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Sub-Lethal Effects of *Lecanicillium lecanii* (Zimmermann)-Derived Partially Purified Protein and Its Potential Implication in Cotton (*Gossypium hirsutum* L.) Defense against *Bemisia tabaci* Gennadius (Aleyrodidae: Hemiptera)

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Abstract: Whiteflies, *Bemisia tabaci* Gennadius (Aleyrodidae: Hemiptera), are a polyphagous economically destructive pest of several solanaceous crops around the world. Many secondary metabolites are synthesized by different biotrophic and necrotrophic fungi which are capable of inducing systemic resistance in plants against various phytophagous pests. The present laboratory work demonstrated the anti-insect impact of a protein extracted and purified partially from an entomopathogenic fungus (EPF) *Lecanicillium lecanii* (Zimmermann) against *B. tabaci*. Three different concentrations (i.e., 7.43, 11.15, and 22.31 $\mu\text{g mL}^{-1}$) of this protein were bioassayed to assess its effect on the fecundity rate of *B. tabaci* on cotton (*Gossypium hirsutum* L.) plants. Furthermore, the possible implication of this fungal protein in defense pathways of cotton plants was evaluated by determining the expression profiles of salicylic acid (SA) and jasmonic acid (JA) pathways related to major genes through reverse transcription qPCR (RT-qPCR). According to the results, all protein concentrations exerted a significant ($F_{3, 252} = 62.51$; $p \leq 0.001$) and negative impact on the fecundity rate of *B. tabaci* females. At the highest protein concentration (22.31 $\mu\text{g mL}^{-1}$), the minimum rate of fecundity (i.e., 2.46 eggs female⁻¹ day⁻¹) of *B. tabaci* was noted on the seventh day, whereas fecundity rates for the other two protein concentrations (i.e., 11.15 and 7.43 $\mu\text{g mL}^{-1}$) were, respectively, 3.06 and 3.90 eggs day⁻¹ female⁻¹. The maximum rate of fecundity (6.01 eggs female⁻¹ day⁻¹) was recorded in untreated (control) treatments. In addition, the foliar application of *L. lecanii* derived protein significantly upregulated all SA linked genes (OPR3, PPO1 and COI1) and slightly triggered up the JA linked genes (LOX1, UBQ7 and AOS) in the cotton plants. These findings revealed that this *L. lecanii* extracted partially purified protein triggered systemic resistance against *B. tabaci* in the cotton plants, proposing its

putative effectiveness as an innovative biological control technique against *B. tabaci* and other phloem-feeding hemipteran pests. Nevertheless, further investigations such as purification and molecular and functional characterization of this *L. lecanii*-derived partially purified protein are required.

Keywords: *Lecanicillium lecanii*; induced resistance; *Gossypium hirsutum*; *Bemisia tabaci*; fecundity; salicylic acid pathway; jasmonic acid pathway

1. Introduction

Sweet potato whitefly, *Bemisia tabaci* Gennadius (Aleyrodidae: Hemiptera), is a highly destructive pest of various agronomic crops, ornamental plants, and vegetables in tropical and subtropical regions [1]. Adults and nymphs of *B. tabaci* damage the plants either by direct sap-sucking and/or indirectly by disrupting normal plant growth and photosynthetic activities and by vectoring different plant pathogens [2]. Combatting *B. tabaci* infestations is primarily dependent on the extensive applications of synthetic insecticides [3] which have caused the development of pesticide resistance in different field populations of *B. tabaci* [4,5]. Moreover, deleterious impacts on other beneficial organisms and problems of environmental contaminations are often associated with chemical insecticides usage [6].

Previous investigations have focused on finding natural compounds from microbes such as entomopathogenic fungi (EPFs) which can serve as alternatives to synthetic pesticides for pest management [7]. EPFs play a significant role as biocontrol agents due to their high host specificity, pathogenicity, and capability to control various insect pests [8–10]. *Lecanicillium lecanii* (= *Verticillium lecanii*) is widely used as an effective biocontrol tool against different sap-sucking and chewing insect pests including *B. tabaci*, the Asian citrus psyllid (*Diaphorina citri*), and the diamondback moth (*Plutella xylostella*) [11–14]. Moreover, EPFs produce several secondary metabolites [15], many of which are biologically active molecules (e.g., depsipeptides and non-ribosomal peptides) with insecticidal, cytotoxic, antiviral, antifungal, anti-proliferative, and immune-suppressant modes of action [16–18]. The crude proteins isolated from *Metarhizium anisopliae*, *Isaria fumosorosea*, and *Beauveria bassiana* produced considerable mortality in cotton leafworms (*Spodoptera littoralis*) [7] and houseflies (*Musca domestica*) [19]. Recently, the infectivity of conidia (spores) and toxicity of culture filtrates of various strains of EPFs *B. bassiana* and *L. lecanii* have been revealed against *Myzus persicae* (green peach aphid) and *B. tabaci* [20–23].

Plant defense against biotic and abiotic stress relies on an array of small endogenous molecules such as salicylic acid (SA) and jasmonic acid (JA). Both of these phytohormones play a vital role in plant defense signaling network. These are the key signaling molecules that are implicated in the mechanisms of plant defense [24]. SA triggers natural biological defense responses in plants such as systemic acquired resistance (SAR) against pathogens, while JA induces plant resistance against insects and necrotrophic pathogens by producing different plant defense compounds such as proteinases and secondary metabolites [24,25]. Although both SA- and JA-related defensive mechanisms are activated in plants in response to different herbivore pests and plant pathogens, JA reaction is primarily related to chewing insects such as lepidopterans [25,26] while the SA pathway is induced by phloem-feeding insect pests such as aphids and whiteflies [27–29].

Taking into account these innovative aspects of entomopathogenic fungi and their secondary metabolites regarding insect pest management, this laboratory work was aimed evaluating the biological action of protein extracted and purified partially from the EPF *L. lecanii* against *B. tabaci* on the plants of cotton (*Gossypium hirsutum* L.). Moreover, the relative expression profiles of major genes possibly involved in SA- and JA-associated plant defence mechanisms were measured through RT-qPCR to investigate the potential implication of this protein extracted from the EPF *L. lecanii* in inducing systemic resistance against *B. tabaci* in cotton plants.

2. Materials and Methods

2.1. Cultures of Plants and Insects

Seeds of cotton were placed in a Petri-plate over a damp filter paper for six to eight days. Later, vigorously germinating seedlings of uniform appearance were transferred to the individual plastic containers ($\phi = 10$ cm) in sterile soil mix and these plastic containers were retained inside an automated growth chamber at 24–30 °C: 20–24 °C (day: night) temperature and 55–65% relative humidity (RH). The adults of *B. tabaci* were captured from the infested plants of cotton growing at the Langfang Research Station, Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. *B. tabaci* population was raised on the cotton plants grown in the plastic containers kept in insect rearing cages at 24 ± 2 °C temperature, 55–65% relative humidity (RH) and 8 h:16 h (D:L) photoperiod. Throughout the study period, cotton plants were exchanged weekly with new ones.

2.2. Culture of *L. lecanii*

Isolated spores of EPF *L. lecanii*, preserved at -80 °C in 20% glycerol, were provided by the State Key Laboratory for Biology of Plant Diseases and Insect Pests (IPP, CAAS, Beijing, China). *L. lecanii* initial culture was prepared growing the above-mentioned fungal spores for 25 days at 25 ± 2 °C in darkness conditions on the potato dextrose agar (PDA) medium (containing potato, dextrose and agar @ 200, 20 and 20 g L⁻¹, respectively) within sterilized glass Petri-plates ($\phi = 90$ mm).

2.3. Extraction and Partial Purification of Crude Protein

For *L. lecanii*-derived crude protein extraction and purification, a previously described method was followed [30]. To prepare the primary culture of *L. lecanii*, 3 mL of the suspension of *L. lecanii* conidia (@ 1×10^8 conidia mL⁻¹) was mixed with 150 mL of PDB (potato dextrose broth) and was incubated for 72 h on a rotatory shaker set at 160 rpm and 25 °C. After that, 25 mL of the primary culture was added in 2.5 L of PDB to prepare the secondary culture which was prepared on a shaker for five days at 200 rpm and 25 °C. Then, after its centrifugation for 40 min at 4 °C and 12,000 rpm, the supernatants were filtered out by a 0.45 μ m membrane filter (Millipore Corporation, Bedford, MA, USA). Then, protein purification system ÄKTA Explorer 10 (GE Healthcare, Waukesha, WI, USA) was used for partial purification of the *L. lecanii* extracted protein. This purification system was based on a 5 mL chromatography column (HiTrap Q HP, GE Healthcare, Uppsala, Sweden). Before purification, this column was calibrated with 50 mM Tris-HCl maintained at pH 8.0 (buffer A) provided by manufacturer. The samples of extracted crude proteins were loaded in the column (@ 2 mL min⁻¹ flow rate). The columns were then cleaned by 50 mM Tris-HCl (buffer A) and proteins bounded within chromatography column were eluted by loading 1 M NaCl and 50 mM Tris-HCl maintained at pH 8.0 (buffer B) at a flow rate of 2 mL min⁻¹. Desalting column (GE Healthcare, Sweden) was utilized to eliminate the salt contents from the eluted proteins. The desalted fractions of *L. lecanii*-extracted partially purified protein was further analyzed by running it on electrophoresis using 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Finally, the eluted protein was preserved at -20 °C in a freezer until further use to conduct the bioassays. The protein concentrations were quantified by the Bicinchoninic Acid Protein Assay (BCA) kit (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Protein Sub-Lethal Activity Bioassay against *B. tabaci*

Laboratory bioassays were carried out for assessing the biological activity of *L. lecanii*-derived partially purified protein against *B. tabaci*. Treatments included three protein concentrations (i.e., 22.31, 11.15, 7.43 μ g mL⁻¹) and one control (Buffer A). Potted cotton plants were used at a five-leaf stage in the bioassays. About 3–4 mL of partially purified protein was sprayed on every plant by a manually operated sprayer bottles. Then, these treated plants were permitted to be drained at 25 °C (room temperature) for 24 h. For

assessing *B. tabaci* fecundity rate, a couple of active and healthy *B. tabaci* adult/plant was restricted on the leaf of potted cotton plant using leaf clip cages. The number of *B. tabaci* eggs/female laid in these clip cages was recorded for the seven consecutive days. For each treatment, 10 independent replications were set. All bioassays were executed at $60 \pm 5\%$ RH and 26 ± 1 °C.

2.5. Extraction of RNA and cDNA Synthesis

Using Plant RNA EasyPure[®] Kit (TransGen Biotech Co. Ltd., Beijing, China), total RNAs were extracted out from the leaf tissues of cotton plants pre-infested with *B. tabaci* and exogenously treated by protein extracted from *L. lecanii* ($@ 22.31 \mu\text{g mL}^{-1}$), and from the leaves of buffer-treated control cotton plants. Extracted RNAs were quantified with NanoPhotometer[®] (NP80 Touch; Implen Inc., Westlake Village, CA, USA). The cDNA was synthesized from the extracted RNA using TransScript[®] One-Step gDNA Removal and cDNA Synthesis Super Mix kit (Beijing, China).

2.6. Reverse-Transcription Quantitative PCR

Relative expression profiles of plant defense mechanisms associated genes were assessed in control (buffer-treated) plants and in protein-treated (pre-infested by *B. tabaci*) plants using reverse-transcription quantitative PCR (RT-qPCR). The genes quantified in this study were OPR3, PPO1, and COI1 of the SA pathway and LOX1, UBQ7 and AOS of the JA pathway. Internal control was comprised of β -actin as reference gene. Nucleotide sequences used as primers for these targeted gene amplifications are detailed in Table 1 and the amplifications were carried out using real-time thermos-cycling PCR System (ABI 7500; Applied Biosystems, Foster City, CA, USA). Twenty microliter of reaction mixture for each sample was consisted of 7 μL of ddH₂O, 0.5 μL of each of the forward and reverse primers, 10 μL of SYBR[®] (2 \times) Premix Ex Taq[®] II (Takara Bio Co. Ltd., Dalian, China) and 2 μL of cDNA template. All qPCR amplifications were carried out using the following thermal protocol i.e., 95 °C for 30 s, 40 cycles with a denaturation, annealing, and extension at 94 °C for 35 s, 60 °C for 41 s and 72 °C for 60 s, respectively. Three independent technical and three independent biological replications were maintained for each sample.

Table 1. Sequences of primer pairs used for RT-qPCR amplifications of major genes possibly implicated in JA- and SA-associated plant defense pathways.

Genes	Forward Primer	Reverse Primer
β -ACTIN	GATTCCGTTGTCCAGAAGTCCT	TACGGTCTGCAATACCAGGGA
OPR3	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT
PPO1	GGTGATAAGCGACAGCCATT	GGAGTGATGAGCGAAGAACA
COI1	ACCACTGCTTGTTAATCCA	CACCTTCTTCATCCTCCATT
LOX1	GCCAAGGAGAGCTTCAAGAAT	TAGGGGTACTTGCGAGAACCT
UBQ7	GAATGTGGCGCCGGGACCTTC	ACTCAATCCCCACCAGCCTTCTGG
AOS	AGGATTACCAGAGACTGTATGAT	ATGCCACCGAATGAATTGAA

2.7. Statistical Analysis

Data were analyzed statistically using Statistix[®] software (V. 8.1; Analytical Software, Tallahassee, FL, USA). All bioassays and determinations were performed in three independent replications. All data values were represented as means with standard errors. One-way factorial analysis of variance (ANOVA) was performed to determine the effect of treatments (time and concentrations) and the treatment means were compared using Fisher's least significant difference (LSD) post-hoc test at standard (5%) probability level. Comparative CT method ($2^{-\Delta\Delta\text{CT}}$) was utilized for determining the relative expression levels of RT-qPCR. Furthermore, data of buffer-treated control and protein-treated plant samples were compared by Student's paired *t*-test ($p \leq 0.05$).

3. Results

3.1. Necrosis Induced in *Nicotiana tabacum* Leaves by the Protein Derived from *L. lecanii*

L. lecanii-derived crude protein was partially purified using ion-exchange chromatography. Finally eluted protein fractions ($44.62 \mu\text{g mL}^{-1}$) were desalted and infiltrated into the *N. tabacum* leaves to evaluate the bioactivity of this partially purified protein. Noticeable necrotic regions became visible on the tobacco leaf areas treated with the partially purified protein after 24 h of infiltration (Figure 1A). Later, SDS-PAGE analysis resulted in 2 clear bands of proteins with molecular weights of 33 and 44 kDa (Figure 1B).

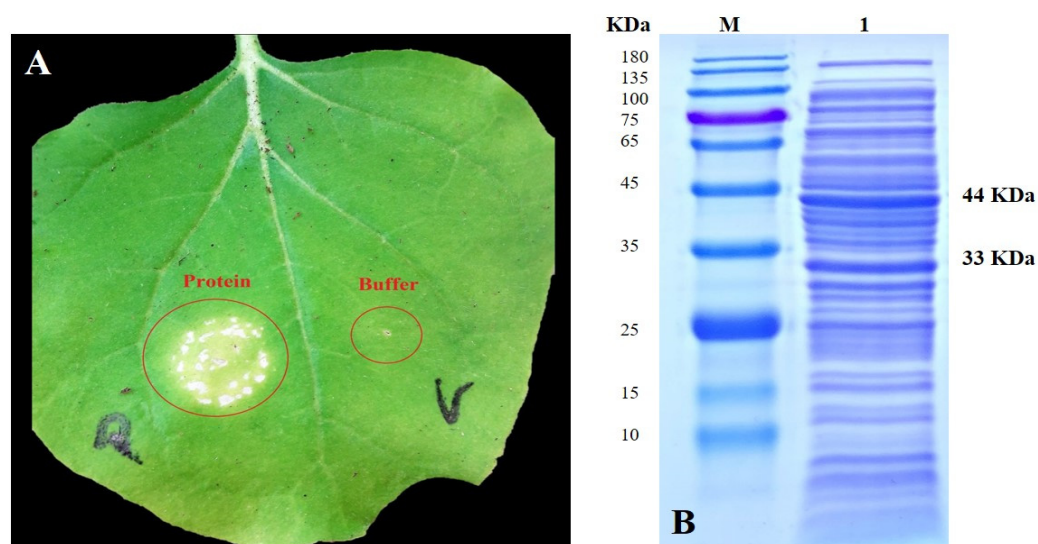


Figure 1. Ion-exchange chromatography exhibited partially purified protein derived by 80% ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$); (A) the necrotic blotch produced by the *Lecanicillium lecanii*-derived protein ($44.62 \mu\text{g mL}^{-1}$) on *Nicotiana tabacum* leaves photographed 24 h post treatment. In the control, *N. tabacum* leaves were treated by the 50 mM Tris-HCL with pH 8.0 (mock buffer); (B) the *L. lecanii*-derived proteins purified by the chromatography column on a 12% SDS-PAGE. Lane M indicates the protein molecular weight (kilodaltons) marker.

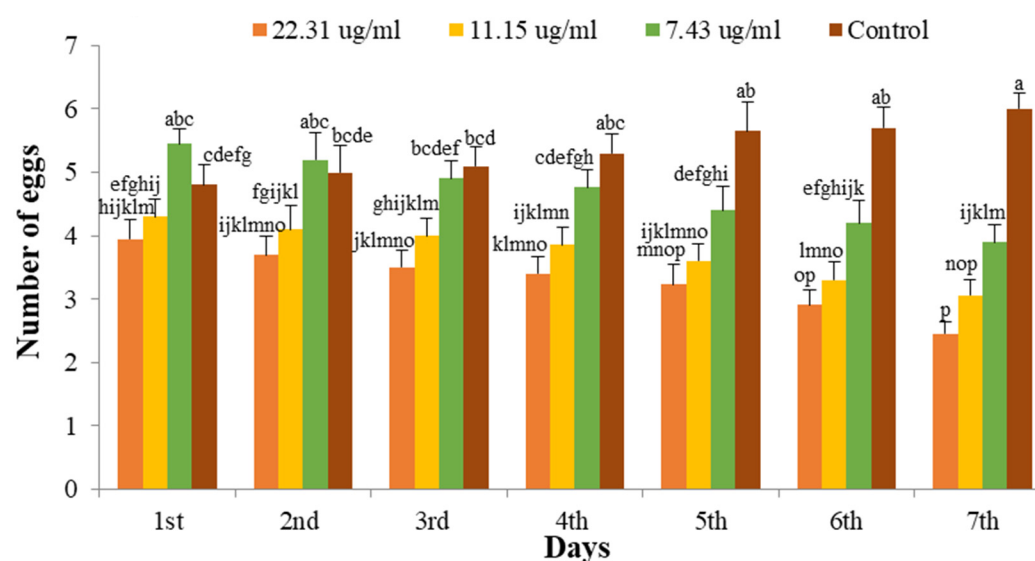
3.2. Impact of Protein Derived from *L. lecanii* on the Fecundity of *B. tabaci*

Bioassays demonstrated a significant impact of the protein extracted from *L. lecanii* on the fecundity rate of *B. tabaci*. The mean fecundity rate of *B. tabaci* compared to the control was inversely associated with the protein concentration. Both factors (i.e., protein concentration ($F_{3, 252} = 62.51$; $p \leq 0.001$) and time ($F_{6, 252} = 2.94$; $p \leq 0.05$)) and their interaction ($F_{18, 252} = 2.24$; $p \leq 0.05$) exerted a significant effect on *B. tabaci* fecundity (cf. Table 2). Minimum fecundity rate (i.e., $2.46 \text{ eggs female}^{-1} \text{ day}^{-1}$) was recorded on the seventh day for the highest concentration ($22.31 \mu\text{g mL}^{-1}$) of protein, while the maximum rate of fecundity (i.e., $6.01 \text{ eggs female}^{-1} \text{ day}^{-1}$) was noted in untreated (control) treatment on the seventh day. Mean fecundity rate recorded for the medium ($11.15 \mu\text{g mL}^{-1}$) and lowest ($7.43 \mu\text{g mL}^{-1}$) protein concentrations were 3.90 and $3.06 \text{ eggs female}^{-1} \text{ day}^{-1}$, respectively (cf. Figure 2).

Table 2. Factorial ANOVA regarding the influence of *Lecanicillium lecanii*-derived partially purified protein on *Bemisia tabaci* fecundity rate (cf. Figure 2).

Source of Variance	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Value	p-Value *
Concentration	3	181.197	60.3990	62.51	<0.001
Time	6	17.043	2.8405	2.94	<0.05
Concentration × time	18	39.008	2.1671	2.24	<0.05
Error	252	243.504	0.9663		
Total	279	480.751			
Grand mean/Coefficient of variation				4.2786/22.97	

* $p < 0.05$ = significant and $p < 0.001$ = highly significant (at $p \leq 0.05$).

**Figure 2.** Mean rate of fecundity (\pm SE; $n = 10$) of *Bemisia tabaci* treated with different concentrations of a protein extracted and partially purified from EPF *Lecanicillium lecanii*. Treatment bars with dissimilar letters differ significantly from each other (one-way ANOVA; LSD post-hoc test ($p \leq 0.05$)) (cf. Table 2).

3.3. Impact of *L. lecanii*-Derived Protein on Expressions of SA and JA Pathway Related Genes

For evaluating the potential impact of *L. lecanii*-derived protein in inducing systemic resistance against *B. tabaci* in cotton plants, the levels of expression of major genes linked with the SA and JA pathways were assessed. The results of this RT-qPCR analysis showed that all genes (i.e., OPR3, PPO1 and COI1) of SA pathway were upregulated significantly ($p = 0.05$) at all of the measured time intervals (i.e., at 12, 24, 48, and 60 h after exposure to *B. tabaci* (cf. Figure 3)), whereas the expression levels of three other important genes related to JA pathway (i.e., LOX1, UBQ7 and AOS) were slightly triggered up (cf. Figure 4). Moreover, all of the targeted genes exhibited their maximum expression levels at 48 h post *B. tabaci* infestation.

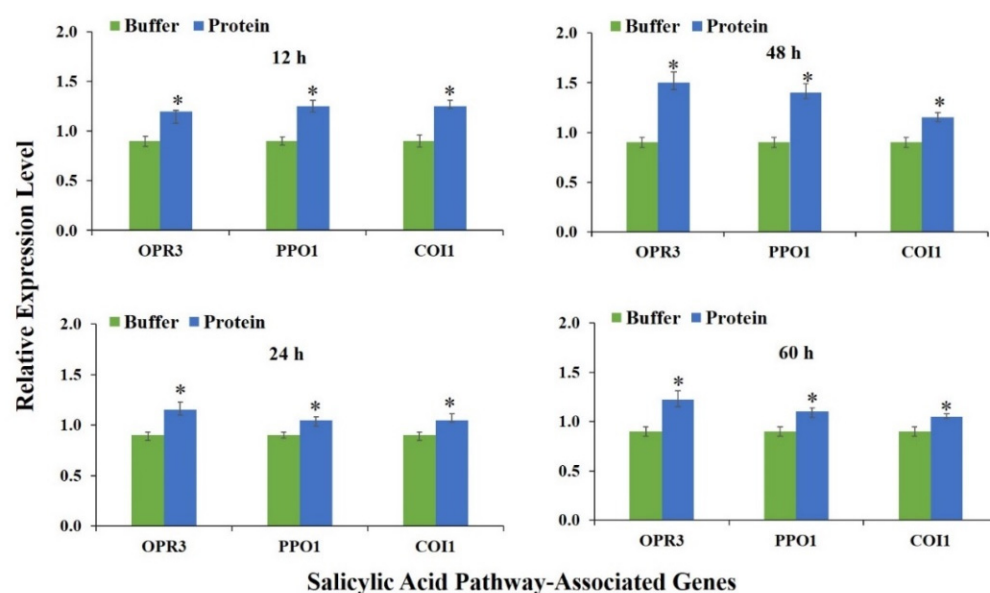


Figure 3. The profiles of relative expression of salicylic acid (SA) plant defense pathway related major genes assessed in the plant leaves treated with a partially purified protein derived from EPF *Lecanicillium lecanii* at various post-treatment time intervals. Green and blue colored columns represent respectively the results of buffer-treated (control) and protein-treated plants. For each target gene, asterisk shows statistically significant change in both of the treatments (Student's paired t -test; $p \leq 0.05$).

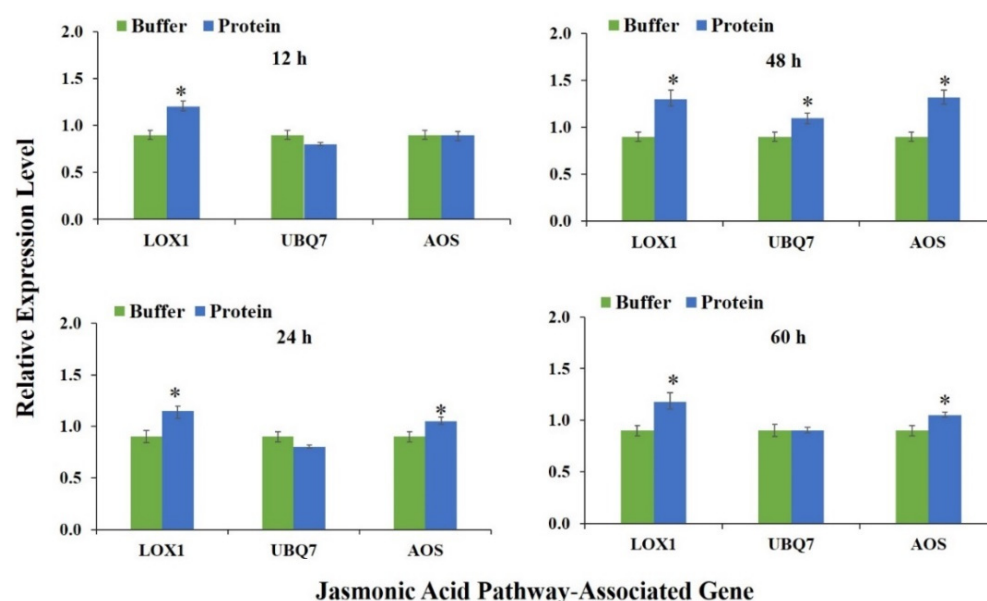


Figure 4. The profiles of relative expression of Jasmonic acid (JA) plant defense pathway related genes assessed in the plant leaves treated with a partially purified protein derived from EPF *Lecanicillium lecanii* at various post-treatment time intervals. Green and blue colored columns represent respectively the results of buffer-treated (control) and protein-treated plants. For each target gene, asterisk shows statistically significant change in both of the treatments (Student's paired t -test; $p \leq 0.05$).

4. Discussion

Many isolates of EPFs produce several proteins which cause induced plant resistance against different insect pests and plant pathogens [31]. This laboratory work was performed in order to evaluate the impact of a protein extracted and partially purified from EPF *L. lecanii* against *B. tabaci*. Usually, EPFs particularly *L. lecanii* produces extracellular pathogenesis-related proteins or insecticidal metabolites such as cyclodepsipeptide

(bassianolide), chitinases, etc. [14,32,33]. In our study, *L. lecanii*-derived proteins would most probably be chitinases corresponding to 44 and 33 kDa [33,34]. Compared to buffer-treated control plants, cotton plants treated exogenously by the foliar application of *L. lecanii* protein exhibited considerable reduction of fecundity rate of *B. tabaci*. Our results substantiate the findings of some previous studies [30–35] who demonstrated the sub-lethal activities of different fungal proteins isolated and partially purified from *B. bassiana* and *L. lecanii* against *M. persicae* and *B. tabaci* on *Solanum lycopersicum* plants. Gurulingappa et al. [36] also reported a significant decrease in the rate of fecundity of *A. gossypii* induced by the fungal filtrates containing secondary metabolites and conidial suspensions of endophytic strains of *B. bassiana* and *L. lecanii* applied exogenously on cotton plant leaves. Similarly, Basit et al. [29] also observed the negative impact of *Botrytis cinerea*-derived elicitor protein on the nymphal developmental period and the fecundity rate of *M. persicae*. Not only against sap-feeding insect pests, *L. lecanii* also produces certain bioactive secondary metabolites such as bassianolide that is toxic to chewing insect pest *P. xylostella* [14,32].

Nevertheless, JA and SA pathways play a vital role in the plant defense response to insect attack [37,38]. The expression profile levels of major JA and SA pathways associated genes showed that the genes associated with SA were considerably and significantly up-regulated, while the genes linked to JA pathway were moderately or slightly upregulated through the foliar application of *L. lecanii* protein. Our results are in line with previous work by [30] who revealed significant sub-lethal anti-insect effects of *L. lecanii* protein on tomato plants against *M. persicae*, concomitantly along with considerable expression levels upregulation of both JA and SA pathway related genes. Furthermore, these findings support that phloem-feeding herbivores, for example whiteflies and aphids, trigger the primary genes related to SA plant defense mechanism more effectively than the genes associated with JA defense mechanism [39,40]. Our results corroborate the findings of Nazir et al. [41] who showed significant anti-insect effects of PeBb1, an elicitor protein derived from EPF *B. bassiana* on *M. persicae* on the *Brassica rapa* plants along with significantly upregulated expression profiles of JA and ethylene (Et) pathways related genes. These findings corroborate those of Basit et al. [42] showing that the exogenous application of PeBA1, an elicitor protein extracted from *Bacillus amyloliquefaciens* NC6 strain, reduced the fecundity and extended the nymphal development time of *M. persicae* on common bean (*Phaseolus vulgaris*) plants. Moreover, this study showed that PeBA1 elicitor induced significant and differential expressions of the genes related to the JA and SA plant defense mechanisms in common bean plants.

Nevertheless, further studies are essential to better understand how this *L. lecanii*-extracted protein affects the defense systems of plant and subsequently impact the insect fitness parameters. Furthermore, although this EPF-derived protein exhibited a considerable suppression of important life trait of *B. tabaci* under the laboratory conditions, considering the population growth rate and high biotic potential of *B. tabaci* more studies are required to characterize the lethal and sub-lethal effects of this partially purified protein so that it can be employed effectively as novel biocontrol tool under the field conditions.

5. Conclusions

We revealed that sap-feeding on the cotton plants treated with a partially purified protein extracted from an EPF *L. lecanii* considerably suppressed the fecundity rate of whiteflies (*B. tabaci*). Moreover, the expression profiles of major genes potentially linked with the plant defense signaling mechanisms were strongly upregulated in exogenously treated cotton plants by this *L. lecanii* extracted protein, suggesting its potential implication as an innovative tool for the biological control of whiteflies (*B. tabaci*) and other sap-feeding insect pests. Nevertheless, the molecular and functional characterization of this fungal protein after its further purification constitutes important future perspective of this work.

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