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Chitinase from *Pseudomonas fluorescens* and its insecticidal activity against *Helopeltis theivora*

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M. Suganthi,[1,*,#] P. Senthilkumar,[2,*] S. Arvinth,[1,3] and K. N. Chandrashekara[1]

[1] *Plant Physiology & Biotechnology Division, UPASI Tea Research Institute, Valparai, Coimbatore 642127, India*
[2] *Department of Genetic Engineering, SRM University, Kattankulathur, Chennai 603203, India*
[3] *Department of Botany, PSG College of Arts and Science, Coimbatore 641004, India*

The tea mosquito bug (TMB), *Helopeltis* spp. (Hemiptera: Miridae) is an insidious pest that poses a significant economical threat to tea plantations. *Pseudomonas* cultures are being used extensively for pest management which, however, resulting in a low mortality rate of insects and which has prompted us to search for a new microbial metabolite for TMB control. A chitinase purified from *P. fluorescens* and partially characterized by our group showed insecticidal activity against TMB. The mode of action behind chitinase toxicity is the enzymatic hydrolysis of chitin, which is a common constituent of the insect exoskeleton and gut lining of the peritrophic membrane. A chitinase-secreting strain MP-13 was characterized based on 16S rRNA sequencing and validated as *Pseudomonas fluorescens*. In the present study, purified chitinase (0.048 units/ml) enzyme from *P. fluorescens* MP-13 revealed 100% TMB mortality under *in-vitro* conditions. The results of this study can be utilized for future crop improvement programs and integrated pest management strategies.

Key Words: biocontrol; chitin; insect mortality; microbial metabolite; tea rhizosphere

Introduction

The tea mosquito bug (TMB), *Helopeltis theivora* Waterhouse (Hemiptera: Miridae), has emerged as a vigorous pest of tea. TMB feeds on young shoots and lays its eggs on the tender stems which leads to about 17% losses in crop yields. Its management has also become a major problem for tea planters. In the case of a severe infestation, the crop loss is near total. Control measures have been totally dependent upon chemical insecticides for many years. Alternate strategies for the management of TMB have also been tried extensively, which include pheromone traps and use of plant-derived agents (e.g. extract of neem and neem kernel) with limited success (Roy et al., 2010). Along with the present efforts in the control of TMB, it is interesting to study the effect of metabolites from biological agents for the control of this pest.

Biological control is an important component of the integrated strategy and is unique because it originates from nature. Bio-pesticides offer an alternative option to chemical pesticides because of their low environmental pollution and low toxicity to human health. Earlier control strategies, such as the direct application of biological control agents, have provided solutions against a variety of pests and diseases (Bassett and Janisiewicz, 2003; Michelsen and Stougaard, 2011). Reports also discuss that the use of biocontrol agents leads to a low mortality of pests (Roobakkumar et al., 2011; Tsagou et al., 2004) and their continuous use leads to the development of resistance to biocontrol agents in the field (Rao et al., 1995; Wirth et al., 2005). Hence, the use of a specific molecule, instead of spraying an entire organism, is an important approach to increase mortality and resistance.

Microbial chitinases are expected to be potential biocontrol agents. The importance of chitinases in the biological control of fungi, nematodes, and insect pests is an

*Corresponding authors: M. Suganthi, Ph.D., Plant Physiology & Biotechnology Division, UPASI Tea Research Institute, Valparai, Coimbatore 642127, India; P. Senthilkumar, Ph.D. (Asst. Professor), Department of Genetic Engineering, SRM University, Kattankulathur, Chennai 603203, India.
E-mail: sugenthi88@gmail.com; mpsenthilkumar@gmail.com
*These authors contributed equally to this work.
#Present address: Department of Biotechnology, School of Life Science, Vels University, Pallavaram, Chennai 600117, India.

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emerging field of research. Chitinase has been isolated from many bacterial strains and reported to be effective against many pests and diseases (Gomaa, 2012; Kramer et al., 1997). The fluorescent pseudomonads are known for an active biocontrol mechanism against many pests, and this strain is already being used in the control of red spider mite in tea (Roobakkumar et al., 2011). Some of the strains of fluorescent pseudomonads have currently received global attention for the production of lytic enzymes, such as chitinase, which is responsible for antagonistic activity. This single molecule is expected to be used in insect pest control. This work is the first to report on the use of chitinase isolated from *P. fluorescens* for the management of TMB.

Chitin is an abundant, insoluble polysaccharide composed of linear chains of β-1,4-*N*-acetylglucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds. Chitin is widely distributed in nature, and is the principle structural component of the outer skeleton (50% of the cuticle is made up of chitin), foregut, hindgut, midgut lining of the peritrophic membrane and, hence, is also essential for the structural integrity of many insects and nematodes (Bhattachrya et al., 2007). Microbial chitinase, which is considered as a potential biocontrol agent, degrades chitin into its monomeric or oligomeric components, thereby degrading the major component of the insect outer skeleton. The application of chitin to the insect enters the gut of the insect larvae and causes significant damage to the peritrophic membrane structure, which prevents the insect from feeding and, consequently, leads to death; or it disrupts the cuticle, which subsequently causes abnormal moulting. Recently, chitinase has received considerable attention because it might play a role in plant defense systems against chitin-containing pathogens and mosquito control (Mendonsa et al., 1996). In the present work, we investigate the development of an enzyme-based pesticide against TMB. In our earlier study, the chitinase enzyme was isolated and characterized from *P. fluorescens* MP-13 (Suganthi et al., 2015). However, chitinase enzyme from *P. fluorescens* has not been reported to control TMB in tea. Hence, the present study reports the phylogenetic identification of the potent chitinase-producing bacterium *P. fluorescens* MP-13, and the evaluation of the insecticidal activity of *P. fluorescens* MP-13 chitinase against TMB.

Materials and Methods

Strain identification using 16S rRNA sequencing and analysis. The total genomic DNA was extracted from strain MP-13 and the 16S rRNA gene was amplified by using the Forward primer (5′-GAGTTTGATCCTGGCTCAG-3′) and the Reverse primer (5′-AGAAAGGAGGTGATCCAGCC-3′). Amplification was performed in a total volume of 25 μl containing 100 ng of genomic DNA, 1 μl of forward and reverse primer (40 pmol/μl), 200 mM of each dNTP (Fermentas), 1 unit of Taq DNA polymerase and 1X Taq buffer (10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.08% Nonidet P40 and 2.5 mM $MgCl_2$: Fermentas). The contents were mixed thoroughly and centrifuged for 5 s at 5000 rpm. The PCR reaction was carried out after incubation at 95°C for 5 min as an initial denaturation, and then were cycled 30 times through the following temperature profile: denaturation for 30 s at 94°C; annealing for 15 s at 56°C; and extension for 1.5 min at 72°C, with a final extension for 5 min at 72°C. The PCR products were purified using Qiagen Q1A Quick Gel Extraction Kit (Qiagen, Inc., Germany) by following the manufacturer's protocol. The purified PCR product was ligated into a T/A cloning vector, pTZ57R/T (Fermentas, USA) and transformed into the *Escherichia coli* DH5α strain as per the manufacturer's protocol. The plasmid was sequenced with an automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Seoul, Korea). The 16S rRNA gene sequence of *P. fluorescens* MP-13 was deposited in the GenBank database with accession number KJ 934371.

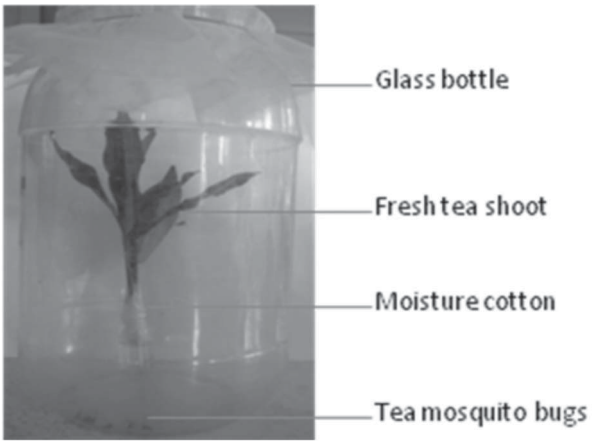

Fig. 1. Bioassay system for evaluating the toxicity of *P. fluorescens* MP-13 chitinase against the tea mosquito bug.

To perform molecular phylogenetic analysis, reference pseudomonas sequences required for comparison were downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genbank. All 16S rRNA sequences were aligned using the multiple sequence alignment program CLUSTAL W, the common gaps from all the selected sequences were removed, and the alignment was checked manually for quality. The final 16S rRNA phylogenetic tree was constructed with the MEGA5 software (Tamura et al., 2007).

Purification of P. fluorescens MP-13 chitinase. A single colony of *P. fluorescens* MP-13 was inoculated in 50 ml of nutrient broth with 1.5% chitin and allowed to grow for 96 h in a shaker at 37°C. The crude enzyme was collected by centrifugation at 5000 rpm for 10 min. The cell-free supernatants were saturated with ammonium sulphate to 50–80% levels, kept at 4°C overnight, and then precipitates were collected by centrifugation at 10,000 rpm for 20 min. The precipitates were dissolved in 50 mM phosphate buffered saline (PBS) pH 7.0 and extensively dialyzed at 4°C against the same buffer for at least 12 h with 2–3 times buffer changes. Chitinase enzyme was purified by following gel filtration chromatography (Suganthi et al., 2015) and the collected fraction was assayed for chitinase activity. Positive chitinase fractions were pooled and lyophilized under 4°C. In our previous

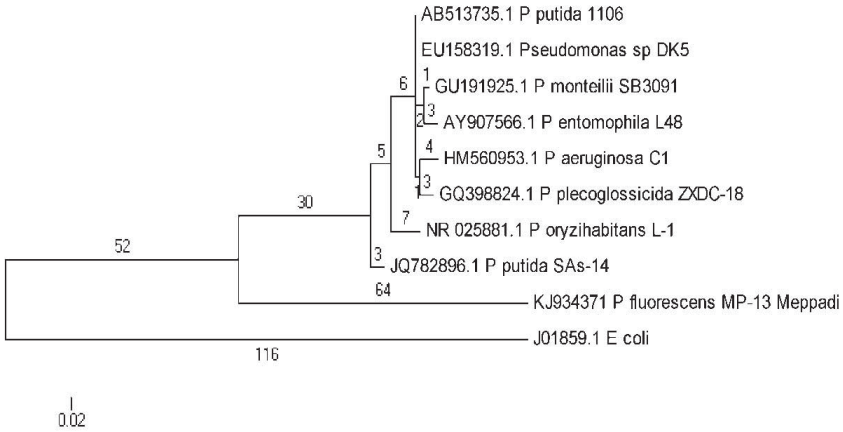

Fig. 2. Phylogenetic relationships of 16S rRNA sequences from *P. fluorescens* MP-13 with other pseudomonas strains.

Maximum Parsimony phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain MP-13 *P. fluorescens* with other related taxa. The tree was rooted by using *E. coli* J01859.1 as outgroup. Bootstrap values are given for each node having 30% or greater support. Bar = 0.02 nucleotide substitution per site. Numbers in parentheses represent the sequence accession numbers in GenBank.

study (Suganthi et al., 2015), SDS-PAGE analysis showed that the molecular weight of isolated chitinase was ~34 k Da.

Chitinase assay. The chitinase activity was determined by using colloidal chitin as a substrate, following the Sun et al. (2006) method. The reaction mixture was prepared by mixing 0.5 mL of enzyme solution and 0.5 mL of 1% colloidal chitin in 1 mL Mcllvaine buffer (100 m mol/L citric acid, 200 m mol/L sodium phosphate) at a pH of 7.0. The mixture was incubated for 30 min at 36°C using a shaking water bath and the reaction was stopped by incubating with boiling water for 10 min. The amount of reducing sugars released in the supernatant was measured by a method using dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 540 nm using a spectrophotometer (Amersham Biosciences). One unit (U) of chitinase activity was defined as the amount of enzymes that liberated 1 μmol *N*-acetylglucosamine (GlcNAc) from colloidal chitin per minute.

Insect toxicity assay. Chitinase enzyme from *P. fluorescens* MP-13 was tested against TMB (*Helopeltis theivora*). For the laboratory bioassay, mature tea (*Camellia sinensis*) shoots were collected from the UPASI experimental farm, Valparai. Shoots were cut to the same size (13 cm from the top) and placed on moist cotton in a glass bottle (101 mm O.D. × 225 mm H, Fig. 1). Laboratory bioassays were carried out on adult females of TMB. TMBs were collected directly from the tea field and cultured in a plastic container. Fresh tea shoots were given as feed, and the shoots were replaced daily. The tea shoots were sprayed with 2 mL of three different concentrations of chitinase (0.012, 0.024 and 0.048 U/ml) using a glass atomizer (50 mL, Vensil). Tea shoots sprayed with distilled water served as an untreated control (Chandrasekaran et al., 2012). The leaf shoots were left to dry for 30 min and then placed in a climatic chamber at 25 ± 1°C, 75 ± 5% RH and a 16L: 8D photoperiod. Ten adults were transferred directly from the colony with a fine brush onto each tea shoot set. The mortality of adults was observed after 24, 48, 72 and 96 h. All experiments were conducted using five replicates of the three concentration observations, with distilled water as the control. The pooled data were subjected to probit analysis (SPSS 10).

Table 1. Insect toxicity of *P. fluorescens* MP-13 chitinase against the tea mosquito bug.

Chitinase U/ml	Percentage of mortality			
	24 h	48 h	72 h	96 h
Control	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}
0.012	24 ± 4.0^{b}	28 ± 4.8^{b}	44 ± 4.0^{b}	44 ± 4.0^{b}
0.024	30 ± 5.1^{b}	50 ± 5.1^{c}	60 ± 0.0^{c}	60 ± 0.0^{c}
0.048	48 ± 4.8^{c}	72 ± 4.8^{d}	100 ± 0.0^{d}	100 ± 0.0^{d}

Values are the means of five replicates ± SE; Means followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's Multiple Range Test (DMRT).

Results

Molecular identification of MP-13

The identification of strain MP-13 was confirmed by 16S rRNA sequencing as *P. fluorescens*. A phylogenetic tree was constructed using 16S rRNA gene sequences of the strain *P. fluorescens* MP-13 (Fig. 2) and the 8 most closely related species of the genus *Pseudomonas* with validly published names, along with an outgroup sequence. On the basis of the 16S rDNA sequence homology, nine bacterial isolates were distributed among two clades of the tree. A phylogenetic tree shows that *P. fluorescens* MP-13 was found in one clade, which could be discriminated phylogenetically from the other different species of *Pseudomonas*. In our earlier report a ~34 kDa chitinase enzyme was isolated and characterized (Suganthi et al., 2015) and used for the present study.

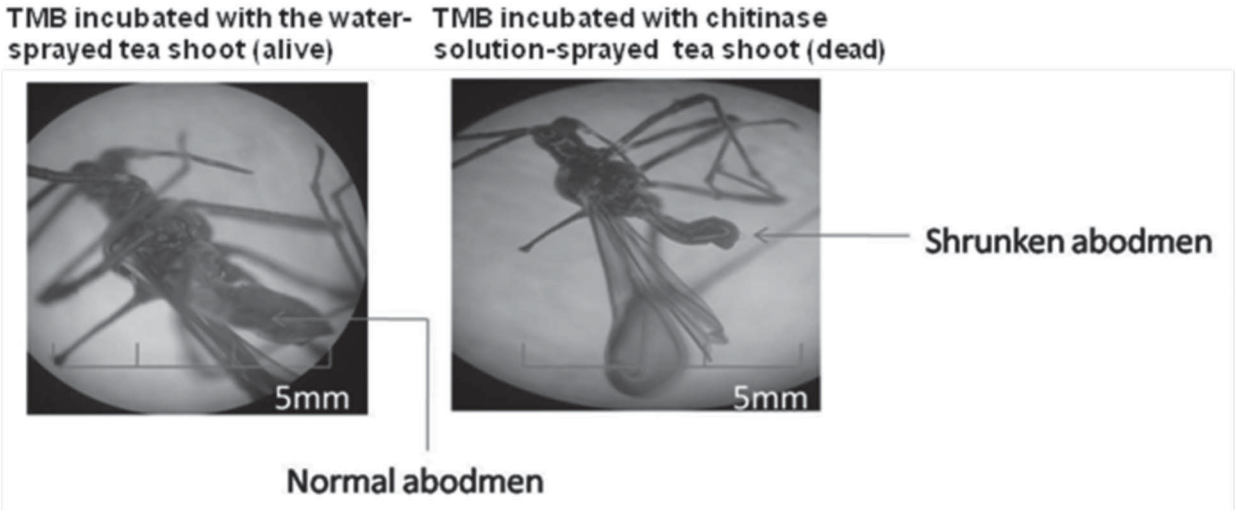

Fig. 3. Toxic effect of *P. fluorescens* MP-13 chitinase on the tea mosquito bug.

Left: Photograph of the tea mosquito bug incubated for 72 h with the water-sprayed tea shoot (control). Right: Photograph of the tea mosquito bug incubated for 72 h with the 0.048 U/ml chitinase solution-sprayed tea shoot. Shrinking of the abdomen was observed in the TMB incubated with the tea shoot treated with the 0.048 U/mL solution of *P. fluorescens* MP-13 chitinase, indicating that the chitinase caused damage to the gut of TMB by digesting the peritrophic membrane. Whereas the TMB treated with water as the control remains normal.

Insect toxicity assay

Purified chitinase from *P. fluorescens* MP-13 reported in our previous study was used for the following insect toxicity assay (Suganthi et al., 2015). The result of the toxicity assay of chitinase *P. fluorescens* against TMB are given in Table 1. The average percent mortality of TMB was 24% after 24 h when chitinase was applied at 0.012 U/ml, after 72 h the percent mortality increased to 44%. But since the percent mortality of TMB increased up to only 60% after 72 h, when chitinase was applied at 0.024 U/ml, a higher concentration of chitinase was required to achieve 100% mortality in the TMB population. The percent mortality of TMB was observed as 100% after 72 h when they were treated with 0.048 U/ml of purified chitinase from *P. fluorescens*. From the results (Table 1), it was clear that no insect mortality was observed in the control, but a stable increase in insect mortality was observed when the concentration of chitinase was increased, indicating that *P. fluorescens* MP-13 chitinase shows insecticidal activity against TMB. A shrinking of the abdomen was observed in the TMB incubated with a tea shoot treated with chitinase (0.048 U/mL), indicating that the chitinase caused damage to the gut of TMB by digesting the peritrophic membrane. This mechanism leads to 100% TMB mortality under *in-vitro* conditions. Whereas no mortality was observed in TMB treated with water as a control (Fig. 3). The results of this study indicate that *P. fluorescens* MP-13 shows a strong efficacy in causing mortality of the phytophagous insect TMB.

Discussion

Due to the high toxicity of chemical pesticides to human beings, animals and beneficial insects, the use of chemical pesticides is being replaced by environmentally friendly biopesticides, which is an alternative approach to control damage caused by insect pests. Native strains of fluorescent pseudomonads were isolated from the tea rhizosphere and evaluated for their insecticidal activity against the red spider mite, *Oligonychus coffeae Nietner* in tea (Tsagou et al., 2004). The bioassay with four hundred *Bacillus thuringiensis* showed that the strains (S29, S40, S616, S1168, S1576), presented the toxicity against *Aphis gossypii*, causing mortality rates greater than 50% on average (Mendonsa et al., 1996).

Instead of using entire living microorganisms as a foliar application, the use of selective metabolites like chitinase which are produced by antagonistic microorganisms, is advantageous (Shternshis, 2005). Chitinase acts as both a contact and a systemic toxic component to kill the insects (Broadway et al., 1998) and hence this study will help in developing new strategies in pest management in tea plantations, as well as opening a new avenue in the formulation of chitinase based biopesticides. Microbes are potential sources of insecticidal enzymes like chitinase. The production of inexpensive chitinase has received attention as a potential biocontrol molecule for the control of many pests. It is an emerging field of research, and has been evaluated for a limited number of pests (Downing et al., 2000; Kramer et al., 1997). Bacterial chitinases are active at an alkaline pH; hence, they may degrade the chitin which is present in the gut lining of insects (Bhattachrya et al., 2007). Chitin metabolism is considered to be an excellent target for selective pest control (Kramer and Muthukrishnan, 1997; Shternshis, 2005). This affects insect digestion, and it directly inhibits the insect growth and development and leads to insect death.

Exotoxins of a variety of bacteria, including *P. fluorescens* (Ajit et al., 2006; Prabakaran et al., 2003), *P. pseudomallei* (Lee et al., 2000) and *P. aeruginosa* (De Barjac, 1989) are known to be toxic to insect pests and plant pathogenic fungi. These exotoxins are reported to

be chitinases. Exotoxins present in the culture filtrate of *P. fluorescens* were found to be effective in killing the pupae of three species of vector mosquitoes, viz., *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes Aegypti* (Kramer and Muthukrishnan, 1997). When an emulsifiable concentrate formulation, prepared with a culture filtrate of this bacterium, was tested against *C. quinquefasciatus* in field conditions, more than 80% mortality was observed after 12 days (Sadanandane et al., 2003). These results indicate that the metabolite present in the culture filtrate of *P. fluorescens* could be a promising candidate for mosquito control. In the present work, the efficacy of chitinase from *P. fluorescens* against TMB has been studied.

A 16S rRNA sequence alignment of *P. fluorescens* MP-13 using BLASTn software for a comparison of up to 1,500 bp gave a high homology (97%) with *P. fluorescens* (HM356022) and (89%) *P. monteilii*-JRI (CP005960). The results of the phylogenetic analysis revealed that *P. fluorescens* MP-13 was separated clearly with a high phylogenetic resolution when compared to other *Pseudomonas* species (Fig. 2). In our earlier study, single step purification of chitinase with a molecular weight of 30 kDa was achieved (Suganthi et al., 2015), and the optimum culture conditions for chitinase production were determined. Reports are available for the degradation of insect gut peritrophic membrane by chitinase. Brandt et al. (1978) observed an interesting note in *Orgyia pseudotsugata*, where chitinase enzyme caused perforations in the gut peritrophic membrane, and thereby facilitated the entry of pathogens into the hemocoel of susceptible insects. *Spodoptera* fifth instar larvae were fed on a diet containing recombinant ChiAII (*Serratia marcescens*) which led to the perforation of peritrophic membranes and further insect death (Regev et al., 1996).

Up till now, no investigations on the effect of bacterial chitinase against tea pests has been carried out. It is evident that, at the specific concentrations tested, chitinase (0.048 U/ml) can induce 100% mortality after 72 h in TMB adults. This study provides evidence for the presence of inducible, extracellular chitinolytic enzymes in *P. fluorescens* that contribute to the strain's insecticidal activity. Based on the observations presented in Fig. 3, the insecticidal activity of *P. fluorescens* MP-13 chitinase is capable of affecting the growth of the insects by digesting its peritrophic membrane, which is a protective sleeve for the gut epithelium of TMB. Chitinase binds to the gut region of TMB, which causes the swelling of mitochondria, endoplasmic reticulum and vacuoles, followed by lysis of epithelial cells due to midgut perforation. This mechanism prevents the insect from feeding on tea leaves and finally leads to death. A recent report discusses that the chitinase of *P. fluorescens* efficiently hydrolyzes cuticular protein chitin as, well as the peritrophic membrane of *Culex quinquefasciatus* (Wang et al., 2010). Mendonsa et al. (1996) reported that enzymes from *Myrothecium verrucaria* bacterium effectively degrade insect cuticles, and 100% mortality of *Aedes aegypti* mosquito within 48 h was observed.

The results of the present work have confirmed that *P. fluorescens* MP-13 strain is the best producer of extracellular chitinase and leads to 100% mortality in TMB. Further field studies required large quantity of chitinase, hence the chitinase gene must be over expressed in the *E. coli* system. The application of chitinase may be achieved by spraying the enzyme directly onto the tea plants. Chitinase-sprayed leaves and fruits of strawberry plants showed no presence of insects or pathogenic fungi (Koga, 2005). In future, chitinase-based biopesticides can be effectively utilized for the management of TMB.

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