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Original article

Do microbial protein elicitors PeaT1 obtained from *Alternaria tenuissima* and PeBL1 from *Brevibacillus laterosporus* enhance defense response against tomato aphid (*Myzus persicae*)?

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ABSTRACT

Tomato aphid (*Myzus persicae*) is a destructive insect pest of tomato responsible for huge losses in the production as well in the vegetable industry. In the present in vitro study two protein elicitors, PeaT1 and PeBL1 were considered to study their efficacies to exhibit defense response against tomato aphid. Three different concentrations of both protein elicitors were applied on the tomato seedlings. After the application of PeaT1 and PeBL1, population growth rates of tomato aphid were decreased as compared to the control treatment. In host preference assay, the tomato aphid showed a preference to build a colony on the control as compared to the treated tomato plant, because tomato leaves provided hazardous surface for aphid after the formation of wax and trichome. The concentrations of protein showed significant ($p < 0.05$) results in life-history traits of the aphid. Jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) showed significant accumulation in tomato seedlings treated with PeaT1 and PeBL1. Elicitors treated plants produced resistance against *M. persicae*. Our finding suggests that PeaT1 and PeBL1 have shown high potentials against the damage of *M. persicae*, and both elicitors could be used as novel biological tools against tomato aphid.
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1. Introduction

Insect pests are one of the significant competitors with human being for the resources generated by agriculture (Oerke and Dehne, 2004). Annually, these pests cause more than 15% loss to global crop production. These losses not only occur in pre harvest but also during the storage of the produce (Oerke 2006; Deutsch et al 2018).

Myzus persicae (Hemiptera: Aphididae) is one of the notorious pest. Because of its feeding behavior it directly affects the quality and yield of various crops including tomato, potato, wheat, maize, cucumber, barley and beans. As a phloem-

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feeding insect, it consumes plant sap and acts as a vector to transmit viruses, which cause severe yield losses. Defense response in plants is activated by various biotic and abiotic elicitors (Zhao et al., 2005). Elicitors are the chemical compounds from biotic and abiotic sources, associated with some micro-organism including viruses, bacteria, fungi and oomycetes which triggers a defense response in host plant (Chen et al 2012). The most commonly known elicitors are proteins, lipids, peptides, glycoproteins and oligosaccharides (Ellis et al, 2009). These elicitors mainly comprise of two major groups; one that activates a defense response in host plants, while the other induces a general defense response in both host and non-host plants (Montesano et al, 2003). Abiotic elicitors are inorganic compounds including, mineral salts, metals and gaseous toxins (Radman et al, 2003), while biotic elicitors are mainly produced by the attack of insect pests and diseases (Dodds and Rathjen, 2010). Currently, due to an increasing demand of food safety and quality standards, elicitors have been studied as an alternative to certain synthetic pesticides (Hael-Conrad et al., 2018).

In nature, plants have to deal with different insect pests and diseases, thus shield themselves using a variety of strategies including chemical and mechanical defenses (Schoonhoven et al., 2005). Chemical defenses depend on secondary metabolites of various chemical origins, which are often described for certain plant taxa and effective against generalist herbivores (Schweiger et al, 2014). Bio-control potential in *Brevibacillus laterosporus* is not solely limited to insects, mollusks and nematodes but also phytopathogenic fungi and bacteria (Ruiu, 2013). Other than constitutive defenses, which are all time present, induction of defenses caused by attack is common (Karban and Baldwin, 1997). Such induction is mediated by phyto-hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Their dependent pathways play a major role in regulation of signaling networks (Pieterse and Van Loon, 1999; Glazebrook, 2001; Salzman et al, 2005). Salicylic acid is a significant signaling molecule, which plays a major role in promoting systemic resistance and local defense reactions against piercing–sucking insects, while jasmonic acid (JA) is involved in defense against chewing insects (Durner et al., 1997; Reymond and Farmer, 1998; Lee et al., 2012). Their defensive signaling pathways are contradictory in dicotyledonous plant species (Tamaoki et al, 2013).

PeBL1 is a broad-spectrum elicitor was studied in *B. laterosporus* strain A60. It plays an essential role in activating early defense response in plants through the SA, JA and ET pathways (Javed and Qiu, 2020). It activates defense enzymes which results in the strengthening the cell wall, and up-regulation genes associated with defense (Wang et al., 2015), while PeaT1 is some sort of general elicitor isolated from *Alternaria tenuissima* (Li et al., 2020). It triggers systemic acquired resistance through salicylic acid pathway in plants which results in the strengthening of cell wall, activation of defense enzymes and up-regulation of defense-related genes (Zhang et al., 2011). In previous studies PeaT1 has been proven to enhance growth and intensification of resistance against abiotic stresses in rice and wheat plants (Wang et al., 2011; Shi et al, 2017), which additionally triggers defense responses against aphids, fungi, viruses, drought and salt (Kulye et al, 2012; Peng et al, 2015; Basit et al, 2020).

In the current study, function and mechanism of both PeBL1 and PeaT1 elicitors were investigated and their effect was evaluated against tomato aphid. Furthermore, growth and development of trichomes on tomato leaves was observed. As well as hormonal contents and gene expression profile of key genes associated with JA, SA, and ET pathways were carried out to evaluate both protein elicitors to assess their potential effect on *M. persicae*.

2. Material and methods

2.1. Plant and insect culture

Tomato plants were grown in growth chamber at 65–70% relative humidity (RH), 21 ± 2 °C and a photoperiod of 14 h: 10 h light/-dark for rearing tomato aphid (*M. persicae*) for 3 months. Seeds of tomato were sterilized with 75% ethanol for 30–50 s, and then used distilled water for washing and put seeds for 2–4 days in presoaked water before sowing. The colony of tomato aphid was established on tomato plants in insect growth chamber, which was established for 2–3 months. To ensure the experiment those aphids were adapted suitably to the chemistry of tomato plants.

2.2. Purification of PeaT1 elicitor

PeaT1 was extracted from *A. tenuissima* culture in 1L of LB medium at 400 rpm for 10–12 h at 36 °C. Pellet was collected after centrifugation; cells were re-suspended with buffer and broken by sonicator mechine. After centrifugation at 10,000 rpm for 25 min, supernatant was collected through a filter paper (size 0.22 μm). This protein elicitor was further purified by column chromatography with a His Trap™ HP column (GX-11860073, GE Healthcare, Munich, Germany). Three different types of loading buffers such as A, B, C were used for the purification of PeaT1. Buffer A (50 mM Tris-HCl, pH 8.0) was used for washing the column from other elicitor, Buffer B (50 mM Tris-HCl, 200 mM NaCl and 20 mM imidazole, pH 8.0) was used to stabilized the columns. Buffer C (50 mM Tris-HCl, 200 mM NaCl and 500 mM imidazole, pH 8.0) was for the elution of elicitor protein. By using desalting column protein was desalted successively and centrifuged at 5000 rpm on 6 °C. Molecular mass of PeaT1 was detected by 12% SDS-PAGE.

2.3. Purification of PeBL1

To express the protein PeBL1, the bacterial strain was grown in 100 ml of LB medium and shaked at 36 °C for 6 h. When its OD value reached 0.6–0.8, 25–30 ml of LB with bacterial spores was put into 1 L of LB medium at 37 °C for 8 h. After adding IPTG, the broth culture was shaking at 16 °C with 200 rpm for 16 h. Centrifugation was constructed at 12,000 rpm for 15 min to get the pellet. The pellet was re-suspended with a buffer (50 mM Tris-HCl, 200 mM NaCl, pH 8.0). The cells in pellet were disrupted by sonicated. After that, the recombinant protein was collected at 5000 rpm for 20 min. Purification was implemented by His-Trap HP column and Hi-Trap desalting column as described by(23).

2.4. Characterization of PeBL1 and PeaT1 elicitor

Protein assay II was conducted to check the concentration of both protein elicitor quantities Kit through (BCA), and then stored at −80 °C for further usage. The elicitor protein was diluted to 25, 50 and 100 times. The concentrations of PeaT1 were observed as 78.68, 64.32 and 43.24 μg/ml, while uses of PeBL1 were 68.72, 54.23 and 36.42 μg/ml.

2.5. Efficacy of PeaT1 and PeBL1 on life cycle of M. persicae

To evaluate the efficacy of PeaT1 and PeBL1 against tomato aphid (*M. persicae*), 5 ml of PeaT1 and PeBL1 with different concentrations were applied on 4 weeks tomato plant. The buffer and water were considered as negative and positive control. The plants were allowed to dry overnight after spraying of protein elicitor. One to three freshly molted larvae of *M. persicae were* released

Table 1
Primer pairs used in the study.

Genes	Forward (5′ → 3′)	Reverse (5′ → 3′)
SOLYC04g079730	GCAATCGGCGATTCTGCTAC	AATGGACTTCGATCCGATGA
SOLYC02g085730	TCCGACTTGACATGCATGAT	TCCGATGGTACAGCATTAGC
SOLYC09g007900	CTCATTGCAATGCATCGAGC	GCATTCTGAGGCGATTCAGC
SOLYC03g036480	ACTCGAGCATCCGTAATCTG	GCGGATCGAAGGCGATCAT
SOLYC12g099000	GCGTGCAGGTCGATCGATT	TCGAGGATCGACGTGTCATC
SOLYC03g043890	CAGTGACCGTGATCGACAG	ATCCGTTAATGCAGCTACTA
Actin	ATCCGGTGACGTAACGTGTC	GCTAACGTACTGCAACGTAC

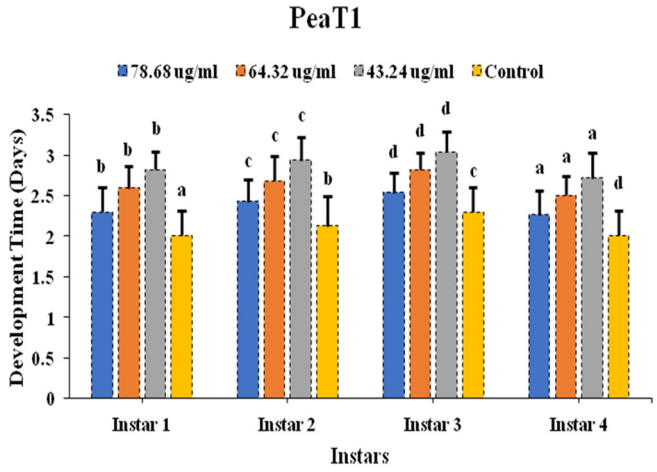

Fig. 1. Mean developmental time of each instars of *Aphid* on tamoto plants after the application of PeaT1 with different concentrations (n = 10). The bars with different color showed significant differences among treatments (one-way factorial ANOVA; LSD at $\alpha = 0.05$).

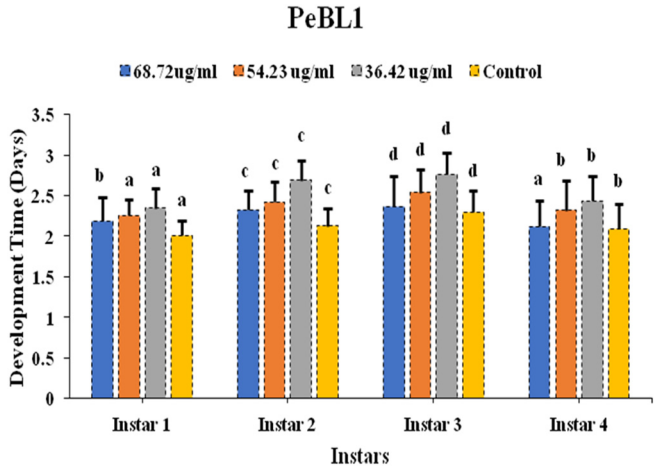

Fig. 2. Mean developmental time of each instars of *M. persicae* on tomato plants after the application of PeBL1, (n = 10). The four color bars indicate significant differences among treatments (one-way factorial ANOVA; LSD at $\alpha = 0.05$).

on tomato leaves but restricted with insect cage. Each developmental time of larval instar was observed after six hours. Each concentration was replicated 10 times, and experiment was repeated thrice.

2.6. Efficacy of PeaT1 and PeBL1 on survival rate of M. persicae

To evaluate the activity of PeaT1 and PeBL1 against tomato aphid, three adults were released on tomato leaves but restricted with an insect cage. One to three freshly molted larvae of *M. persicae* was maintained on each leaf. For each larvae data of survival rate was collected on daily basis. Fecundity of *M. persicae* was observed for seven consecutive days and average fecundity was calculated (reference). Each proteins concentration was replicated 10 times.

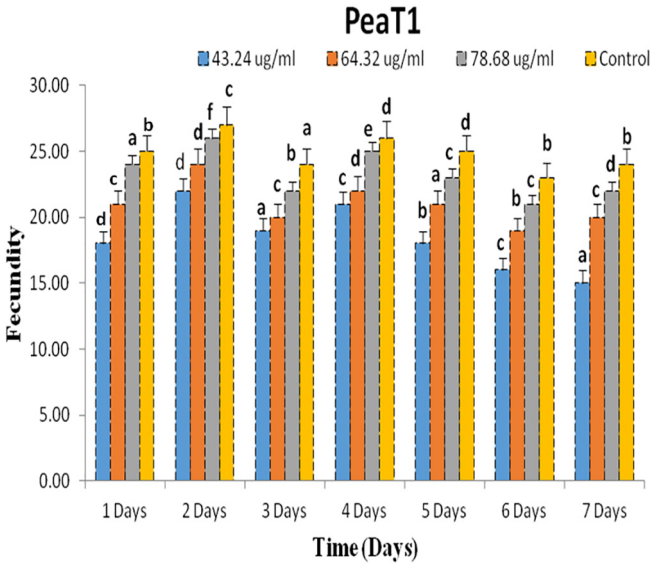

Fig. 3. Average fecundity of *M. persicae* after the treatments with different concentrations of PeaT1 (n = 10). Letters on each bar showed the differences among concentrations (one-way ANOVA; LSD at $\alpha = 0.05$).

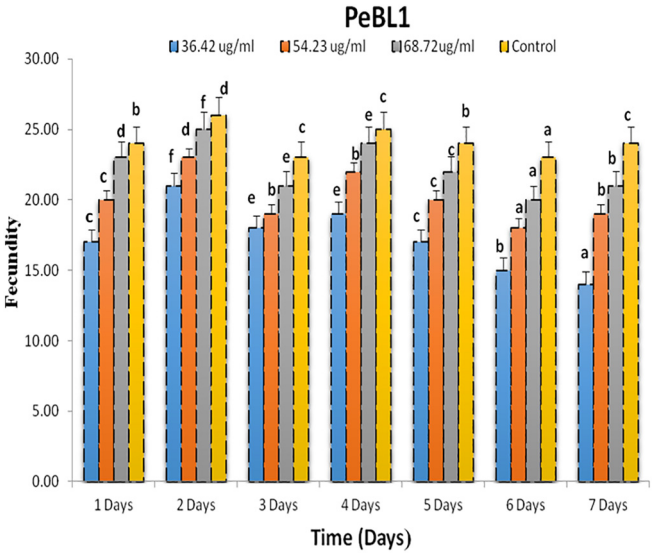

Fig. 4. Average fecundity of *M. persicae* treated by different concentration of PeaT1 (n = 10). Letters on each bar show the changes among different concentrations (one-way ANOVA; LSD at $\alpha = 0.05$.

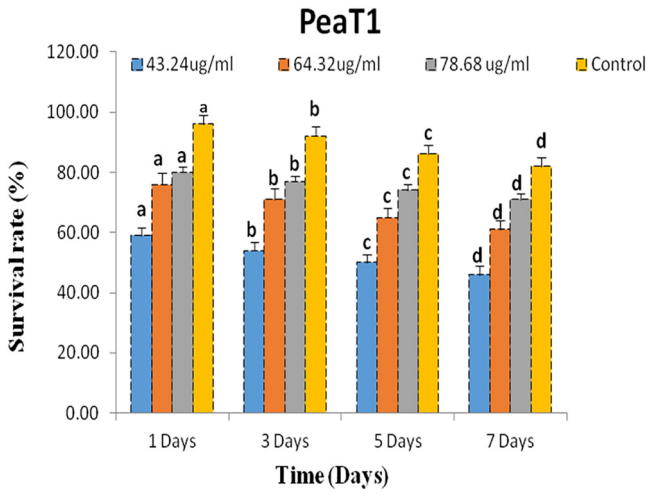

Fig. 5. The mean survival rates of the tomato aphid after the application of PeaT1 (±SE; n = 10) at different concentration levels (One-way ANOVAanalysis; LSD test at $\alpha = 0.05$).

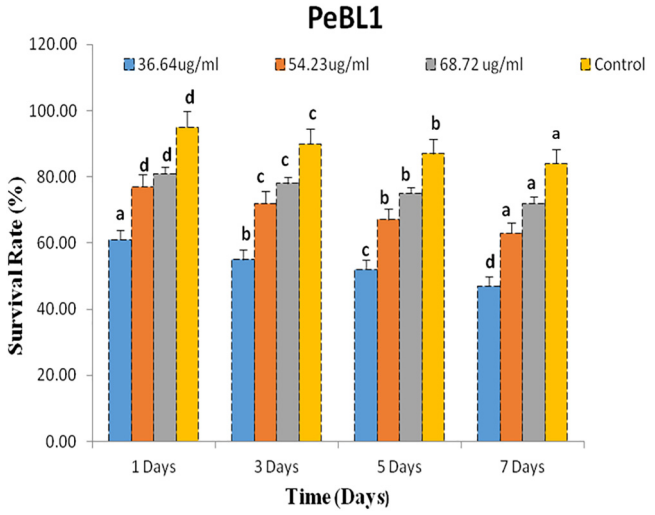

Fig. 6. The mean survival rate of the tomato aphid after the application of PeaT1 (±SE; n = 10) at different concentration levels (One-way ANOVAanalysis; LSD test at $\alpha = 0.05$).

2.7. Extraction of RNA and cDNA synthesis

Extraction of RNA from the leaf samples was followed by Kit, Total RNA Extraction Reagent (Nanjing NOVIZAN Biotechnology Company, China) according to the manufacture protocol. The RNA quality was observed by nano photometer (Please provide the company information of this machine). After extraction, RNA was transcribed into cDNA by HiScript II 1st Strand cDNA Synthesis Kit (Nanjing NOVIZAN Biotechnology Company, China) and the concentrations of the cDNAs were adjusted to be same.

2.8. Quantitative real-time PCR analysis

RT-qPCR was performed to quantify the expression profile of the key genes associated with JA, SA, and ET pathways. The feeding of tomato aphid on plants treated with protein elicitors was considered a treatment, and plants treated with buffer were considered controls. For amplification of RT-qPCR, 12 gene primer pairs (Table 1) were used with the Applied Biosystems, USA (ABI 7500) system. All reactions were performed using the SYBR Premix Ex Taq II kit (TransGen Biotech, Beijing, China), in a 20 μL total sample volume (2.0 μL cDNA, 10.0 μL SYBR Premix Ex Taq II, 1.8 μL of primers, and 6.2 μL of distilled deionised water). The qRT-PCR procedure was as follows: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing at 60 °C for 15 s, with a total of 40 cycles. Standard curves were run simultaneously PCR are shown in Table 1.

2.9. Statistical analysis

The data regarding concentration of protein elicitors and time were subjected to analysis of variance (ANOVA) with factorial arrangement using Statistics 8.1 software (Tallahassee, FL, USA). Means were compared using least significant difference (LSD) test at 5% level of probability. Gene expression profile was analysed by CT method. Student's *t*-test was used to assess the fold changes between buffers and treated sample ($p < 0.05$) (30).

3. Results

3.1. Influence of PeaT1 and PeBL1 on developmental time of M. persicae

Analysis of variance showed a significant effect at different concentration of protein elicitors. Different trends were found on the developmental time by applying the different concentrations of elicitors. Nymphal development time of each instar was increased with the increase of concentrations of the each protein elicitor (Fig. 1). After the application of PeaT1, it was observed that the first instar took two days to moult in control while in elicitor treated the same instar took 2.3, 2.5, and 2.8 days respectively according to the concentration of elicitor. Generally second instar took 2.1 days in buffer treated plant while it extends the time to 2.4, 2.6 and 2.9 days respectively according to the concentrations of PeaT1. Normally third instar took 2.3 days in buffer treated plant while the times are 2.5, 2.8 and 3 days in treated plants at various concentrations of PeaT1. Similar trends were found in fourth instar larvae, normally it took two days to moult but after feeding on treated plants with different PeaT1 concentrations it tooks 2.2, 2.5 and 2.7 days. However various concentrations of PeaT1 showed a significant effect on the developmental days for each instar, viz. F1 = 1.64 $p < 0.02$, F2 = 1.08 $p < 0.03$, F3 = 1.59 $p < 0.02$ and F4 = 1.04 $p < 0.03$ respectively.

Similar trends were found after the application of PeBL1 (Fig. 2), if 1st instar takes 2 days in control condition, it takes 2.1, 2.2, 2.3 days respectively according to PeBL1 concentrations. The 2nd instar spends 2.1 days in treated tomato plant with buffer while in treated plants with different PeBL1 concentrations; it takes 2.3, 2.4 and 2.6 days respectively. For the 3rd instar, it takes 2.3 days in buffer treated plant but 2.4, 2.5 and 2.7 days with three .PeBL1 concentrations. The 4th instar larvae keeps a similar trend, that is the normal developmental time is 2 days, corresponding to 2.1, 2.3 and 2.4 days after feeding at treated tomato plants with PeBL1 However, the effect of PeBL1 concentrations showed a significant effect on the developmental time for each instar, viz. F1 = 0.29 day $p < 0.03$, F2 = 0.67 day $p < 0.04$, F3 = 0.62 day $p < 0.01$ and F4 = 0.54 days $p < 0.000$, respectively.

3.2. Influence of PeaT1 and PeBL1 on fecundity of M. persicae

Factorial analysis showed a significant effect at the concentration F1 = 5.84 $p < 0.05$ and time F2 = 1.18 $p < 0.01$ that influence the fecundity of *M. persicae* on tomato plants. The aphids produce fewer off springs after feeding on treated plant with PeaT1 as com-

pared to the buffer treated plant (control). Maximum fecundity (value) of *M. persicae* corresponding to maximum concentration of PeaT1 (value) was shown in the Fig. 3.

After the application of PeBL1 protein, similar trends to PeaT1 were found (Fig. 4). Maximum fecundity was observed at maximum concentration while minimum fecundity appeared at minimum concentration.

3.3. Influence of PeaT1 and PeBL1 on fecundity of M. persicae

In comparison to control the bioassay results showed a downward trend in overall survival rate of *M. persicae* fed on plants treated with PeaT1 and PeBL1. Significant effect was quantified by mean survival rate of *M. persicae* on tomato plants at different concentrations of protein elicitors PeaT1 ($F1 = 18.33$, $p < 0.00$). Time interval (Days) showed a significant effect on the survival rate ($F2 = 24.55$, $p < 0.00$) while its intraction with concentration showed non significant effect on the survival rate ($F3 = 0.3$, $p < 0.97$). PeaT1 showed a significant effect on the treated plant as compared to the control andthe survival rate of aphid was decreased as compared to the control at different concentrations (Fig. 5). The maximum survival rate (value) was observed at 1st day while minimum (value) appeared after 7 days of feeding

The effect of PeBL1 on the treated plant as compared to the control was shown in Fig. 6. The survival rate of aphid was decreased as compared to the control at different concentration. Survival rate was decreased with the increase of time, thus maximum survival rate (value) was observed at 1st day, and minimum (value) was also observed after 7 days.

3.4. Influence of PeaT1 & PeBL1 related to JA, SA, and ET pathways

To assess the key mechanism of recombinant protein elicitor whetherPeaT1 and PeBL1 play any role to enhance resistance in tomato plant, protein elicitors (PeaT1 and PeBL1) were sprayed on the six week old tomato seedling. Expression of different defense related genes in the plant were quantified. We found that all key genes participating in the defence mechanism were up-regulated as compared to the control except SOLYCO3g036480. It was quantified that enhancing resistance was influenced on aphid infestation. All the marker genes associated with JA pathway were slightly up-regulated while all the marker genes associated with SA and ET pathway were strongly up-regulated after 1,2,3 and 4 days of post inoculation of aphid feeding (Figs. 7 and 8). Maximum up-regulation was observed after second and third day of post inoculation while minimum up-regulation was observed at one day of post inoculation (dpi).

4. Discussion

After feeding of sucking insect pest potential use of bio-pesticides such as protein elicitors used as a novel biological tool in pest management strategies, play a vital role in signalling mech-

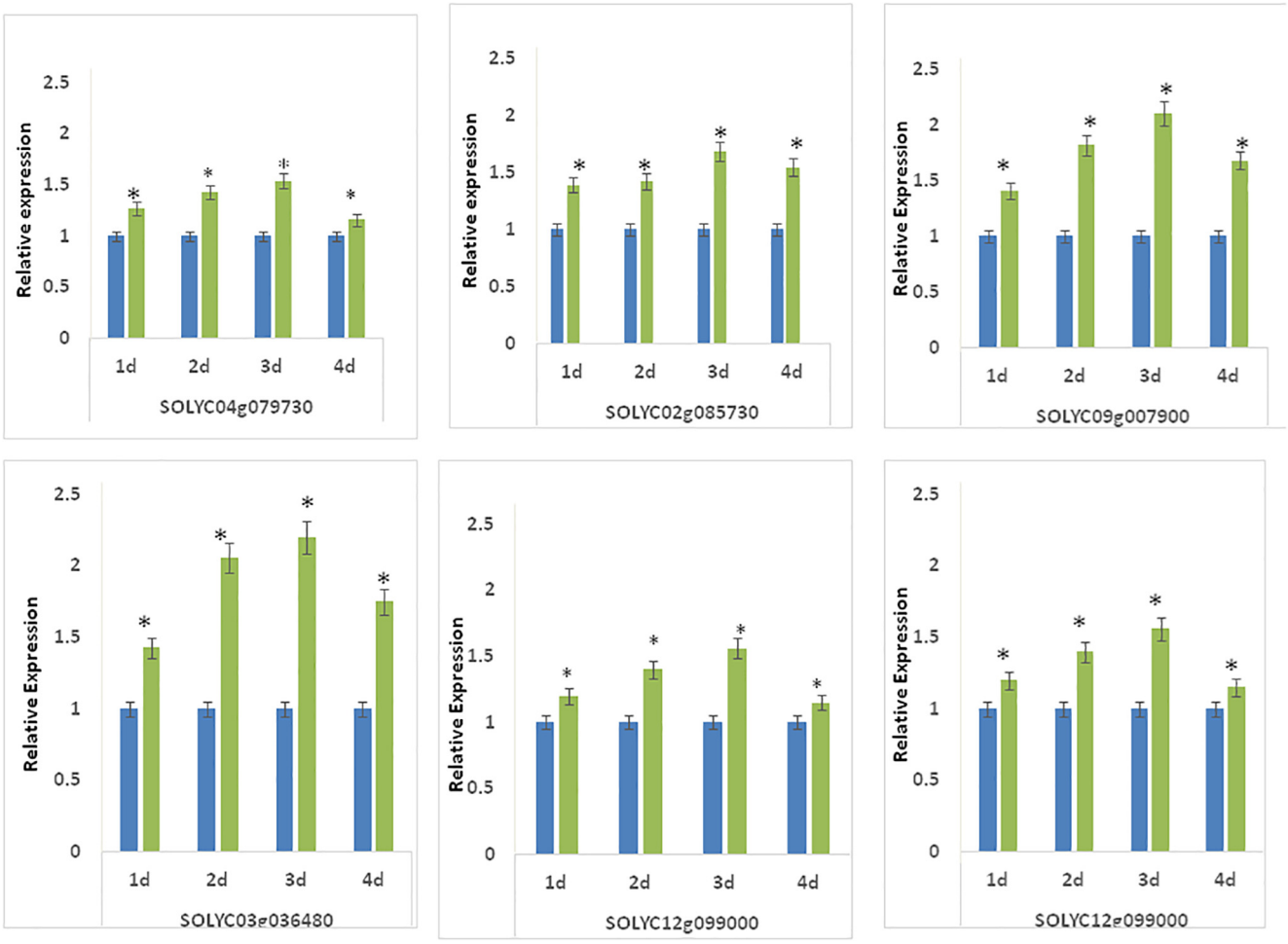

Fig. 7. The expression profile of key genes associated with JA, SA and ET pathways. Blue colour was showed as a control and green colour represented treatment with PeaT1 after aphid feeding. The asterisk on bar indicated a significant difference from buffer control by Student's *t*-test ($p < 0.05$) for each genes.

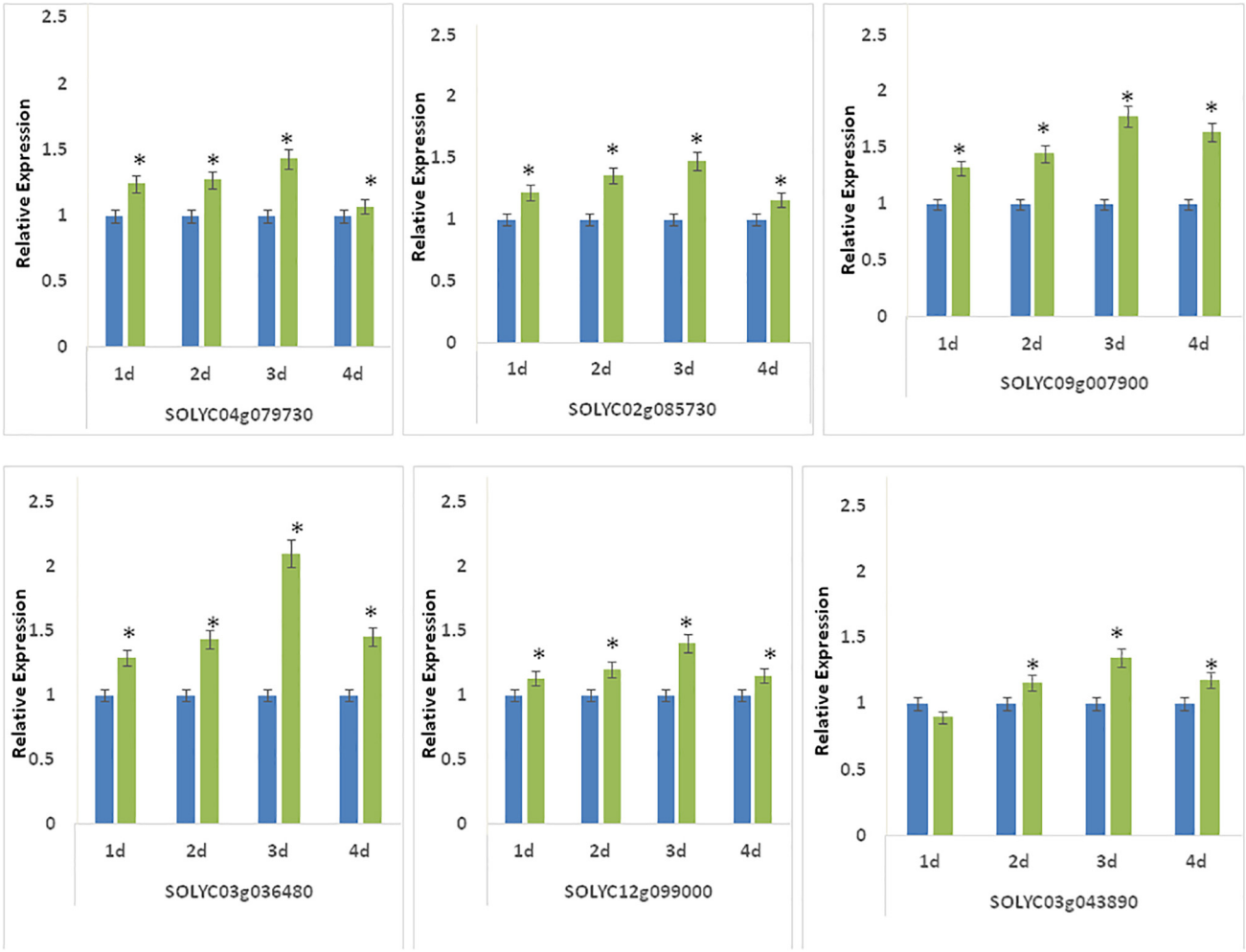

Fig. 8. The expression profile of key genes associated with JA, SA and ET pathways. Blue colour showed as a control and green colour represented treatment with PeBL1 after aphid feeding. An asterisk on bar showed a significant difference from buffer control by Student's *t*-test ($p < 0.05$) for each genes.

anism of plant defense (Bale et al., 2002). Strains of *Alternaria tensimia* and *Brevibacillus laterosporous* have been shown a wide range of antimicrobial activities for the control of microbial agents just like fungi and bacteria. It can enter the cell and position in the nucleus and cytoplasm where it disrupts the production of protein by reallocating of RNA and DNA (Brogden, 2005). Entomopathogenic fungi and bacteria, either biotrophic or necrotrophic contents, are an important source of protein elicitors such as PAMPs and MAMPs (Boughton et al, 2006). This in vitro study showed a putative role PeaT1 and PeBL1 protein elicitors for the control of tomato aphid. Previously, various studies showed chemical elicitors application just like benzothiadiazole, methyl jasmonate and numarious other plant defence related protein like proteinase that significantly inhibited the activity of sucking insect pest of tomato (Mallinger et al, 2011). Beside this, it is common how protein elicitors assemble induced systemic resistance in case of piercing-sucking insect pests (Maffei et al, 2012; Bostock et al, 2001).

Our results are in agreement with (Boughton et al, 2006), which indicate that by the use of protein elicitors such as methyl salicylate reduce the population of soybean aphid up to 40%. Bioassay result showed that after the treatment of PeaT1 and PeBL1, population development of *M. persicae* was slower as compared to the control, which was similar to the negative effect of external application of various protein elicitors just like benzothiadiazole and JA, methyl jasmonate on fitness and growth population of different aphid species (Millinger, 2001). Maximum elongation was observed at 3rd instar with the minimum concentration. Just like a potential biocontrol of this entomo-pathogenic fungi and bacteria has been observed in Lepidpotera coleopteran and Diptera as well as different nematodes and mollusks (Ruiu et al, 2013), PeaT1 and PeBL1 played a putative role for the repression of sap sucking insect pest by quantifying survival rate, population performance and growth parameter. In our current study, we observed an increase in the production of trichomes on PeaT1 and PeBL1 treated leaves seedling produced more trichomes in comparison as compared to the control, which restricted the colonization and reproduction of aphid. This structure had a vital effect on physical resistance against sap sucking insect and pathogens (Javed and Qiu, 2020). Moreover, SA, JA and ET enhanced transcription to enhance resistance against tomato aphid, and in *Arabidopsis* aphid infestation enhanced significantly the transcript of SA marker genes PR1, PR5 and BGL2 and JA associated marker genes LOX, LOX12, and PDF1.2 (Javed and Qiu, 2020). All the marker genes in the present study associated with JA, SA and ET defence related pathway were strongly up-regulated particularly for related pathway genes of SA (SOLYC09g007900, SOLYC03g036480) and JA (SOLYC04g079730, SOLYC02g085730). These results were resemblance with the previous study (Basit et al, 2019). The findings from this study confirmed the activation by *M. persicae* of JA, SA, and ET pathways-associated genes (Cooper and Goggin, 2005; Ali and Agrawal, 2012).

5. Conclusion

Our findings showed that PeaTI and peBL1 had resistance against *M. persicae* and a great increase of developmental time of each instars and reduction of the survival rate and fecundity was observed. Disturbance of aphid colonization was also observed by the increment of PeaT1 and peBL1 concentrations. Molecular mechanism induced by PeaT1 and PeBL1 such as JA, SA and ET pathway is participated worldwide to assess the physical response of tomato plant. Importantly, the present study provided us strong evidence that PeaT1 and PeBL1 could be applied to protect tomato seedling against the attack of *M. persicae*.

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Declaration of Competing Interest

The authors declare no conflict of interest exists.

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