Differential Responses of Ceratitis Capitata to Infection by the Entomopathogenic Fungus Purpureocillium Lilacinum.

Wafa Djobbi  
National Centre of Nuclear Sciences and Technologies

Meriem Msaad Guerfali (✉ msaad_tn@yahoo.fr)  
National Centre of Nuclear Sciences and Technologies

Agnès Vallier  
Institut National des Sciences Appliquées de Lyon

Kamel Charaabi  
National Centre of Nuclear Sciences and Technologies

Justin Maire  
Institut National des Sciences Appliquées de Lyon

Niclas Parisot  
Institut National des Sciences Appliquées de Lyon

Hubert Charles  
Institut National des Sciences Appliquées de Lyon

Haytham Hamden  
National Centre of Nuclear Sciences and Technologies

Salma Fadhl  
National Centre of Nuclear Sciences and Technologies

Abdelaziz Heddi  
Institut National des Sciences Appliquées de Lyon

Ameur Cherif  
Manouba University

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Abstract

*Ceratitis capitata* (medfly), is one of the most injurious pests of fruits with quarantine importance because of its extremely wide host range. The use of entomopathogenic fungi constitutes a promising approach for potential applications in integrated pest management. Nonetheless, developing methods of insect control can also involve the use of fungal machinery to produce metabolic disturbance that can increase its effectiveness by producing a detrimental effect on insect development. Insect species, such as *Ceratitis capitata*, depend on reproduction potential, nutrient reserves, metabolic activities and immune response for their survival. Accordingly, the purpose of this study is to use the entomopathogenic fungus *Purpureocillium lilacinum* to investigate, its sublethal effects on *Ceratitis capitata*. Laboratory bioassays were conducted on medfly V8 strain. The bioassays were monitored to determine the virulence of *P. lilacinum* on the fruit fly. *P. lilacinum* was tested against 5 days-old males and females, through abdominal topical applications. Following the fungal inoculation, we showed (i) a significant increase of sugar amount in tissues, (ii) a significant decrease in carbohydrase activities, digestive glycosyl hydrolase and proteinase activities in whole midguts of treated flies, (iii) an over-expression of *Takeout* and *Attacin-A* genes induced by infection. Moreover, the up-regulations observed for *relish, cecropin 1, ceratotox-A* and *defensin* genes are due to physiological mechanisms occurring during infection.

Introduction

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*Ceratitis capitata* (medfly) (*Diptera: Tephritidae*), is a cosmopolitan invasive agricultural pest that affects a wide host range of fruits and has a worldwide distribution. Chemical treatments constitute the useful tool in Tunisia for the control of medfly, however repeated pesticide use exerts a specific selection pressure which is pesticide resistance. Environmentally friendly control procedures were developed for the control of medfly, using microorganisms such as bacteria, fungi and viruses. Over
the last years, the use of entomopathogenic fungi constituted a promising agent of control for insect pests in agriculture. The use of entomopathogenic fungi as biocontrol agents plays a crucial position in enhancing integrated pest management (IPM) efficiency.

Entomopathogenic fungi (EPF) have been shown to be potent biocontrol agents. Furthermore, they have been attested to induce a large scale of sublethal effects within infected insects. Accumulating evidence were reported that EPF profoundly affect host biological traits including mating behavior alteration, food consumption reduction, immune reaction alterations, developmental rates, longevity and fecundity reductions. However, over time Purpureocillium lilacinum has been described mainly as nematode pathogen but also have shown clear potential as insect-pest control agent. The adult Mexican fruit fly Anastrepha ludens, the melon fly Bactrocera cucurbitae, the brown flour beetle Tribolium confusum, the cotton aphid Aphis gossypii, the cotton bollworm Helicoverpa zeamays, the fruit fly Bactrocera spp., the greater wax moth Galleria mellonella, the tomato moth Tuta absoluta, the Asian citrus psyllid, Diaphorina citri and the Sweet potato whitefly Bemisia tabaci.

P. lilacinum is a slowly killing pathogen able to survive for longer periods within hosts. This strategy allows P. lilacinum to be more efficient and to have a higher transmission rate. It has now been confirmed that protease, phospholipases, chitinase, mannanase, beta-glucanase and lipase hydrolyzing enzymes, leucinostatins and paecilomide secondary metabolites play crucial roles in P. lilacinum pathogenicity. Many of these secondary metabolites showed immune system modulatory activity, as well as insecticidal, cytotoxic, and antimicrobial activities.

An increasing number of studies have proven that P. lilacinum is negatively affecting the host’s biological parameters, in addition to causing death due to mycosis. Recent advances have revealed many effects on reproduction, growth, food consumption and sexual behavior. Meanwhile, the mechanisms of host-fungus interaction are still understudied and poorly understood. P. lilacinum mediates various toxicological effects making it useful for control strategies. Consequently, this study is the first report on applying the fungus P. lilacinum as a novel and promising biocontrol agent against C. capitata. We have examined the role of P. lilacinum in transformation response elaboration within C. capitata. We investigated in-vitro, the sublethal activities of P. lilacinum and analyzed biologically susceptible activities, (i) fertility and fecundity parameters, (ii) nutrient reserves, (iii) biochemical enzymes activities, (iv) defense-related genes.

Results

Fecundity and fertility parameters. After the inoculation of the spore suspension, treated flies laid lower average of eggs per female when compared to untreated individuals. A significant reduction in fecundity and egg fertility of treated females was observed. About only 133.50 ± 1.08 eggs were laid by treated female, whereas control flies laid 245 ±4 eggs per female (Table 1). In addition, P. lilacinum inoculation
reduced significantly egg fertility by 62.9%; 28.8±4.4% of the eggs hatched from treated flies compared to 91.7 ± 2.7% from non-treated flies (F$_{3,2}$= 44.82, P< 0.0005) (Table 1).

**Nutrient reserves measurement.** Treatment with *P. lilacinum* has resulted in a varying composition of larvae nutrient reserves. The amount of sugar increased significantly (P=0.007) in tissues of third instars larvae from treated flies 0.521 ± 0.002 µg/ mg of Protein, compared to larvae from non-treated flies 0.332 ± 0.001 µg/ mgP. Moreover, glycogen and lipid amounts remained the same, less than 0.05 µg/ mgP, there was no significant difference between larvae from infected and control flies (Fig. 1).

**Enzymatic assays.** The carbohydrase activity in the whole midguts of treated flies showed a significant decrease post-inoculation for starch 487.04 ± 41.98 AU/mgP (P<0,00001) and for Pectin 130.48 ± 51.95 AU/mgP (P= 0.009), compared to controls 1055.68 ± 44.29 AU/mgP; 468.37 ± 32.23 AU/mgP; respectively (Fig. 2).

The digestive glycosil activity seems to be affected as well during infection. After 72 h of fungal inoculation, treated flies showed a significant decrease in specific α-glucosidase activity 18.43 ± 0.14 AU/Gut compared to control flies 24.53 ± 0.955 AU/Gut (P=0.0001) and in β-galactosidase activity 9.10 ± 0.75 AU/Gut, compared to control flies; 16.33 ± 1.24 AU/Gut (P< 0.0001) (Fig. 3).

The digestive proteinases (measured by the amounts of azocasein and hemoglobin in the midgut) are less active in infected adults of *C. capitata* than controls. Higher capacity to digest protein for control flies midgut was observed. For azocaseinase and hemoglobinase, infection induced a significant decrease (P=0.0001; P=0.003, respectively) in activity 2 ± 0.2 AU/Gut, and 5.66 ± 2.11 AU/Gut, respectively compared to controls 4.93 ± 0.58 AU/Gut; 13.64 ± 0.50 AU/Gut; respectively (Fig. 4).

**Molecular assays: steady-state levels of gene transcripts.** Box-plots (Fig. 5) revealed that the expression profile of some AMPs encoding genes depends significantly on fly gender for both infected and control flies. The sex seems to strongly influence the expression of AMPs regardless of the infection status. A significant change in expression levels of *Attacin-A* (F = 19.06; P=0.001), *Ceratotoxin-A* (F = 3.76; P=0.05), and *Takeout* (F = 4.66; P=0.001) within females rather than males was observed.

The variable status (Virgin, mature and mated) influenced the expression profile of some AMP encoding genes (Fig. 6). We noticed a significant activation of immune-response related genes: *Attacin-A* (F = 19.06, P<0.001), *Takeout* (F = 4.66, P = 0.05), *Ceratotoxin-A* (F = 3.76, P=0.01), and the IMD pathway transcription factor *Relish* (F = 12.85, P<0.001) for both sexes. For females (Fig. 6B), the expression profile of *Attacin-A* and *Ceratotoxin-A* increases with maturity compared to their counterparts virgin, immatures, and mated flies. Males showed a profile that remains similar to that expressed by females (Fig. 6A). Mature males express more AMPs than virgin immature and mated ones.

Times after infection induce a significant increase on 5 of the 7 transcripts studied: *Attacin-A* (F = 11.76, P<0.001), *Ceratotoxin-A* (F = 6.44, P<0.001), *Takeout* (F = 11.33, P<0.001), *Defensin* (F = 4.21, P = 0.01),
and Relish (F = 15.44, P<0.001) for both males and females infected, compared to uninfected flies (Fig. 7).

After 72 h of inoculation, infection variable modulated significantly Attacin-A (F = 6.49, P = 0.01), and Takeout (F = 13.01; P<0.001) expression levels for the times analyzed (0h, 24h, 72h and 144h). For the other examined genes: Defensin, Relish, PGRP-LC, Ceratotoxin-A, and Cecropin-1, there was no significant evidence for specific changes on expression levels after the fungal inoculation (Fig. 8). For Virgin flies profile, a significant increase on transcript levels of Takeout (F_{15,32} = 7.12; P<0.005), and Attacin-A (F_{15,32} = 2.1; P=0.05) was observed by 3.56-fold and 6.68-fold; respectively with infected females (Fig. 8B), and by 9.26-fold and 1.13-fold, respectively with infected males (Fig. 8A).

A Significant status x time interaction (F = 3.13; P=0.007), and sex x status x infection (F = 7.04 P=0.001) were obtained as well with the transcriptional activity of Cecropin-1 (F_{15,32} = 3.66; P<0.001) by 52.80-fold and 9.90-fold within males and females, respectively (Fig. 8).

After maturation, a transcriptional activity of immune-response-related genes was observed for both males and females inoculated with P. lilacinum in comparison to their counterpart control flies. A significant overexpression of Attacin-A (F_{15,32} = 2.17; P=0.03) by 3.17-fold for males, and 1.80-fold for females was detected. Likewise, significant status x sex x time variables interaction (F_{6,6} = 2.71; P=0.01) was obtained with Ceratotoxin-A (F_{15,32} = 4.24; P<0.001) for infected females (Fig. 8).

Once mated, infected flies profile shows that immune response transcript levels increase by 1.80-fold within males and by 7.50-fold within females for Attacin-A (F_{15,32} = 8.54; P<0.005), and by 2.41-fold within males and 39.38-fold within females for Takeout (F_{15,32} = 4.72; P<0.005) (Fig. 8). Moreover, the significant upregulation observed within Relish, Ceratotoxin-A, and Cecropin-1 genes after 72 h post-inoculation are due to the time, sex, infection and status different interactions (Table 2).

Discussion

The purpose of this study was to identify biological processes by which C. capitata attempted to defend itself against P. lilacinum infection challenge. The host-pathogen interaction involved defense mechanisms which may include subsequent behavioral and/or physiological responses of the host to fungal pathogenesis. For this reason, we screened reproductive-based response (fecundity and fertility parameters), metabolic-based response (enzymatic activities), and molecular-based response (transcriptional profiles) of C. capitata. The expression approach to determine the effects of infection on transcript abundance in C. capitata focused on differentially represented transcripts related to immune system processes. Accordingly, qRT-PCR analyses were performed on infected and control virgin immature, virgin mature, and mated individuals of both sexes in order to determine whether genes related to the immune response show changes in transcriptional regardless gender (males or females) and sexual status (maturation and mating).
*P. lilacinum* expresses a broad range of sublethal effects. The results showed that female progeny production and egg fertility are affected by infection with *P. lilacinum* isolate. Our findings are in pars with those demonstrated by Toledo-Hernández *et al.*\(^8\) who worked on the evaluation of the virulence and sublethal effects of *P. lilacinum* against the Mexican fruit fly *Anastrepha ludens*. Similar effects on *C. capitata* were obtained after infection with the fungus: *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus*\(^{25,26,27}\). In addition, we demonstrated the increase in sugar reserves during the third larvae instars of all sampled larvae from infected flies compared to similar low lipids and glycogen levels. In fact, insects metamorphic processes require the consumption of their carbohydrate reserves for the synthesis of pupal cuticle and imaginal tissues\(^{28}\). This suggests that the high sugar content can be related to a lack of energy expenditure or to a deficiency in sugar digestion machinery (inhibition of the assimilation of sugars). This hypothesis, however, needs further investigation.

Additionally, enzymatic activities from *C. capitata* adults were significantly susceptible to infection under the *in vitro* assays (optimum pH). *Ceratitis capitata* rely on specific α- and β-galactosidases associated with enzymatic activities on maltose and sucrose to digest carbohydrates with complex proteolytic systems for protein digestion, especially the serine endoproteinase\(^{29}\). We were able to demonstrate that the capacity to digest carbohydrates, evaluated by the amounts of di and polysaccharide in midguts, seems to be disturbed by infection. Treated flies always show a significant decrease in their enzymatic activities, compared to untreated flies, by the third day of infection. These findings are in agreement with those of Malaikozhundan and Vinodhini\(^{30}\) obtained with the cowpea weevil *Callosobruchus maculatus* infected with *Bacillus thuringiensis* and therefore suggest the susceptibility of *C. capitata* enzymes to *P. lilacinum*.

Sugars and proteins are macronutrients that play critical physiological roles in diptera resource allocation, longevity\(^{31}\), reproduction\(^{32,33}\), larval development\(^{34}\), mating success\(^{35}\), and in sex pheromone production\(^{36,37}\). Digestion and absorption of carbohydrates stimulate the lipogenic activity (incorporation of lipid reserves). Lipids modulate hormonal changes such as juvenile hormone (JH)\(^{38}\) which regulates mature sexual behavior in both males and females of diverse insect orders\(^{39}\). In addition, it has been proven that the egg-laying abilities of laboratory reared females of the Mediterranean fruit fly *Ceratitis capitata* (Wiedmann) and the melon fly *Bactrocera cucurbitae* (Coquillett) are delayed or suppressed by limiting access to host fruits and dietary protein\(^{40}\). To compensate these nutritional deficiencies, adults of *C. capitata* are able to allocate energetic reserves to be used slowly under a "reproductive waiting mode"\(^{41}\) or/and to proceed to egg-reabsorption in reproductive females observed in the olive fly *Bactrocera oleae*\(^{33}\). Based on these results, we can thus suggest that the low carbohydrate digestion (after enzymatic inhibition) caused by infection with *P. lilacinum*, is linked to the fertility and fecundity reduction in *C. capitata*.

Immune system is a key barrier against infectious microorganisms (virus, bacteria, fungi and parasites). Therefore, nutrition is essential to pathogens resistance in insects\(^{42,43,44}\). Gomulski *et al.*\(^{45}\), suggested that the abundance of immune gene transcripts might be related to the nature of the food sources within
C. capitata. In our case, the apparent lack of immune response after fungal inoculation in C. capitata within some of the studied genes could, however, be related to the enzymatic activities decrease in inoculated flies. Additionally, fungi are known to produce secondary metabolites that display various immunosuppressive properties capable to disrupt the host’s physiological processes. Several fungi have proven the potential to modulate the immune response of different insects as the large pine weevil, Hylobius abietis, the greater wax moth, Galleria mellonella, and the tobacco caterpillar, Spodoptera litura. P. lilacinum is known to produce leucinostatins, a family of lipopeptide antibiotics with a broad range of biological activities, including mortality and reproduction inhibition in nematodes. These results drew our attention to the relationships between the lack of immune gene expression and leucinostatins. We hypothesize that P. lilacinum uses leucinostatins as immune system inhibitors to maximize its proliferation rate.

Physiological attributes (e.g., sex, maturation, mating) impact the immune system function of C. capitata. The up-regulation observed with Relish, Cecropin-1, Ceratotoxin-A, and Defensin genes is probably in case of being implicated in other physiological mechanisms occurring during the same time of infection, rather than the proper effect of infection. The effect of sex (gender) and status (virgin, mature and mated) variables seems to strongly influence the expression of some C. capitata AMP genes regardless of the infection challenge. Females and males express different profiles levels of Attacin-A, Ceratotoxin-A, Relish, and Defensin. This sex-specific response was demonstrated previously with C. capitata and Drosophila suzukii. During maturation, C. capitata expressed a significant increase in immune-response related genes (Attacin-A, Ceratotoxin-A, Relish, and Defensin) compared to their counterparts immature virgin. This up-regulation may be explained by the process of immunity anticipation prior to mating which was previously demonstrated in Ceratitis capitata.

Following fungal inoculation, the immune system of C. capitata involved the activity of Takeout and Attacin-A genes displayed to overcome the fungal infection within virgin immature, virgin mature, and mated flies. Accordingly, Attacin-A is an antimicrobial peptide implicated in the Imd signalization pathway, and Takeout is a circadian clock-regulated output gene involved in the control of different aspects of insect behavior and physiology such as aging, longevity, feeding behavior, locomotion, circadian behavior, and insecticide susceptibility. This finding provides a first evidence for the involvement of Takeout gene in C. capitata immune response against entomopathogenic fungi.

The effects cited above, in our opinion, may lie beneath the remarkable effect caused by P. lilacinum. A cause-and-effect relationship could be established between the fungal inoculation and the resulting metabolic and molecular disorders. These findings provide a comprehensive insight on the dynamics of C. capitata's response to P. lilacinum infection revealing that metabolic processes, rather than immune functions, played a key role in the response. Furthermore, metabolic and molecular information about C. capitata's response systems to P. lilacinum proliferation, of which little is known to date, could lead to a better understanding of the mechanism in controlling plant pests, as well as improve its capabilities as a bio-control agent.
Methods

Flies. The Mediterranean fruit flies used in the experiments were from a colony of the VIENNA 8 (V8) genetic sexing strain (GSS) maintained in the Tunisian Medfly rearing facility situated in the National Centre of Nuclear Sciences and Technology (CNSTN). This strain has two mutations with two markers: white pupae (wp) and temperature-sensitive lethal mutation (tsl). The mass-rearing was maintained under optimal conditions\(^59,60\). Wild flies were obtained after sampling infested fruits hosting the fly. The fruits were then collected and placed on a mesh screen in a plastic container, allowing larvae to emerge from the fruit and pupate at room temperature. The pupae collected in this way are placed in bottles for the emergence of adults, which will then be used for bioassays.

Fungal isolate. \textit{Purpureocilium lilacinum} was isolated from soil samples collected from a citrus orchard in Tunisia.

Bioassays. Laboratory bioassays were conducted on medfly V8. The bioassays were monitored to determine the virulence of \textit{P. lilacinum} on fruit fly. \textit{P. lilacinum} was tested against 5 days-old males and females, through abdominal topical applications. The bioassays were carried out with five replicates under optimal conditions 20 ± 2 °C, 50-60% RH with a photoperiod of 15:9 (L:D) h. Fifteen adults (males and females) were individually treated with 5 µl of designated suspensions and placed together in a ventilated Petri dish (89 mm diameter, 23 mm height). Adult flies were fed with sugar: yeast (3:1) and water. Control flies were treated with Tween 80 (0.02%) free of fungal suspension.

Fecundity and fertility parameters. Adult fecundity was measured to evaluate the effect of medfly adult exposure to \textit{P. lilacinum} treatment at \(10^8\) conidia/ml. Each replication consists of a group of five females and five males placed in a ventilated box under optimal conditions 20 ± 2 °C, 50-60% RH with a photoperiod of 15:9 (L:D) h and flies were fed with sugar: yeast (3:1) and water. The combinations studied are: \(\square\) control \(\times\) \(\square\) control; \(\square\) treated \(\times\) \(\square\) treated. Eggs are collected 48 hours after the first day of treatment. The number of eggs laid per replication was then counted\(^27\).

Egg fertility was realized by lining up one hundred eggs in a Petri dish. Petri dishes are then incubated at 26 °C with 60% RH and a photoperiod of 16:8 (L: D) h for 48h. The number of larvae hatched was counted for 7 days\(^27\).

Nutrient reserves measurement. For the measurement of larvae nutrient reserves, three third-instar larvae were homogenized in 0.2 ml of 2% Na\(_2\)SO\(_4\) with the addition of 1.3 ml chloroform-methanol (1:2) (vol: vol) solution. After 10 min of centrifugation at 5,200 g, the supernatant was used for lipid quantification and the pellet, was further treated for glycogen and sugars estimation\(^33\). Lipid quantification was assayed as described previously by Warburg and Yuval\(^61\). After Chloroform: methanol (1:2) total evaporation at 90 °C, lipids were resuspended in 0.3 ml H\(_2\)SO\(_4\). Lipids were hydrolyzed at 90 °C for 10 min and an aliquot (30 µl) was reacted with the 270 µl vanillin reagent for 30 min at room temperature. Quantity of total lipids was estimated by measuring absorbance at 490 nm and using a vegetable oil as a
standard. Carbohydrates content was estimated using the method in Warburg and Yuval \textsuperscript{61}. After the addition of 200 mL of anthrone reagent and 1 mL of water to 300 µl of the supernatant obtained from Chloroform: methanol extraction, the mixture was kept reacting for 10 min at 90 °C. Total carbohydrates in the sample were estimated by measuring the absorbance at 630 nm and using anhydrous glucose as a standard. Glycogen quantification was carried out according to Tolmasky and Krisman \textsuperscript{62}. The pellet obtained was digested with 33% KOH for 15 min followed by ethanol 96% precipitation. After overnight incubation at 4 °C, glycogen was precipitated at 2,000 g and the pellet obtained was resuspended in 0.1 ml of water. The total amount of glycogen was then determined by the addition of I$_2$/KI/CaCl$_2$ reagent.

**Enzymatic assays.** Assays were realized after digestive tract dissection of 50 adult flies in NaCl (0.15 M). The intestines are then homogenized in 50 mM Tris-HCl buffer (pH 8.0). After centrifugation at 10,000 g for 30 min at 4 °C, the supernatant obtained was recovered and kept at -20 °C to be used as a source of enzymes\textsuperscript{29}.

Disaccharide and polysaccharide activity was determined according to Baker \textit{et al.}\textsuperscript{63}. Briefly, 80 µl of intestinal homogenates were incubated 30 min at 37 °C with 60 µl of 1 % substrate solutions (maltose, sucrose, pectin and starch) and 400 µl of sodium acetate buffer 0.1 M (pH = 5.5). The aliquots were heated in boiling water for 10 min then 100 µl of DNS solution was added. The absorbance was measured at 540 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 540 nm. Monosaccharide activity was determined by the estimation of p-nitrophenol (PNP) released by hydrolysis of the corresponding p-nitrophenyl conjugates used as substrates: PNP α-glu and PNP β-gal dissolved in sodium acetate buffer 0.1 M, pH = 5.5\textsuperscript{29}. The substrate solution 3 mM (3 µl) was incubated with 50 µl of the intestinal homogenates and 450 µl of sodium acetate buffer at 37 °C for 10 minutes. The reaction was stopped by the addition of 130 µl of acetic acid (30%, p/v). The amount of nitrophenol released was estimated by measuring the absorbance at 405 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 405 nm.

Azocaseinase activity was determined according to Silva \textit{et al.}\textsuperscript{29}. Briefly, aliquots of 50 µl of intestinal homogenate were incubated with 450 µl of buffer solution (50 mM Tris-HCl, 20 mM CaCl$_2$, pH 8.0) and 500 µl of azocasein solution (1.5%) at 37 °C for 30 min. To stop reaction, 150 µl of TCA solution (20%) was added. The samples were centrifuged at 10,000 g for 10 min. The obtained supernatant was alkaline with NaOH (0.2 N). Soluble peptides were measured by absorbance at 440 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 440 nm.

Hemoglobinase activity was performed as described in Silva \textit{et al.}, \textsuperscript{29}. Intestinal homogenates (100 µl) were incubated with 50 µl sodium acetate buffer solution (0.2 M, pH 4.5) and hemoglobin solution (1%) at 37 °C for 60 min and then we added 100 µl of a TCA solution (40%). Samples were centrifuged at 10,000 g for 10 min and the recovered supernatant was alkaline with NaOH (2 N) solution. Soluble peptides were then measured according to Lowry \textsuperscript{64} at 750 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 750 nm. All enzymatic assays were
performed in triplicate. Gut homogenate was replaced by saline solution for blank and gut activities were expressed as activity units per gut (AU/gut).

**Molecular assays.** RNA extraction was performed using trizol reagent (Invitrogen) following the manufacturer’s procedures. RNA concentration and purity were assessed at 260 and 280 nm absorbance using a Nanodrop® spectrophotometer (Thermo Scientific). Total RNA (1µg) was treated using the RQ1RNase -Free DNase kit (Promega). Purification of the RNA was subsequently performed using the Nucleospin RNA Clean-up kit (MACHEREY-NAGEL). The synthesis of the cDNA was carried out with the Kit iScriptTM cDNA Synthesis (Bio-Rad) using 1 µg of RNA in 20 µl of reaction volume. The reaction program comprises a primer hybridization step (oligo (dT) and random) at 25 °C for 5 min, a second RT step at 46 °C for 20 min and finally inactivation of the reaction at 95 °C for 1 min.

Quantitative Real-Time PCR (qRT-PCR) analysis was performed to identify transcript changes in response to infection of selected genes, using cDNA derived from total RNA of flies samples. Five genes encoding antimicrobial peptides (AMPs), namely Ceratotoxin-A, Attacin-A, Cecropin-1, Takeout, Defensin, and two additional immune genes, namely Relish and PGRP-LC, were analyzed, as well as two reference genes, namely Gapdh and G6pdh, for the normalization (Table S1). Conventional PCR reactions were first performed for the preparation of the standard ranges (making the standard curve). The PCR products were subsequently purified using the GenElute TM PCR Clean-Up Kit (Sigma-Aldrich). The real-time quantification was performed in a CFX Connect TM Real Time System (Bio-Rad) using the MasterCycler® 480 SYBR Master I Master (ROCHE). Cycling conditions involved an initial 95°C for three minutes, 40 cycles of 10 seconds at 95 °C, 30 seconds at 57 °C and 30 seconds at 68 °C. A fluorescence reading was made at the end of each extension step. Three replicates were performed and specificity of the amplification products was assessed by melt-curve analysis. The standard curves were constructed with serial 6-fold dilutions for each AMP genes studied, ranging from 2000fg/µl to 0.2 fg/µl. PCR efficiencies were above 94% for all primer pairs (Table S1). A fluorescence reading was performed at the end of each step of extension.

**Statistical analysis.** All the parameters were statistically processed by analysis of variance with a single-factor ANOVA test followed by Dunnett test. Results are reported as means ± standard error (SE). To determine the significant enrichment of differentially expressed immune related genes, analysis was performed using multi-way Analysis of Variance (ANOVA). Furthermore, to identify variables effects (sex, maturity and mating) on immune response of Ceratitis capitata V8, R programming language was used to reveal interaction between Status, Sex, Time and Infection factors.

**Declarations**

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results”.

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**Tables**

**Table 1.** Fecundity and Fertility parameters (± SE) of *C. capitata* adults infected by *P. lilacinum* isolate.

<table>
<thead>
<tr>
<th></th>
<th>Fertility</th>
<th>Fecundity/fly</th>
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</thead>
<tbody>
<tr>
<td>C x C</td>
<td>91.7± 2.7</td>
<td>245±4</td>
</tr>
<tr>
<td>Inf x Inf</td>
<td>28.8*±4.4</td>
<td>133.5±1.08</td>
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</tbody>
</table>

(*) indicates a significant difference in the between infected adults and controls (1-way ANOVA, p<0.05).

**Table 2.** Variance Analysis table on Statut, Time, infection and infection variables interaction.
<table>
<thead>
<tr>
<th></th>
<th>Attacin-A</th>
<th>Ceratotoxin-A</th>
<th>Cecropin-1</th>
<th>Defensin</th>
<th>Relish</th>
<th>PGRP-LC</th>
<th>Takeout</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Statut</strong></td>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
<td><strong>Time</strong></td>
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<tr>
<td><strong>Infection</strong></td>
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<tr>
<td><strong>Statut x Sex</strong></td>
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<tr>
<td><strong>Statut x Time</strong></td>
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<tr>
<td><strong>Statut x Infection</strong></td>
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<td><strong>Sex x Time</strong></td>
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<tr>
<td><strong>Sex x Infection</strong></td>
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<td><strong>Time x Infection</strong></td>
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<tr>
<td><strong>Statut x Sex x Time</strong></td>
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<td><strong>Statut x Sex x Infection</strong></td>
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<td><strong>Sex x Time x Infection</strong></td>
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<tr>
<td><strong>Statut x Sex x Time x Infection</strong></td>
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</tr>
</tbody>
</table>

Signification codes. * p , 0.05, **p, ≤ 0.01, ***p, ≤ 0.001. ANOVA test.

**Figures**
Figure 1

Average amounts of lipid, glycogen and sugar in infected larvae (white columns) and control larvae (black columns) of Mediterranean fruit flies C. capitata. Bars represent standard errors. (*) indicates significant difference between columns (P<0.005).

Figure 2

Digestive disaccharide and polysaccharide activities after fungal inoculation to Ceratitis capitata (24 to 144 hours post-inoculation); Black and Gray bars represent enzymatic activities per gut for control and infected flies respectively, (---) and (…) represent enzymatic activities per mg protein for control and
infected flies respectively. (A) represent enzymatic activities on Starch; (B) represent enzymatic activities on maltose (C) represent enzymatic activities on sucrose; (D) represent enzymatic activities on Pectin. Bars represent standard errors. (*) indicates significant difference between control and infected flies (P<0.05).

Figure 3

Digestive glycosyl hydrolase activities after fungal inoculation to C. capitata (24 to 144 hours post-inoculation). Black and Gray bars represent glycosyl hydrolase activities per gut for control and infected flies respectively, ( - - - ) and ( . . . ) represent glycosyl hydrolase activities per mg protein for control and infected flies respectively. (A) β-galactosidase activities; (B) α-glucosidase activities; (C) α-galactosidase activities and (D) β-glucosidase activities. Substrates used: PNPaglu, PNPbglu, PNPagal, PNPbgal. Bars represent standard errors. (*) indicates significant difference between control and infected flies (P < 0.05).

Figure 4
Digestive proteinase activities after fungal inoculation to C. capitata (24 to 144 hours post-inoculation). (A) Dark bars represent azocaseinolytic activities per gut for control flies; gray bars represent azocaseinolytic activities per gut for infected flies; (- - -) represent azocaseinolytic activities per mg protein for control flies; (…) represent azo-caseinolytic activities per mg protein for infected flies. (B) Dark bars represent hemoglobinasic activities per gut for control flies; gray bars represent hemoglobinasic activities per gut for infected flies; (- - -) represent hemoglobinasic activities per mg protein for control flies; (…) represent hemoglobinasic activities per mg protein for infected flies. Bars represent standard errors. (*) indicates significant difference between control and infected flies (P < 0.05).

Figure 5

Characteristics of transcript levels for 7 immune genes in C. capitata inoculated with P. lilacinum. Red boxplot: females; blue boxplot: males; M.I: male inoculated; F.I: female inoculated; M.NI: male not inoculated; F.NI: female not inoculated. Each value is plotted as a dot. Boxplots represent 3 independent biological replicates, p-values are reported topleft on each panels when significant (ANOVA F-test).
Figure 6

Characteristics of transcript levels for 7 immune genes in *C. capitata* inoculated with *P. lilacinum* based on status and infection variables. (a) transcript levels of immune genes in *C. capitata* males; (b): transcript levels of immune genes in *C. capitata* females. Red boxplot: inoculated flies; blue boxplot: control flies; V.I: Virgin inoculated; A.I: Mated inoculated; Mat.I: mature inoculated; V.IN: Virgin not inoculated; A.NI: Mated not inoculated; Mat.NI: mature not inoculated. Each value is plotted as a dot. Boxplots represent 3 independent biological replicates, p-values are reported topleft on each panels when significant (ANOVA F-test).
Figure 7

Characteristics of transcript levels for 7 immune genes in C. capitata inoculated with P. lilacinum based on times variable. (A) transcript levels of immune genes in C. capitata males; (B): transcript levels of immune genes in C. capitata females. Red boxplot: inoculated flies; blue boxplot: control flies; 0.I: 0 h after inoculation; 24.I: 0 24h after inoculation; 72.I: 72h after inoculation; 144.I: 144h after inoculation. Each value is plotted as a dot. Boxplots represent 3 independent biological replicates. Each value is plotted as a dot. Boxplots represent 3 independent biological replicates, pvalues are reported topleft on each panels when significant (ANOVA F-test).

Figure 8

Characteristics of transcript levels for 7 immune genes in C. capitata inoculated with P. lilacinum. qPCR analysis of expression levels of AMPs in the (A) males and (B) Females of inoculated at 0, 24, 72 and 144 hpi (hours post inoculation). Three biological replicates were conducted. Error bars indicate SD. Asterisks above bars indicate for each gene a significant difference with untreated control (ANOVA, Tukey adjusted p < 0.05).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.doc