Zagazig University
Faculty of Science

Evaluation of Some Fungal Metabolites as Alternatives Pesticides in Control *Aphis gossypii* Glover

A Thesis **Submitted**

By

Noha Hassan Essam El-Din Metwally Lokma

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Botany Department
Faculty of Science
Zagazig University

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Summary and Conclusion

As recent approaches to select new agents of potential entomopathogens for aphid biocontrol; these trials were carried out to determine the efficacy of some natural isolates of fungi associated with aphids or found on infested plants of aphids host or plant root zone soil ; against cotton aphids, *Aphis gossypii* (Glov.),in comparable with some bio and conventional aphicides. The biological control must be widespread but chemical one must be restricted in narrow limits. Therefore, there is considerable interest in the explanation of microorganisms including entomopathogenic fungi for biocontrol of different pests.

Microbial degradation of insect cuticle components such as lipid, protein and chitin components and production of cuticle degrading enzymes particularly lipases,protease and chitinases have captured the world wide attention of biocontrol studies. But unfortunately the degradatives ability of insect cuticle fungal species have little attention and are not being completely investigate in Egypt. Therefore, the present investigation is directed to study the possible use of lipase, protease and chitinase enzyme by some local fungi. Selecting the most potent fungal species and optimization of the culture conditions to reach optimum growth as well as lipase protease and chitinase production and its potentiality to degrade cuticle. Some physicochemical properties of lipase protease and chitinase were also revealed. Moreover, the present work extends to investigate the effect of coindial suspensions and metabolites of selected organism on biological aspects of some developmental stages of cotton aphids to biocontrol this insect.

The obtained results can be summarized in the following:

1. Fungi recovered in the present investigation belonging to 3 classes namely; Zygomycetes; Ascomycetes and Deutromycetes represented as 52 isolates . Ascomycetes represented by 4 genera and the most frequent genera were *Aspergillus* represented by 22 isolates and *Penicillium* represented by 8 isolates. Deutromycetes was the most representable class according to number of genera, it comprised 8 genera *viz*., *Alternaria*; *Cephalosporium*; *Cladosporium*; *Fusarium; Humicola*; *Hypomyces*; *Stachypotrys* and *Trichoderma*. Zygomycetes represented by only one fungal genus, *Mucor* in 2 isolates.
2. Twelve fungal isolates were chosen for the further biocontrol test in this investigation. These isolates were isolated from different sources where, *Acremonium* sp.; *Cladosporium* sp.; *Humicola* sp2 and *Stachypotrys* sp1 were isolated from sandy; clay and salty soil samples, respectively. While the other isolates were isolated from insects where, *Cephalosporium* sp1; *Cephalosporium* sp2and *Trichoderma* sp1 were isolated from bees and *Humicola* sp1; *Stachypotrys* sp2 and *Trichoderma* sp2were isolated from the cotton aphids while *Chaetomium* sp. was isolated from the house fly.
3. The biological parameters of *A. gosypii* on the mean duration of immature stages (nymphal instars periods). Data as follow in mean of first, second, third and fourth nymphal instars were 1.63 ± 0.08, 2.083 ± 0.094, 1.83 ± 0.08 and 1.7 ± 0.06 days respectively. The total mean duration of nymphal instars were 7.243 ± 0.09 days.
4. The longivety of apterous (adults) was 11.25 ± 0.86. The longevity of apterous divided in to three periods, pre-parturation, parturition and post parturition periods were 1.01 ± 0.05, 8.45 ± 0.81 and 0.785 ± 0.09 respectively. The mean total longevity of *A. gossypii* (life span)

was 17.49 ± 0.86 days. The data also show the mean number off spring per female were 29.47 ± 1.94 progenies.

5. This work was extend to evaluated the efficacy of 12 selected isolated fungi against cotton aphid *A. gossypii*. Data showed that more than half of tested fungal species revealed obvious aphicidal effect expressed as mortality percentages at experimental concentration in comparison with control. Maximum inhibition of aphid growth was observed with used spore suspension of *Trichoderma* sp_2(*Trichoderma hamatum*) recording mortality percentage (65.15 %), followed by that of *Acremonium* sp_1, *Trichoderma* sp_1, *Hypomycetes* sp, *Cephalosporium* sp_2. While the minimum inhibition was observed with spore suspension of *Stachypotrys* sp_1 and sp_2, *Humicola* sp_1 and sp_2 recording mortality percentage 28.92%, 27.91%, 24.41%,and 22.12 %, respectively.

6. The effect oflatent effect of selected isolated fungi using 10^4 and 10^8 spore/ml conidial suspension compared by bio and chemical insecticides (at recommended concentrations) on biological aspects of adult stage of cotton aphids under laboratory conditions was investigated. All tested fungi using 10^4 and 10^8 spore/ml conidial suspension recorded shorter longevity period (summation of three periods, preparturation, parturation and postparturation) and less mean number of nymphal female of the tested cotton adult aphids as compared to control.

7. *Trichoderma* sp_2 (*T. hamatum*) recorded the shorter longevity period (5±2.79 and 8.31±0.09) and less mean number of nymphal female (6.33±0.03 and 16.00±1.00) between all the tested fungi, at both10^8 and 10^4 spore/ml conidial suspension, respectively. While, *Humicola* sp_1 and sp_2 and *Stachypotrys* sp_1 and sp_2 recorded the highest mean of longevity period and nymphal female number of the tested cotton

adult aphids as compared to other tested fungi at both two tested conidial suspension concentrations.

8. The biocontrol test extended to investigate the latent effect of two bionsecticides (Biovar, Bioranza) and one chemical insecticide (acetambride) by using different concentrations of them at recommended concentrations. At the two tested concentrations of the three tested insecticides, the longevity period of adult aphids was shorter than that of control. And by detecting the number of nymphal female we noted that all of them are lesser than in control.It was also, found that, the used bio and chemical insecticides were more effective than the tested fungi except *Trichoderma* sp_2 (*T. hamatum*) which also, still less effective than the chemical insecticide acetambride.

9. Since the use of chemical insecticides for controlling this insect pest is undesirable and integrated control programs necessitate the integration of several systems, it is important to investigate a new mean of pest control. So, we use a mixture of the conidial suspension of the more active isolated fungi at two concentrations $10^8$and 10^4 spores/ml and the smallest concentration (0.313g/L distilled water) of chemical insecticides, Acetambride, at which it gave mortality as control agent against *A. gossypii*. At first, we determined the mortality percentages in the cotton aphids post-treatment separately with 0.313g/L concentration of Acetambride; $10^8$and 10^4 spores/ml conidial suspension of the most active six fungal species from the previous experiment namely; *Trichoderma* sp_1, *Hypomyces* sp*; Trichoderma* sp_2*; Chaetomium* sp*; Cladosporium* spand *Cephalosporium* sp_2 after 24; 48 and 72hrs. It was observed that, Acetambride gave the highest mortality even at its smallest concentration as compared to control and other tested fungal species after 72hrs post-treatment. It was also, observed that, *Trichoderma*

sp_2 (*T. hamatum*) gave the highest mortality after 72hrs post-treatment. Then, we determined the mortality percentages in the cotton aphids after treatment with a mixture of 0.313g/L concentration of Acetambride and the conidial suspension of the most active six fungal species at two concentrations $10^8$and 10^4 spores/ml, after 24; 48 and 72hrs. It was found that, the mixture of Acetambride and conidial suspension of *Trichoderma* sp_2 (*T. hamatum*) recorded the highest mortality (52.92±2.02 and 41.43±1.03) at both tested concentrations (10^8 and 10^4 spores/ml conidial suspension), respectively after 72hrs post-treatment. Finally, we determined the Co-Toxicity factors which showed that, the combination of tested fungi at 10^8 and 10^4 spore/ml conidial suspension with 0.313g/L concentration of Acetambride revealed antagonism action not additative action.

10. From all previous results, the fungus, *Trichoderma* sp_2 were identified as *Trichoderma hamatum* and used for future investigations in this study.

11. The biochemical responses of adult *A. gossypii* expressed as total soluble protein; transaminase enzymes (glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT)) and carbohydrate hydrolyzing enzymes (amylase, trehalase and invertase) were assayed by using the same treatments used for biological studies with LC50 of Acetambride, recommended concentration of Biovarand Bioranzaand *T. hamatum* conidial suspension alone or mixed with the chemical insecticide Acetambride (at lower concentration give mortality ratio, the biochemical responses to these treatments were applied on adult. These attributes measured at the intervals of 48, 72 and 96 hours after treatments.

- It was found that, all tested treatments showed a disturbance in the total soluble protein of the adult cotton aphids. All treatments caused a decrease in total soluble protein of the adult cotton aphids after 96hrs post-treatment as compared to control (untreated aphids). It was also observed that, each of acetambride+ *T. hamatum* conidial suspension (10^8 spore/ml); *T. hamatum* conidial suspension (10^8 spore/ml) and biovar caused an increase in total soluble protein of the adult cotton aphids after both 48 and 72hrs post-treatment, while, acetambride and bioranza caused a decrease in its amount after the same time intervals as compared to control.
- All treatments caused a decrease in GPT relative activity of adult *A. gossypii* after 72hrs post- treatment, as compared to control. Then the GPT relative activities started to increase again after 96hrs post-treatment in case of acetambride+ *T. hamatum* conidial suspension; *T. hamatum* conidial suspension; bioranza and biovar in increase percents 78.25; 108.04; 97.25 and 101.01%, respectively as compared to control except in case of acetambride the GPT disappeared after this time (100% decrease percent).
- All treatments caused an increase in the relative activity of adult *A. gossypii* GOT after 48hrs post-treatment as compared to control but after this time it was found that, there was a disturbance in GOT relative activities with these treatments where, acetambride caused a decrease in GOT relative activity as 55.93% decreasing percent after 72hrs post-treatment, while GOT disappeared after 96hrs post-treatment as compared to control. It was also, observed that, each of acetambride+ *T. hamatum* conidial suspension; *T. hamatum* conidial suspension and bioranza caused a decrease in GOT amounts in 4.24; 5.46 and 16.25% decreasing percentages after 96hrs post-treatment as compared to control. On the other hand

biovar still causing an increase in GOT amount as 18.63% increasing percent after 96hrs post-treatment as compared to control.

- All treatments caused an increase in adult cotton aphid amylase relative activity after 48hrs post-treatment then caused a decrease in its relative activity after 72hrs post-treatment, as compared to control. It was also, observed that the adult cotton aphid amylase relative activity started to increase again after 96hrs post-treatment in 298.17; 327.96; 317.17 and 271.13% an increasing percentages in case of acetambride; acetambride+ *T. hamatum* conidial suspension; *T. hamatum* conidial suspension and bioranza, respectively as compared to control (untreated aphids) while the amylase enzyme disappeared in case of biovar treatment after 96hrs post-treatment.
- In general all treatments caused a decrease in *A. gossypii* trehalase enzyme relative activity after 48 and 72hrs post-treatment as compared to control. It was also, observed that, the trehalase enzyme disappeared in case of acetambride; acetambride+ *T. hamatum* conidial suspension and bioranza treatments while in case of *T. hamatum* conidial suspension and biovar treatments, it started to increase in 39.24 and 51.93% increasing percentages, respectively after 96hrs post-treatment as compared to control.
- Acetambride; acetambride+ *T. hamatum* conidial suspension; *T. hamatum* conidial suspension; bioranza and biovar treatments of adult *A. gossypii* caused a decrease in invertase enzyme relative activity in a decreasing percentages 63.74; 26.58; 5.55; 58.41 and 76.56%, respectively after 72hrs post-treatment as compared to control, but the adult aphid invertase relative activity started to increase in increasing in case of *T. hamatum* conidial suspension;

bioranza and biovar treatments but, disappeared in case of acetambride and acetambride+ *T. hamatum* conidial suspension treatments after 96hrs post-treatment

12. The effect of some environmental and nutritional factors on mycelial growth and lipase, protease and chitinase activity of *T. hamatum* were investigated.

- 7 days, 6 days and 5 days were the optimum incubation periods, for lipase, protease and chitinase, respectively.
- Initial pH5 was found to be optimal for lipase and chitinase activity while pH8 was found to be optimal for protease activity and growth of *T. hamatum*.
- the incubation temperature 30ºC was the optimum for lipase and protease while 25ºC was the optimum for chitinase.
- The optimum output of lipase, protease, chitinase and biomass of tested organism were recorded on using 50 ml of fermentation medium dispensed in 250 ml fermentation flask (1/5 v/v).
- The emulsified olive oil (the carbon source of the basal medium) was the most favorable carbon source for lipase activity of *T. hamatum*. While glucose was the best carbon source for biomass production. And the best concentration of olive oil for *T. hamatum* was 6.0%.
- Glucose was the best carbon source for biomass yield and protease activity of tested fungus and the best concentration of glucose for *T. hamatum* was 2.0%.
- Colloidal chitin (the carbon source of the basal media) was the best carbon source for biomass yield and chitinase activity of tested fungus and the best concentration of colloidal chitin for *T. hamatum* was 1%.

- Peptone, casein and yeast extract were the best nitrogen sources in the case of lipase, protease and chitinase, respectively. And 0.5 % peptone; 1.50 % casein and 0.5 % of yeast extract were the best concentrations.
- |The concentrations 1.5, 2 and 1 g/ L of KH_2PO_4 were the best phosphorous source in the fermentation medium, for lipase, protease and chitinase, respectively. The optimum KCl (0.025, 0.075 and 0.05) for lipase protease and chitinase, respectively. And 0.075%, 0.075% and 0.05% (w/v) $MgSO_4.7H_2O$ for lipase, protease and chitinase medium, respectively.
- The addition of 10 mg/ L of $FeSO_4.7H_{2O}$ and $ZnSo_4.7H_2O$,$Cocl_2$ to the experimental medium had stimulatory effect in lipase, protease, chitinase, and biomass production by *T. hamatum*. However, the supplementation of $CuSo_4.5H_2O$ and $Mncl_2$ and to the tested culture medium led to decrease in biomass, lipase, protease and chitinase production of *T. hamatum* led to decrease in biomass, lipase, protease and chitinase activities of the experimental organism. On the other hand, the addition of NaCl and $CaCl_2$ to the fermentation medium give enzymes as well as biomass yields relatively as the same in the case of control (basal medium without micro elements).

13. When the protein fractions of *T. hamatum* salted out at different ammonium sulphate ratio of reactions, the highest fraction activity for each enzyme was salted out at 60% ammonium sulfate (for lipase) and 70% ammonium sulfate (for both protease and chitinase) and showed highest recovered activity as well as relatively high protein content,also ammonium sulphate saturation showed that the highest specific activity of *T. hamatum* lipase, protease, chitinase reaching

(1.48 U/mg/m, 7.43 U/mg /m, 1.34, U/ mg protein., respectively were higher than that of the crude enzyme preparation.

14. Some physical chemical properties as well as enzymatic reaction conditions of crude lipase, protease and chitinase of *T. hamatum* were studied.

- The optimum temperature of the enzymatic reaction of *T. hamatum* lipase, protease and chitinase will be assayed at 40ºC, 40ºC and 50ºC, respectively. However, the lipase, protease and chitinase retained about 77.90, 59.13 and 21.47% reduction in its activity respectively after heating at 60ºC for 30 minutes and almost lost their original activity as heated at the same temperature for 120 min.
- pH6, pH8 and pH5 can be considered as the optimum pH values for lipase, protease and chitinase activity respectively. However, the data appeared that, there were more than 92.34, 91.57, 76.57 and 51.40, 12.43, 80.92% loss in their activities in 2hrs at pH3.0 and pH 9.0 for lipase, protease and chitinase respectively.
- The optimum reaction time for crude lipase, protease and chitinase is 60, 90 and 90 minutes, respectively.
- 1ml (0.19 mg protein / reaction mixture) and 1.5 ml (0.18 mg / protein / reaction mixture) and 1.5 ml (0.14 mg protein / reaction mixture) were the optimum for lipase, protease and chitinase, respectively.
- The protease, lipase and chitinase enzymes had high affinity and catalytic activity for casein**,** olive oil and chitin respectively (km 0.27, 0.14 and 1.43 mM, Vmax 1.26, 0.137 and 0.09U/mg/min, respectively.

- Lipase was activated by addition of $Znso_4.7H20$, $CoCl_2$, $MnCl_2$, $MgSO_4.7H_2O$. While protease and chitinase activities were increased by addition of first for protease $MgSO_4.7H_20$, $CaCl_2$, KCl, $Cuso_4.5H_20$ and second for chitinase $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSo_4.7H_2O$ were increase the activity of chitinase. However, some other tested substances such as $FeSo_4.7H_2O$, Nacl, $Cacl_2$, Kcl, $CusSo_4.5H_2O$ for lipase and $ZnSO_4.7H_2O$, $CoCl_2$, Nacl, $MnCl_2$,EDTA, $Feso_4.7H_2O$ for protease and $cocl_2$, $CaCl_2$, Nacl, Kcl, $Mncl_2$ for chitinase causing inhibitory effect on lipase, protease and chitinase enzymes activities that showing decreasing in its ratio of the original activity (control) respectively. On the other hand, the crude, lipase, protease and chitinase were completely inactivated by Hg^{2+} after incubation period for each enzymes. The opposite were also true for Na^{2+}, K^{2+} and Co^{2+} ions which n't significantly for tested *T. hamatum* enzymes.

15. Protease enzyme produced by *T. hamatum* was subjected to purificational steps by applying fractional precipitation by ammonium sulphate saturation level, dialyzation against tap water and sucrose as well as applying on column chromatography technique containing firstly Sephadex G_{-100} and socondly $G_{50.}$

16. Enzyme activity, protein content and specific enzyme activity were 42.07 (U/ml), 0.42 (mg/ml) and 3.34 (U/mg protein/m), respectively. Ammonium sulphate was added to CFF in order to achieve 70 % saturation levels. the purity of protease enzyme increased by 2.22 fold.

17. The most active protease enzyme fractions previously obtained at 70% ammonium sulphate saturation was dialyzed against tap water followed by sucrose until a constant volume achieved.

18. Two active peak was obtained at fraction No.(26, 34) showed highest specific activity(8.71,9.19) U/mg/m protein) and the purity of protease increased by 2.61 and 2.75 fold respectively.

19. Second purification was achieved by using sephadex G50, a very active peak was obtained at fraction No.(28,35).

20. SDS-polyacrylamide electrophoresis was applied to examine the efficiency of the purification steps, using the SDS-PAGE approach, the two sharp fraction protease (proteaseI and proteaseII) that recovered from gel filteration, were fractionated as two distinctive bands, for protease enzyme. The protein profile show that, the molecular weight of proteaseI was 31.101 kDa while the proteaseII has molecular weight 34.622 kDa.

21. The biocontrol potential of *T. hamatum* conidial suspension and its proteases (I and II) against the adult cotton aphids as compared to two commercial bioinsecticides (Biovar and Bioranza) was studied. Each of biovar and bioranza were more effective against *A. gossypii*than *T. hamatum* conidial suspension and proteases (I and II) as biocontrol agents.

22. The toxicity index values at LC_{50} revealed that the tested fungus conidial suspension and proteases (I and II) had less effect on *A. gossypii* than the two commercial bioinsecticides. The results of tolerance rate of*A. gossypii* as response to tested materials revealed that the tested aphids were more susceptible to Biovar, at LC_{50}, followed by Bioranza and (10^2,10^3,10^4 and 10^8 spores/ml) concentrations of conidial suspension of *T. hamatum* with 35.89 fold of most potent compound, Biovar. Also, LC_{90} and both toxicity index and tolerant rate at $LC_{90,}$ the tested compounds recorded the same trend at the LC_{50}. So, it could be concluded that the cotton aphid was

more tolerant to natural isolate, *T. hamatum* and its proteases (I and II) than the two commercial bioinsecticides.

23. The effect of highly two concentrations with highly mortality percentages of tested fungal species (*T. hamatum*) and its proteases I , and Π enzymes on longevity of adults and number of nymphs of tested insect was investigated. The mean longevity of tested aphids at 10^8 and 10^4 spore/ml, were shorter than control.

24. *T. hamatum* conidialsuspensionhave significant effect on the number of off spring compared to the experimental control value. The number of nymphs were 13.87 and 19.17 lesser than control 28.67, respectively.

25. The biocontrol test extended to investigate the latent effect of purified *T. hamatum* proteases (I and Π) on longevity and number of nymphs of adult tested aphids during life span using two concentrations (2.5 and 5.0 ml/Petri dish). Values of the mean longevity periods in all experimental treatments were shorter than control. Also, the results showed that, the purifiedproteases (I and Π) treatments have latent effect at 0.5 ml/Petri dish more than at 2.5 ml concentration compared to control on the longevity as well as the number of offsprings.

26. The microscopic examination (light and electron microscopy) of adult *A. gossypii* in this study revealed that the*T. hamatum* spore suspension brought about massive disintegration and deformation of the aphid's body and tissues. Also,it revealed the development and the colonization of the fungus inside the insect.

- The light and transmission electron microscope examination of untreated and treated adult cotton aphid showing different histological changes between them. The untreated adult cotton aphid semi-thin section showed the normal structure of the aphid's body with normal intact cuticle which clearly differentiated into

epicuticle and endocuticle layers. Normal adipose tissue (fatty tissue beneath the cuticle); normal gut epithelial cells surrounding the lumen gut; normal entire salivary gland surrounded by its membrane and normal ovary tissue with embryo were also observed. The basement membrane that surrounded the all insect organs in the haemcoel of the insect also appeared normal and intact.

- The treated adult aphid semi-thin section showing many abnormalities in the insect's body. There is a disorganization of the cuticle which appeared not differentiated into its epicuticle and endocuticle layers and become black-spotted due to fungal invasion as compared to the untreated one. It was found that the treatment of adult *Aphiss gossypii* with *T. hamatum* spore suspension resulted in: lyses of the ovarition follicles in the ovary; deformation of hind gut epithelial cells and the lumen area under these disintegrated epithelial cells became wider as compared to the untreated insect and deformation of the salivary gland which appeared losing its surrounding membrane. Also, several vacuoles were found inside the insect tissues and located the adipose tissue. The adipose tissue was totally occupied by the fungus hyphae and spores. It was observed that the fungus was able to penetrate the insect cuticle and passed through the subcutaneous muscle which appeared deformed and disintegrated. The adipose tissue which appeared disintegrated and more vacuolated. It was seen that the adipose tissue; lumen and other disintegrated insect structures and organelles were totally occupied by the fungus hyphal growth; yeast-like hyphal bodies and spores.