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Induction of Biochemical Changes in Santa Teriza Lime Leaves by Chitosan Application Influence Citrus Leaf Miner Damage

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Abstract

The effect of foliar spray of young lemon trees with Chitosan (CH) (200 - 300 - 400 ppm), on antioxidant enzymes activities (peroxidase POD, polyphenol oxidase PPO) and total phenolic content, and their influence on leaf miner (*Phyllocnistis citrella*) activity (tunnel length and damage density) under field condition was investigated. The results showed that treatment with chitosan 300 ppm enhanced the total phenolic content (5.975 mg/g) and the POD activity (0.533 min^{-1} mg^{-1} protein), while PPO activity was increased by chitosan 200 ppm (1.394 ΔA/min/g FW). The results demonstrated as well that chitosan treatment has a beneficial effect in reducing leaf miner activity, by decreasing tunnel length and damage density.

Keywords

Citrus, Chitosan, Peroxidase, Polyphenol Oxidase, Total Phenolic Content, *Phyllocnistis citrella*

1. Introduction

Citrus which belongs to the family Rutaceae, is one of the most major fruit crops, widely distributed over the world, and includes oranges, lime, and grapefruits… and is native to tropical and sub-tropical regions of the world [1].

Citrus fruits are characterized by their nutritional value and special flavor, they are consumed mostly as fresh or as juice, they are rich in carbohydrates (such as sucrose, glucose and fructose), and vitamins such as V. C and B, they have low content of protein and fats. They are considered as an important source of fiber, which makes them important to prevent digestive diseases [2].

Citrus fruits are rich as well in minerals such as Fe, Mg, K and Na… [3], and antioxidants [4] and flavonoids [5], which gave a valuable medical importance in preventing influenza and cancers [6].

However, citrus cultivation always faces several pest attacks. The infection of citrus leaf miner *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) CLM, is one of the most harmful pests that mostly attacks nurseries, young plantations and tender flushes. CLM is an epidermal tissues herbivore feeding, forming serpentine tunnels on leaf surface. As a mining result, the mesophyll cells, (which are photosynthetic area) are destroyed [7] [8], and leaf surface is deformed [9]; this ultimately reduces tree photosynthetic capacity [10]. CLM is considered as the primary responsible for spreading bacterial citrus disease *Xanthomonasaxonopodis* (*Hasse*) *pv. Citri* [11] [12] [13].

There is a worldwide trend to explore new solutions for controlling plant diseases, giving priority to methods that are efficient, reliable and safe to the environment.

Chitosan (CH) is a natural biopolymer (a deacetylate of chitin), a compound eco-friendly and characterized by unique chemical properties, found in fungal cell walls, and can be easily obtained from shellfish wastes. It can be used in different industries, medicine and agriculture [14].

Plants can protect themselves by morphological and biochemical mechanisms against herbivores, which are mediated by direct and indirect defenses [15]. Direct defense is intervened by plant features that affect the herbivore's biology (mechanical protection such as cell wall strength), or harmful chemical productions such as (alkaloids, phenols, quinones…) which prevent or stop the insects attack [16].

Host plant interaction with herbivore attack is very complicated. Many researches on this relationship of host plant with its natural enemies depended on internal personal bodyguards such as enzymes (antioxidants enzymes (POD, peroxidase, PPO polyphenol oxidase, and catalase CAT…)), that guide critical stage of plant defense.

Under various stresses, plants use an important policy to defend themselves. They generate ROS (hydroxyl radical, OH^-, superoxide radical anion O_2^-, hydrogen peroxide H_2O_2), as a common phenomenon due to abiotic and biotic factors ROS play a signaling role which mediate several responses, and act as toxin [17].

The antioxidants enzymes (such as POD, PPO) and phenolic compounds act as protection system in plants against reactive oxygen species, phenolic compounds have the ability to scavenge free radicals, giving hydrogen atoms or electrons [18].

PPO is considered as a plant defensive protein, it causes anti-nutritive modification of plant proteins upon cell herbivores invasion [19].

On other hand, PPO regulated plastid oxygen levels, and contributed in phenolics biosynthesis and wounds remedial [20] [21].

Chitosan enhances plant growth, yield and stimulates defense mechanisms, it is natural elector of plant defense against pathogenic attack before and after harvest and considered as antifungal [22], antibacterial [23], antiviral [24], and could be used as bio insecticides [25].

Its effects on plant defense mechanisms are still obscure. It depends on chitosan structure and concentration [26] [27] [28], plant species [29] and developmental stage [30].

Chitosan may play a role in phenolic syntheses, by enhancing chitinase and chitosanase synthesis (PR: they are members of a group of plant pathogenesis related proteins), and could damage pathogenic cell walls and may encourage plant defense [31], and can induce many defense genes in different plant species (such as rice) [32]. Reference [33] demonstrated its insecticidal activity against herbivores (Lepidopterous and Homopterous), and its application encourage the mortality of *Plutellaxylostella*, which reached 80%. Chitosan application at (5000 mg/g) was affective in stopping larval weight gain of *Spodoptera littoralis* (cotton leaf warm) after 4 days of feeding [34], and inhibited as well *Tuta absoluta* development and degraded eggs lying [35].

Foliar application of chitosan enhanced cucumber growth parameter affected by cucumber mosaic virus, and promoted leaf chlorophylls content, phytohormones (*i.e.*, indole acetic acid, gibberellic acid, salicylic acid and jasmonic acid), non-enzymatic antioxidants (*i.e.*, ascorbic acid, glutathione and phenols) and enzymatic antioxidants (*i.e.*, superoxide dismutase, peroxidase, polyphenol oxidase, catalase, lipoxygenase, ascorbate peroxidase, glutathione reductase, chitinase) [36].

When *Pinus koraiensis* seedlings were grown for eight days in DCR media under different chitosan concentrations, it showed an improvement of seedling polyphenols accumulation [37].

Foliar application of chitosan increased H_2O_2 in *Dracocephalum kotschyi*, and enzymatic activities (guaiacol peroxidase, catalase and phenylalanine ammonium lyase) and non-enzymatic (total phenols and flavonoids) and stimulated nutrient absorption which encourage the accumulation of macro elements [38].

This study aimed to improve physiological characteristics of santa teriza young trees sensible to CLM by enhancing defense enzyme activities (POD and PPO), and leaf total phenolic content, which is considered of a great importance in limiting CLM infection.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted under field condition during 2021 in Tartus suburb (Syria), on Santa Teresa citrus lemon young trees (4 years old), young trees of Santa Teresa were brought from the nursery, and planted in field in Tartus suburb, and let to grow normally until they reached 4 years old, during this time, nutrients like nitrogen, phosphor and potassium were added to the

soil. The young trees were irrigated when needed.

2.2. Chitosan Treatment

Different chitosan concentrations were applied four times in February by 15 days interval. Treatments were: T0 Control (treated with distilled water), T1 Chitosan 200 ppm, T2 Chitosan 300 ppm, T3 Chitosan 400 ppm, and Tween-20 was added (2 ml/l).

Complete random design was used, and one young tree was considered as replicate, repeated 3 times.

2.3. Enzyme Supernatant Preparation

Fresh leaves affected by CLM larval, were washed with distilled water and kept frozen until analysis at Tishreen university labs., 2 g was homogenized in 7 ml cold potassium phosphate buffer (pH = 7, M = 0.1), and centrifuged at 10,000 rpm for 10 min at 4˚C, and the supernatant was used to determine POD and PPO activity. All steps of the extraction procedure were carried out at 1˚C - 4˚C. PPO and POD activity was determined using a spectrophotometric method. Blank sample contained the same mixture solution without enzyme extract.

2.4. POD Activity

Peroxidase activity was determined using guaiacol as hydrogen donor, according to [39]: 200 µL of the supernatant was added to 3.5 ml of potassium phosphate buffer, 200 µL of (0.25% v/v) guiacol and 200 µL 0.1 M H_2O_2, the reaction was followed calorimetrically at 470 nm in spectrophotometer (JASCO-Japan) for 3 minutes. POD activity was expressed as increasing in absorbance at 470 nm min^{-1} mg^{-1} protein.

2.5. PPO Activity

1.95 mL of Phosphate buffer solution (0.1 M, pH 7), 1 mL of 0.1 M catechol as a substrate and 50 µL of the enzyme extract were pipetted into a test tube and mixed thoroughly. The mixture was rapidly transferred to water path (25˚C). The absorbance at 410 nm was recorded continuously for 5 min. PPO activity was expressed as increasing in absorbance at 410 nm (ΔA/min/gFW), according to [40] [41].

2.6. Determination of Total Phenolic Content TPC

Fresh infected leaves were washed with distilled water, and 2 g was homogenized in 15 ml of 80% (v/v) ethanol and centrifuged for 15 min at 10,000 rpm. The extract was infiltrated, and the residue was extracted again with 80% (v/v) ethanol, centrifuged again for 15 min at 10,000 rpm. 40 µl of extract was added to 200 µl Folin-Ciocalteu reagent + 3.16 ml distilled water and 600 µl of 20% Na_2CO_3. Then, the mixture was shaken and kept in dark for 1.5 h. The absorbance at 720 nm (at 25˚C) was recorded with spectrophotometer, using glass cuvettes at 720

nm (25˚C) and Catechol standard curves (from 0 - 400 mg /l). Blank tube contained 40 µl of methanol. Total phenols were expressed as milligrams of Catechol acid equivalents per gram of fresh extract, according to [42].

2.7. Tunnel Length

Using (Scale Master Pro-China) devise, leaves with full tunnels (the larva reached up the pupae) or leaves with dead larva tunnels were used.

2.8. Damage Density

Using the formula:

Number of tunnels/number of injured leaves.

The number of tunnel was counted on each injured leaf, on both sides, on all branches.

2.9. Statistical Analyses

Analysis of variance (One way ANOVA) was applied using the Gen stat 12 statistical program, and differences between means were evaluated with Duncan's test (LSD 0.05).

3. Results and Discussion

3.1. Chitosan Effect on POD Activity

POD activity was affected by chitosan treatments, T2 (CH 300 ppm) enhanced significantly enzymatic activity (0.533 mm^{-1} mg^{-1} protein), compared to control (0.237 mm^{-1} mg^{-1} protein), followed by T1 (CH 200 ppm) (0.483 mm^{-1} mg^{-1} protein), T3 (CH 400 ppm) (0.341 mm^{-1} mg^{-1} protein), **Figure 1**.

Plant under biotic stress start to generate ROS, this is an effective mechanism against the pathogenic infection, which induce oxidative stress, that could generate specific genes, responsible on antioxidant response [43], by activating antioxidant enzymes (such as Superoxide dismutase (SOD), Peroxidase (POX) and Catalase (CAT)) responsible on scavenging ROS.

According to [28], chitosan increased the accumulation of H_2O_2, improved the activities of phenylalanine ammonialyase and chitinase, improved as well

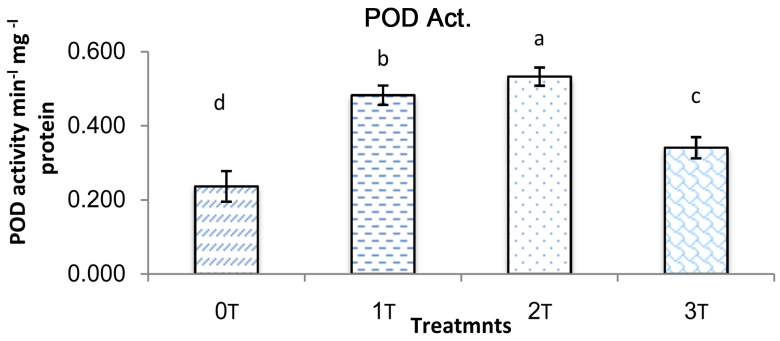

Figure 1. Chitosan effect on POD activity, LSD = 0.05808.

transcription of defense related genes (b-1,3-glucanase and chitinase) and accumulation of pathogen related protein (PR1). The increase of POD activity in our results, may be due to the increase of H_2O_2 concentration, as a result of CLM attack in lemon leaves, this is considered as a major plant defense mechanism [44] [45] [46].

These results are in consistent with [47], which demonstrated that treatment of wheat seedling with Oligochitosan promoted POD, CAT and SOD.

3.2. Chitosan Effect on PPO Activity

PPO activity was increased by Chitosan treatments, T1 (CH 200 ppm) enhanced PPO activity (1.394 ΔA/min/gFW) compared to control (1.325 ΔA/min/g FW), while other treatment didn't record any enhancement in PPO activity, **Figure 2**.

PPOs are known to oxidize phenols to quinones, which is implicated in pest resistance.

It was found that feeding ability of cotton bollworm (*Helicoverpa armigera*) on foliage transgenic tomato, which contain an overexpressing PPO was less than those feeding on non-transformed tomato, which indicate that tomato PPO plays an important role in cotton bollworm resistance [48].

PPO catalyze two distinct reactions: the o-hydroxylation of monophenols to o-diphenols and the oxidation of o-dihydroxy phenols to o-diquinones [49], these quinones have a high activity and are quickly polymerized leading to form brown and black pigments that affect the quality and nutritive value of fresh plants [50] [51].

Quinones as well at pH < 4 submit to reactions that results semiquinone leading to ROS generation, which may perform as part of signal transduction pathways for establishment of plant immunity [52]. Reference [53] found that H_2O_2 is able to encourage several defense genes, including PPO and proteinase inhibitors I and II, that could lead to improved levels of disease and insect resistance.

Figure 2 showed that chitosan (T1) improved PPO activity compared to the control and other treatments, which is in accord with [54], who indicated an increase in PPO and POD enzymes activity in tomato fruit. Reference [55] showed as well, that treatment with chitosan increased PPO and POD activity in palm roots.

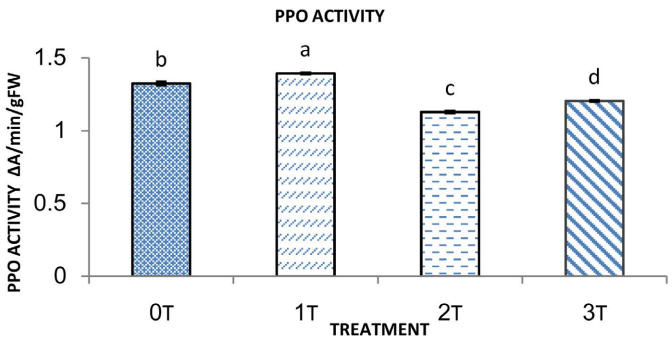

Figure 2. Chitosan effect on PPO activity, LSD = 0.01597.

3.3. Chitosan Effect on Total Phenolic Content TPC

Total phenolic content in lemon leaves was stimulated by chitosan treatment, T2 (CH300 ppm) compared to the control (5.975 mg/g; 5.373 mg/g) respectively, while TPC in other treatment T1, T3 were not affected, Figure 3.

Chitosan is known to regulate gens, which are responsible on phenylalanine ammonia-lyase (PAL) biosynthesis [56], this enzyme controls phenolic biosynthesis [57]. In our results, chitosan at 300 ppm stimulated TPC in lemon leaves, referring to Figure 2, chitosan decreased PPO activity at the same concentration, this indicates that phenolics didn't oxidize by PPO and accumulated in the leaves. T1 (200 ppm) stimulated PPO (Figure 2), phenols content didn't increase, it is possible that PPO oxidize phenols to quinone or other products. Chitosan at (400 ppm T3) decreased PPO activity (Figure 2), and phenolic didn't affect, it is possible that there are other factors that influenced TPC.

This result is in agreement with [58], who indicated chitosan enhanced cytosolic H^+, oxidative bursts and phytoalexins (such as terpenoids, isoflavones, alkaloids and phenolics).

3.4. Chitosan Effect on CLM Biology (Tunnel Length, Damage Density): Tunnel Length

Chitosan application at different concentrations decreased significantly tunnel length, the heights reduction in tunnel length was at T3 (CH 400 ppm), followed by T1 and T2 (49.75, 61.5, 66.2 mm) respectively, compared to the control (89.2 mm), Figure 4.

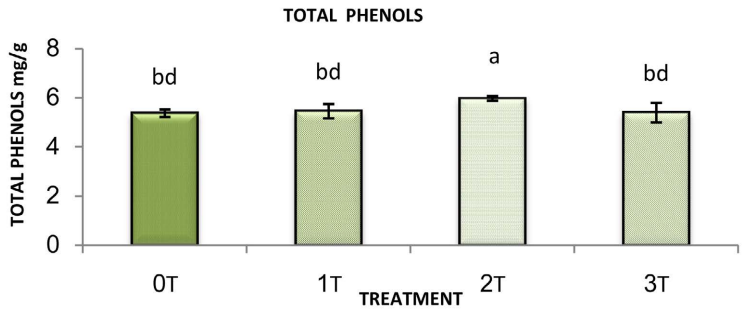

Figure 3. Effect of chitosan on total phenolic content, LSD = 0.4959.

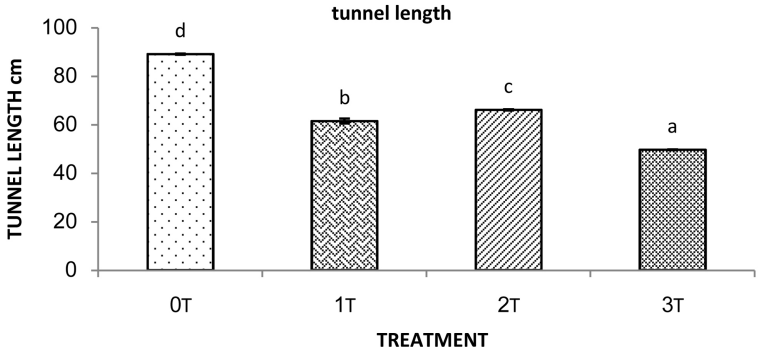

Figure 4. Effect of chitosan on tunnel length, LSD = 1.00.

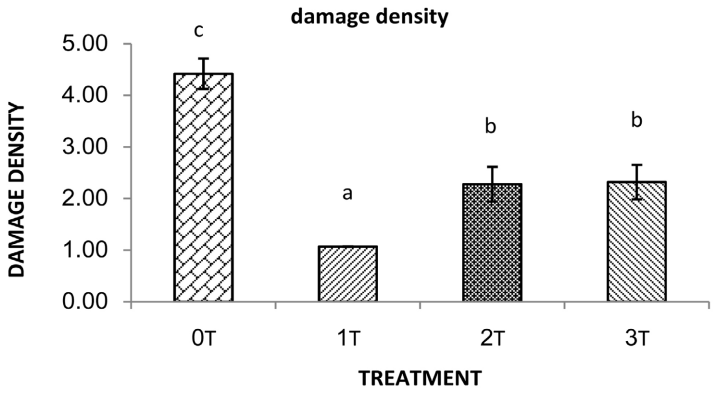

Figure 5. Effect of chitosan on damage density, LSD = 0.526.

The effect of chitosan may be due to the reduction in larva diet [34]. Chitosan effect on phenolic content may have an influence on the nature of cell compounds which may be not favorable to the larva nutrition, it is possible as well that chitosan has toxic effects, this results in accord with [33], and with [59] who showed that chitosan increased phytoalexins compounds which play a major role in plant defense.

3.5. Damage Density

Chitosan treatment was effective in reducing damage density, T1 CH 200 ppm was the most effective in damage density reduction (1.067), compared to the control (4.423). T2 CH 300 ppm and T3 CH 400 ppm were effective as well (2.28 - 2.323) alternatively, **Figure 5**.

Damage density results of tunnel numbers and not of tunnel length. Chitosan treatments decreased tunnel length which may due to the reduction of larva nutrition; this may reflect on larva activity and as consequence tunnel numbers and damage density. Reference [60] showed that PPO and POD enzymes oxidize phenolic compound to quinones, which may interact with leaves proteins, and affect the amino acids availability. Amino acids may be converted to alkaloids (which lower the proteins nutritional value in plants) which may affect the insects feeding [61] [62]. Other studies indicated that quinones have a toxic effect on herbivores [63]. Chitosan stimulated PPO and POD activities which explain its effect on damage density.

4. Conclusions

We can conclude that chitosan treatment by stimulating PPO and POD, and other factors not studied in this experiment, affected TPC which reduced CLM attack by decreasing tunnel length and damage density.

To our knowledge we are the first to study the effects of chitosan on citrus leaf miner CLM.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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