata*, *Penicillium Roqueforti*, larvae, pupae, biopesticide

INTRODUCTION

The Mediterranean fruit fly *Ceratitis capitata* (Wied.) (Diptera: Tephritidae), is one of the most important pests of fruit. It is a polyphagous insect that causes huge economic losses (Lux et *al*., 2003). It is the main obstacle to production and export of fruits. It has a wide distribution in the Mediterranean from where the name of the Mediterranean fruit fly (Lekchiri, 1982; Nunez, 1987). In Algeria, especially in Kabylia, the medfly causes much damage to fruit crops (Sadoudi Ali Ahmed et *al*., 2012) because the climate associated with biotic and cultural factors offer favourable conditions for its development. The damages of this fruit fly are of two types: on the one hand, those due to the bites of female caused by nesting attempts which give the fruit a bad appearance. On the other hand, the damages due to the larvae which cause fruit rot and result in mold in particular *Penicillium digitatum* (Cayol et *al*., 1994).

Several control methods are recommended against this pest but the fight remains essentially chemical despite the adverse effects of organophosphate pesticides on the insect itself which develops resistance.

The Autocide fight, also called the sterile insect technique (SIT), allowed the eradication of this pest in some areas such as California and Southern Mexico (Riba et Silvy, 1989; Arevalo-Galarza et Follet, 2011).These same authors found that the treatment of infested fruit papaya *Carica papaya* by the SMDD technique makes ethanol vapor highly lethal to the larvae and adults of the fruit fly, when the insects were exposed to low doses. Similarly, they produced fewer pupae.

Current researches have used biological control including the use of microorganisms such as entomopathogenic fungi as an alternative to chemical fight against *C. capitata*. *Metarhizium anisopliae*, with its asexual reproduction is a fungus that has been the subject of many biological control trials against the Mediterranean fruit fly. This is related to its infestation mode (Meyling and Eilenberg, 2007) and the insect's toxicity mechanisms (Clarkson and Charnley, 1996). It was demonstrated that the penetration of spores varies with the degree of contamination and the thickness of the cuticle (Brooks et *al*., 2004).

Biopesticides, which are soluble crude extracts, produced from the fermentation of pathogenic fungi *M. anisopliae* and *Bauveria sp*, are considered as promising insecticides for the control of the Mediterranean fruit fly (Lopez and Orduz, 2003; Dimbi et *al*., 2009; Amora et *al*., 2009; Ortiz-Urquiza et *al*., 2010 a). Several of these biopesticides intended for the control of fruit fly were extracted from *Metarhizium* (Butt et *al.*, 1994; El Akhdar and Houda, 2009; Ortiz-Urquiza et *al*., 2009; Molina et *al.*, 2010; Ortiz-Urquiza et *al.*, 2010 b; Ortiz-Urquiza et *al*., 2010a). These products are characterized by the presence of toxins called destruxins secreted by this fungus (Vey et *al*., 2001).

It is in this context that this study is registered with aims to evaluate the biopesticidal effect of a conidial suspension of *Penicillium roqueforti* on *C. capitata* larvae and pupae and highlight its entomopatogenicity.

P. roqueforti is an ascomycete fungus saprophyte widespread in nature. Its main industrial use is the production of blue cheese like "Roquefort", "Fourme d'Ambert", "blue Auvergne" etc. (Moreau, 1980). It is the main cause for the maturation of cheeses in which it operates through its proteolytic and especially lipolytic enzymes (Assenat, 1967; Gripon & Berger, 1972).

Enzyme production by *P. roqueforti* varies depending on culturating conditions (Stepanaik et *al*., 1980). It's an interesting fungus, mainly because of its various substrates on which it develops and also for metabolites that may develop.

MATERIAL AND METHODS

Animal material

The third larval stage of *C. capitata* are recovered from infested fruit from different orchards of Kabylia (Algeria).

Isolation of *Pinicillium Roqueforti*

We put the roquefort in a pasteurized transparent jar in which we can observe it and provide it with water to keep it wet. This jar is placed in a drying oven in complete darkness at a temperature of 20 ° C. After two weeks we have noticed the rapid development of the fungus on the surface of all the cheese which we sampled and seeded on the Potato Dextrose Agar (PDA) maintenance medium.

Maintenance of the mycelium and transplanting

After solidification of the PDA culture medium in Petri dishes, *P. roqueforti* was seeded from previously prepared cultures of 8 days.

In order to multiply the mold cultures, we made new samples of inoculums rings implants of 1cm diameter which we planted on two medium Potato Dextrose Agar (PDA) and Rapper Complete (RC).

Determination of the average sporulation's intensity of the fungus in both culture media

The intensity of sporulation is estimated after 8 days of incubation on both PDA and RC environments. Three washers inoculum of 1 cm diameter are removed from the edge of each culture medium. The spores laden surface is scraped aseptically using a metal spatula, which we put in 1 ml of distilled water. An agitation during 2 minutes makes it possible to release the spores of the conidiophore. At the end of agitation, we added 9 ml of distilled water to achieve a dilution of 10^{-1} followed by homogenization of the tubes for 3 minutes. After counting using a cell Malassez, we determined the number of spores / ml in the stock solution for each medium. The latter is considered as the reference concentration in the biological treatment of larvae.

Treatment of the third larval stage by the conidial suspension at various concentrations (spores / ml)

In Petri dishes of 9 cm diameter, the larvae of the third stage of the fruit fly (20 larvae / Petri dish) are sprayed with a 10 ml spray conidial suspension at different concentrations : d1 (2×10^6), d2 (3.2×10^6), d3 (4×10^6) and d4 (5×10^6spores / ml) with 4 repetitions for each concentration. A control is performed in parallel, sprayed with 10 ml of distilled water. To assess the effect of the fungus on the larvae, we calculated the rate of larval mortality, the rate of larvae trasformed into pupae, the pupae malformed rate and the emergence rate of the adult of the fruit fly resulting from pupae.

On the other hand, we put the third larval stage of *C. capitata* in plastic trays of 13 × 10 × 23 cm containing 2 cm of soil from the orchard where we recovered infested fruit (20 larvae / tray). We sprayed soil of each container with 10 ml of each conidial suspension (d1, d2, d3 and d4) with 4 replicates for each concentration. The trays are placed in a dark and wet environment. We recall that the larvae are introduced into the contaminated soils after allowing the fungus to incubate in soil for 8 days.

A control is performed in parallel and under the same conditions by spraying the soil with distilled water. After 10 days we calculated the rate of emergence. Pupae which did not give adults are transferred into Petri dishes containing a wet filter paper to observe possible development of a fungus on their cuticle and determine if they are really attacked by *P. roqueforti.*

The results are analyzed by the software R. When normality was verified by the Shapiro- wilk test we conduct an analysis of variance. In the contrary case we submitted our results to the Kruskal -Wallis test.

RESULTS

Rate of Larval Mortality

From the results shown in Figure 1, we notice that the larvae mortality of *C. capitata* is 5% for the control (d0). This mortality increases with the number of spores in the conidial suspension to reach a maximum rate of 21.25% to d4 dose (5×10^6spores / ml).

The analysis of variance at 5% shows that the larval mortality rate differs significantly depending on the doses used (P = 0.0117 *). Newman and Keuls test class d4 dose in the group (a) and d0 and d1 in the group (b).

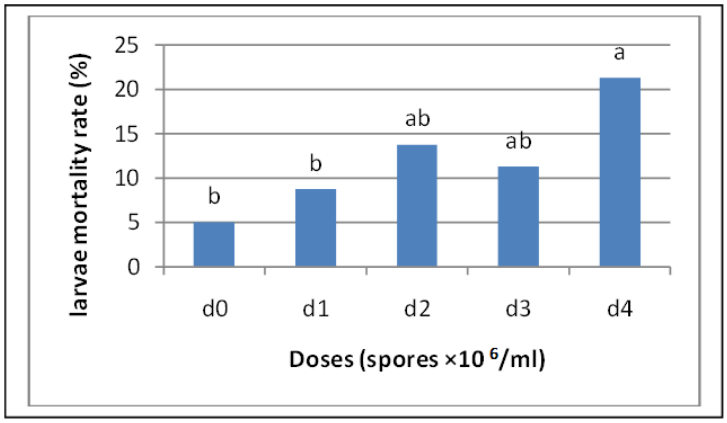

Figure 1: Mortality rate of the third larval stage of *C. Capitata* according to conidial suspension of *P. roqueforti* after 24 hours of treatment.

Larvae rate transformed into pupae.

According to the results shown in Figure (2), 95% of control larvae (d0) are transformed into pupae after 48 hours. At the dose d2 , we obtained 85% of pupae from treated larvae. This rate decreases to 77.5 % at the dose d3 which is of the order of 4 x 10 ^{6} spores / ml.

The Kruskal -Wallis test shows a significant difference in the rate of pupae transformed into larvae according the different doses (p -value = 0.03249).

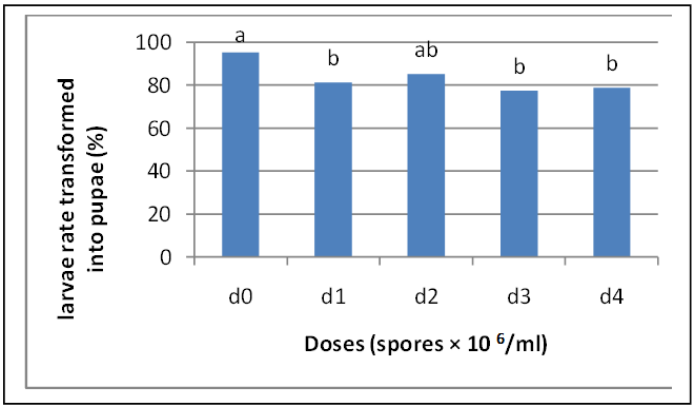

Figure 2: Rate of the third larval stage of *C. Capitata* transformed into pupae after 48 hours of treatment with different doses of the conidial suspension of *P. roqueforti*

Malformed pupae rate

According to the results presented in Figure 3, the control has not malformed pupae. On the contrary, the pupae malformed rate increases by increasing the number of spores in the conidial suspension. At the dose d1 which is of 2 × 10^{6}spores / ml, we recorded a malformed pupae rate of about 5%. This rate increases to 71.25% at the dose d4 which is of the order of 5 × 10^{6}spores / ml.

The Kruskal -Wallis test shows a significant difference in rate of malformed pupae according to the different doses of the conidial suspension (p -value = 0.002776).

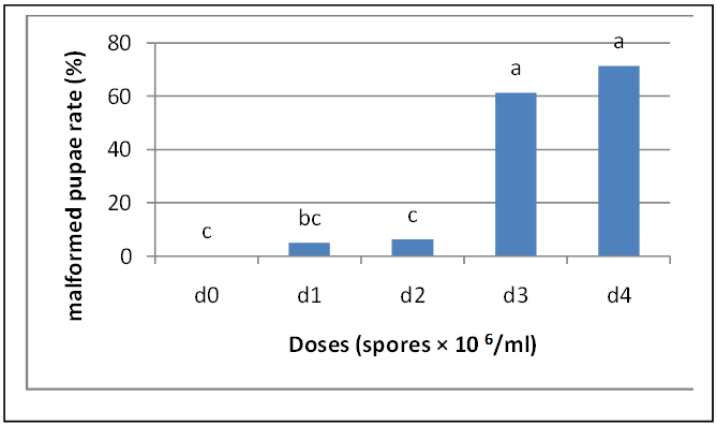

Figure 3: Malformed pupae rate of *C. capitata* according to doses of conidial suspension of *P. roqueforti* after 48 hours of treatment

Adult emergence rate of the medfly resulting from treated larvae

According to the results presented in Figure 4, the control has a maximum emergence rate of about 80%. However this rate decreases by increasing the number of spores in the conidial suspension. At the dose d3 we record an emergence rate of 4.95 %. This rate vanishes at the dose d4 which is of the order of 5×10^6 spores / ml.

The Kruskal -Wallis test shows a significant difference of emergence rates according to the different doses (p -value = 0.001584).

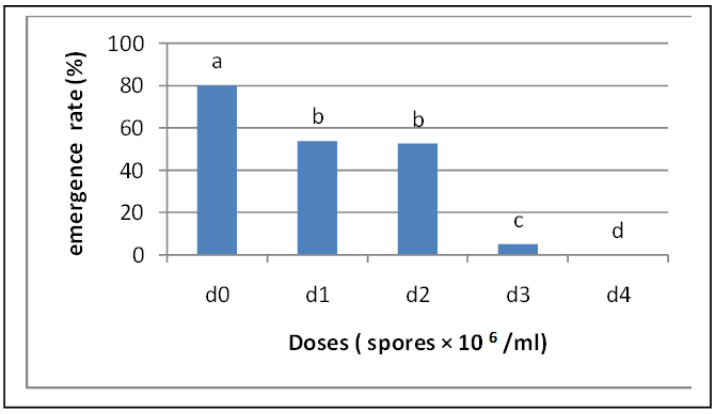

Figure 4: Adult emergence rate from *C. capitata* pupae treated with conidial suspension of *P. roqueforti.*

Adult emergence rate of *C. capitata* in the soil contaminated with the conidial suspension of *P. roqueforti*

According to the results presented in Figure 5, the control has a maximum rate of adult emergence 72.5%. This rate decreases by increasing the number of spores of *P. roqueforti*. At the dose d2 which is 3.2×10^6 spores / ml, we record a rise of 12.5 %.

The Kruskal -Wallis test shows a significant rate of emergence according to different used doses (p -value = 0.0218).

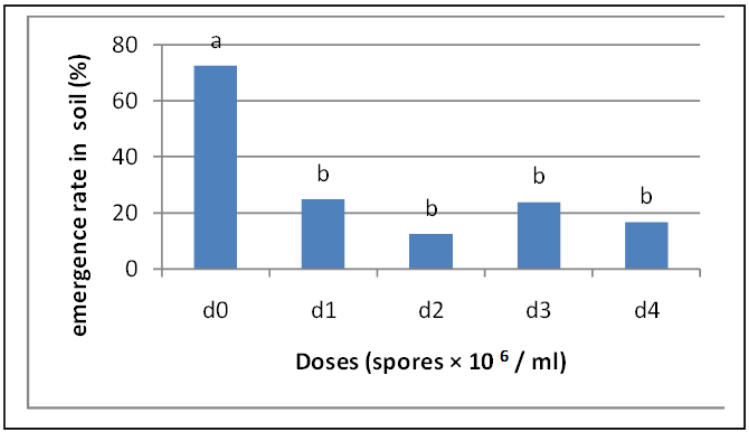

Figure 5: Adult emergence rate of *C. capitata* from pupae placed in a contaminated soil according to the different doses of the conidial suspension of *P. roqueforti*

DISCUSSION

The results show a decrease in larval mortality compared with the control. We recorded a larval mortality rate of 21.25% at a dose of 5 x 10^6 spores / ml against 5 % for the control after 24 hours of treatment.

Similarly, the result shows a decrease in the number of healthy pupae from larvae treated with conidial suspension of *P. roqueforti* after 48 hours. Thus we obtained 77.5% of healthy pupae whith the dose 4 x 10^6 spores / ml against 95% of healthy pupae in the control. In addition, we observed a high number of malformed pupae by increasing the doses. in fact we recorded, 71.25% of pupae malformed at the dose of 5 × 10^6 spores / ml contrariwise the control presented only healthy pupae. The spores of *P. roqueforti* therefore exert an insecticidal effect on larvae and pupae of *C. capitata.*

Our results are in accordance with those of Boudjlidae and Soltani (2011) which showed the larvicidal activity of the entomopathogenic fungus *M. anisopliae* (Metsch) against the fourth larval stage of *C. capitata*. The accumulated percentage of mortality after 7 days of inoculation was 26.13 % for the lower dose of about 6,5.10^5 spores / ml and increased to 89.05 % for the higher dose represented by 52.10^5 spores / ml.

According to Gallo et *al.*, (2002) the third larval stage leave the fruit to pupate in the soil. At this stage, they become vulnerable before entomopathogenic fungi (Gutierrez et *al.*, 2000; Bissoli, 2004).

Our results are also in accordance with those of Bissoli (2004) who found that *M. anisopliae* caused mortality of 66, 5% of larvae of *C. capitata*. In the other hand, they do not agree with those of Dinalva et *al*., (2006) who reported that the conidia of *M. anisopliae* do not affect larva survival in the soil with or without fungus.

Studies have shown that the toxicity of the fungus *M. anisopliae* (Metsch), used in the fight against fourth stage larvae and adults of *C. capitata*, is due to the selective action of enzymes secreted by the fungus itself act on the compound of the insect cuticle and facilitates the penetration of the hyphae through the cuticule (Butt et *al*., 1994; Ekesi et *al*., 2003; Yee and Lacey, 2005).

Nguyen et *al*., (2008) reported a high larval mortality of the fifth stage of the Lepidoptera *Helicoverpa armigera* (Noctuidae), by the contamination of three entomopathogenic fungi *M. anisopliae*, *Paecilomyces fumosoroseus*, *Beauveria bassiana* with a dose of 1 × 10^7 conidia / ml for each of fungal species.

Our results are in line with those of Riba et *al*., (1984) who found that strains of *M. anisopliae* and *P. citrinum* have been very aggressive against larvae of *Culex pipiens*, *Aedes aegypti* and *Anopheles stephenis.* They manifest their insecticidal effect is through toxin, either by colonizing the hemocoel.

The same author notes that the strains of *P. citrinum* kill mosquito larvae without multiply in the host, probably through a process toxemic. In contrary, Roberts, (1967) showed that *M. anisopliae* attack larvae by respiratory siphon. That is why this germ kills more mosquitoes when applied to the surface of the water. But indeed, if larvae are placed in conidial suspensions of high concentration (10^7 spores / ml) the fungus can kill it before colonizing the hemocoel.

Laboratory studies have showed that *M. anisopliae* is pathogenic to eggs of *C. capitata* (Caslilho, 2000), larvae (Gutierrez et *al*., 2000) and adults (Gracia et *al.*, 1984, 1985; Dimbi et *al.*, 2003; Boudjlidae and Soltani, 2011).

We observed a decrease in the rate of emergence in the case of larvae treated only with conidia of *P. roqueforti* where the rate of emergence vanishes at a dose of 5 x 10^6 spores / ml against control who has 80%. Similarly in the case where the larvae are placed in contaminated soil, the emergence rate decreases up to 12.5% against a control which represents 80% emergence.

Our results are in line with those of Dinalva et *al*., (2006) who obtained an emergence rate of 44.2% from medfly larvae treated with *M. anisopliae* against a control who has given 87% of adults. When they treated the fruit fly pupae with the same fungus, they got 22.3% of adults emerged from pupae treated against 89.5% for the control.

Similarly, our results corroborate those of Ekesi et *al*., (2003) who found a decrease of adult emergence rate of the medfly compared to the control, after inoculation of spores of *M. anisopliae* in soil.

The decrease in the rate of emergence of adults resulting from larvae treated with *P. roqueforti* could be due to the effect of the fungus on the larva before pupation.

According Ferron et *al.*, (1975) the larvae were probably contaminated with spores at pupation; these had already sprouted and started the infectious process that led to the death of the nymph.

Quentela et *al*., (1994) shown that the soil sprayed with conidia solution of *M. anisopliae* and *B. bassiana*, control 30% to 50% larval mortality of *Chalcodermus bimaculatus* (Fiedler). Conidia have a significant effect on adult emergence compared with their effect on the larvae.

According Mochi et *al*., (2006) toxic synthesized by *M. anisopliae* interfere especially with the emergence of adults rather than the larval mortality.

According Castilho (2000), these results may be influenced by environmental conditions such as humidity and temperature which affect the spore germination. The influence of climatic conditions on insect mycosis has been mentioned many times (Macleod et *al.*, 1966; Moor, 1973; Riba et *al.*, 1984; Mochi et *al*., 2006; Hassan, 2014).

Ferron et *al*., (1975) report that the relative humidity is also often mentioned as a key factor in the success of the infection. They accepted, generally, that the pathogenic fungi spores can germinate in the presence of a water film or water-saturated atmosphere.

Riba et *al.*, (1984) find that a few hours of exposure to relative humidity between 10 and 90% significantly reduce

the activity of entomopathogenic hyphomycetes. Thus, the germination of conidia of *B. bassiana* does not exceed 20% if they were placed 25 days at 30% of humidity. In addition, the duration of the pathogenesis of *B. bassiana* against corn borer larvae is increased about 3 days when the relative humidity decreases from 100 to 30%. Under these conditions the fungus can not sporulate on the surface of cuticles.

If *M. anisopliae* is placed less than 90% humidity, it loses infectivity against eggs of the corn borer. Moreover, an exposure of from one day to the humidity below 90% is sufficient to reduce by 85% the number of eggs attacked by this fungus.

According to our results, we noticed that after the death of the fruit fly pupae, *P. roqueforti* continues its development saprophytically and colonize different tissues. After complete invasion of the dead pupa, further development of *P. roqueforti* is conditioned by ambient moisture. Therefore, we put the pupae that did not give adults in Petri dishes containing a wet filter paper in order to have high humidity. The mycelium after having again get through the cuticule, but this time from the inside outwards, grows on surface to form conidiophores.

According Prasertphon and Tanada (1968), the entomopathogen could be conserved as chlamydospores. As the fungus multiplies on or inside the dead pupae, we can consider that each pupae killed by the fungus is a potential source of infection likely to maintain the pathogen inoculum from one generation to the next or from one year to another. According Ferron (1977), it is possible to infect a pest population by artificial dispersing an inoculum multiplied laboratory regardless of the relative humidity values of the atmosphere.

CONCLUSION

We conclude that *P. roqueforti* affects significantly the survival of third larval stage of *C. capitata.* It would be interesting in the future to determine by which process this pathogenicity is done. Is that only by multiplying itself in the host as *P. citrinum* or secreting toxins or both mechanisms at the same time? Likewise, is it as *M. anisopliae* attacking the larvae through the respiratory siphon?

We also retain that *P. roqueforti* affect pupation decreasing the larvae rates transformed into pupae and increasing the number of malformed pupae.

This is possible thanks to its enzymatic mechanism and different secreted toxins, which degrades the protein cuticles pupae of the Mediterranean fruit fly. Thus, indirectly, it affects the rate of emergence of the flies in both cases (with or without soil).

We got a decreased rate of emergence from control in the two previous cases (with or without soil). The emergence rate is zero in the fourth dose (5×10^6 spores / ml).

P. roqueforti therefore affects the last phase of the cycle of the medfly as the adult emergence phase. This fungus, used extensively in the food industry especially in the cheese dairy, is appears as an interesting entomopathogenic fungus in biological control against *C. capitata*.

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