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Proceeding Paper

Response of *Cucumis sativus* to Spirotetramat Application Leads to Changes in Salicylic Acid, Antioxidative Enzymes, Amino Acids, Mineral Elements, and Soluble Carbohydrates †

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Abstract: Plants are influenced by pesticides in terms of various physio-biochemical parameters. This study provides initial evidence on the effect of the insecticide spirotetramat on plant physiological characteristics as a non-target organism. Cucumber plants (*Cucumis sativus* L.) exposed to spirotetramat were studied 10 days after treatment. There was an increase in the activity of antioxidant enzymes including superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, and phenylalanine ammonia-lyase. Malondialdehyde, total chlorophyll, hydrogen peroxide contents, and electrolyte leakage index was not affected by spirotetramat. Further biochemical analyses revealed an increase in the content of some free amino acids, as well as sucrose, glucose, and fructose. The amount of salicylic acid and also minerals calcium, manganese, copper, zinc, iron, nitrogen, and magnesium, were elevated in spirotetramat-treated plants. Results have shown that spirotetramat can manipulate cucumber plant physiology through induces biochemical responses that are reflected in changes of antioxidative enzymes, amino acids, soluble carbohydrates, salicylic acid, and mineral elements. The findings of this study provide an insight into the side effects of spirotetramat as a chemical with no specific target site in plants that show no adverse effects on plant health indices. This study focuses on observed physiological changes related to toxicity in plants exposed to hazardous substances present in the environment that can improve our knowledge and understanding of the underlying effects of xenobiotics on plants.

Keywords: antioxidative system; biochemical changes; host plant; oxidative stress; physiological responses

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1. Introduction

Control of crop pests mainly depends on pesticide utilization. Being absorbed by plants through the leaf surface, pesticides may influence plant immune system [1]. Spirotetramat, a tetramic acid derivative, is an effective insecticide against sucking pests such as whiteflies that translocate in xylem and phloem (ambimobile) [2]. Like other insecticides, spirotetramat is synthesized based on insect physiology and has no known recognized site of action in plants. Having absorbed by the plant, the active ingredient is transformed into a biologically active form named spiroteramat-enol that is the most prominent compound [2].

As important inducers of abiotic stress, pesticides have profound effects on plants physiology in agricultural ecosystems [3] and trigger a wide range of defense reactions that provide the regulatory potential to conserve plant fitness [4]. However, there are few studies that have evaluated the plant immune systems in response to insecticides. The present study attempted to investigate the biochemical responses of cucumber plants, as a non-target organism, to spirotetramat by exploring various physiological mechanisms.

2. Materials and Methods

The seeds of greenhouses cucumber (cultivar hybrid super N3) were planted in sterilized soil composed of 1/1/2 cocopeat/peat moss/perlite and then were grown in a greenhouse under controlled conditions. They were irrigated every three days up to the soil capacity with distilled water. Cucumber seedlings were grown for 30 days untill they reached the intended phenological stage (6–8 true leaves), at which stage they were sprayed with the insecticide spirotetramat (Movento® SC 100, Bayer CropScience, Germany) at the recommended dose for the tobacco whitefly in the greenhouse (50 mg a.i./L). Simultaneously, control plants were sprayed with distilled water. After spraying the whole cucumber leaf surfaces to the point of runoff, all of leaves were harvested at 10 days after treatment, as the most efficient time for whiteflies control.

For superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPX) activity, the method of Homayoonzadeh et al. [5] was used. The SOD activity which measures inhibition of the photochemical reduction of NBT spectrophotometrically at 560 nm was carried out for 20 min at 25 °C both under a fluorescent light and in the dark. CAT activity was assayed using H_2O_2 as substrate and measurement at 240 nm. For GPX activity, absorbance of the formed tetraguaiacol was determined at 470 nm using guaiacol as a substrate. Assessment of ascorbate peroxidase (APX), glutathione reductase (GR), and phenylalanine ammonia-lyase (PAL) activities was done based on the method of Homayoonzadeh et al. [6]. For APX activity H_2O_2 and ascorbic acid were used as substrate and reductant, respectively. Decrease in absorbance at 290 nm was recorded due to oxidation of ascorbic acid. GR activity was carried out with recording absorbance at 412 nm, due to formation of TNB resulted from the reaction of DTNB with GSH. PAL activity was determined based on the rate of production of cinnamic acid (CA) and phenylalanine was used as the substrate and after incubation at 37 °C. The extraction of CA product by ethyl acetate was performed and then resuspended in sodium hydroxide and measured at 290 nm. Contents of malondialdehyde (MDA), total chlorophyll, and salicylic acid (SA) were measured using the method as claimed by Homayoonzadeh et al. [7]. MDA content was quantified using the TBA test and products were analyzed colorimetrically at 532 nm. Total chlorophyll were extracted using 100% acetone and quantified with read of absorbance at 662 nm for Chl *a*, 645 nm for Chl *b*, and 470 nm for Car. SA extraction was carried out using methanol and subsequently quantified with LC-9A HPLC system equipped with a UV/VIS detector. The separation was performed on a GLC-ODS C18, 150 mm × 6 mm i.d. column, at 40 °C and 24 psi internal pressure with a 10 min run time. Separation was carried out isocratically with a mobile phase composed of methanol/water (70/30) with a flow rate of 1 mL min^{-1}. The detection wavelength was 235 nm. The concentration of H_2O_2 as well as electrolyte leakage index (ELI) was estimated according to the method as described by Homayoonzadeh et al. [8]. H_2O_2 content measurement is based on potassium iodide oxidation by H_2O_2 in acidic medium. The absorbance of the reaction mixture was measured at 390 nm. Assessment of ELI was done using a platinum electrode, and then the percentages of initial to final conductivity were recorded.

Water extraction of amino acids was carried out based on the method of Wayne [9] using sterile deionized water. After extraction, analysis was carried out by HPLC with ortho-phthalaldehyde precolumn derivatization, separation on a 120-3-C18 H column (250 mm × 3 mm i.d.) and monitored with a fluorescence detector. The solutions were detected using the following conditions: 30 min run time, 0.6 mL min^{-1} flow rate, column

temperature 30 °C and λ excitation at 330 nm and λ emission at 450 nm. Individual sugars including sucrose, glucose, and fructose were extracted based on the method of Meyer and Terry [10] with 62.5% methanol. Contents of sucrose, glucose, and fructose were measured using an HPLC system. The samples were injected into a Eurokat H column of 300 mm × 8 mm i.d. The mobile phase was sulfuric acid 0.01 N at a flow rate of 0.5 mL min^{-1}. The column temperature was held at 30 °C. Elemental analysis was carried out following the method of Zafar et al. [11]. According to this method, samples were chemically digested using a mixture of nitric acid, sulfuric acid and perchloric acid (5/1/0.5) and then analyzed for the elements of interest utilizing atomic absorption spectrophotometer AA-680 with suitable hollow cathode lamps. Contents of different elements were determined using calibration curves constructed using standard solutions of the elements calcium (Ca), manganese (Mn), copper (Cu), zinc (Zn), iron (Fe), and magnesium (Mg). Nitrogen (N) content was determined by the Kjeldahl method [12].

Experiments consigned to a completely randomized design with three independent biological replicates. After the data passed Shapiro–Wilk's test for normality and Levene's test for equality of variances, an unpaired *t*-test was used to analyze. All analyses were carried out in GraphPad Prism version 8.2.0.

3. Results

The activity of antioxidant enzymes generally increased in response to spirotetramat treatment. Specific activities of SOD, CAT, and GPX were higher in treated plants compared to control counterparts, in 1.61-, 1.21-, and 1.33-fold, respectively. In addition, results for APX, GR, and PAL specific activities showed a similar increasing trend with 1.43-, 1.29-, 1.44-fold, respectively (Table 1). There were no significant changes in contents of plant health indices consisting of MDA, total chlorophyll, H_2O_2 in response to treatment. In contrast, SA amount in treated plants increased 1.37-fold (Table 2).

Further analyses on sugar also showed accumulated more sucrose (2.54-fold), glucose (2.51-fold), and fructose (2.59-fold) (Table 3). Also, treated plants had higher contents of Ca (1.05-fold), Mn (1.63-fold), Cu (1.58-fold), Zn (1.71-fold), Fe (1.48-fold), Mg (1.05-fold), and N (1.19-fold) than the control (Table 4). Measurement of amino acids content demonstrated that spirotetramat-treated plants had more arginine (2.47-fold), cysteine (2.23-fold), GABA (1.91-fold), glutamic acid (2.38-fold), glutamine (3.49-fold), glycine (2.47-fold), isoleucine (1.63-fold), lysine (3.06-fold), methionine (2.07-fold), ornithine (5-fold), phenylalanine (2.89-fold), tryptophan (2.57-fold), and tyrosine (2.2-fold) contents than the controls. However, concentrations of alanine, asparagine, aspartic acid, histidine, leucine, serine, threonine and valine did not differ between treatment and control (Table 5).

4. Discussion

Treatment with the spirotetramat, Movento® SC 100, induces physiological and biochemical responses in cucumber plants at 10 days after treatment and that is reflected in changes in the metabolism of antioxidant enzymes, salicylic acid, amino acids, mineral elements, and soluble carbohydrates. The activity of SOD, CAT, GPX, APX, GR, and PAL (Table 1) was elevated in spirotetramat-treated seedlings. As with our findings, Homayoonzadeh et al. [8] demonstrated that application of imidacloprid and dichlorvos at the recommended dose in cucumber plants improved SOD, CAT, APX, GPX, GR, and PAL specific activities. Also, Shakir et al. [13] reported increased activities of SOD, GPX, CAT, and APX in tomato shoots subjected to different levels of the emamectin, cypermethrin, and imidacloprid. Moreover, the application of imidacloprid and phosalone at recommended doses is reported to enhance levels of SOD, CAT, APX, GPX, GR, and PAL in *Pistachio vera* seedlings [6].

Plant health indices containing MDA, chlorophylls, H_2O_2, and ELI [14] that were not influenced by spirotetramat application (Table 2) illustrated the key issue that Movento®

has no adverse effect on cucumber seedlings at the recommended dose. These results also show spirotetramat has no unfavorable impacts on membrane permeability, cell viability, and photosynthesis system. The metabolism of signaling molecules such as SA can also be regulated by xenobiotics [15]. In the present study, SA level increased in cucumber seedlings exposed to spirotetramat (Table 2). According to Szczepaniec et al. [16] the application of imidacloprid on tomato seedlings as well as thiamethoxam on cotton plants results in increased levels of SA. Ford et al. [17] also observed that the content of SA increased in *Arabidopsis thaliana* in response to application of clothianidin.

Soluble carbohydrates contribute significantly to plant responses to the stresses and therefore are commonly recognized as giving rise to the concept of sweet immunity due to their crucial functions in the plant defense system [18]. Cucumber plants showed an increase in soluble carbohydrates content in response to spirotetramat (Table 3). The findings of our study corroborate the pervious findings that the application of imidacloprid and phosalone showed a rise in the content of soluble carbohydrates in the pistachio seedlings [6]. Also, Homayoonzadeh et al. [8] reported an enhanced level of total soluble carbohydrate in cucumber plants in response to imidacloprid and dichlorvos at the recommended dose.

The toxic effects of pesticides can be mitigated by nutrients [19]. In this study, the contents of Ca, Mn, Cu, Zn, Fe, N, and Mg were elevated upon exposure to spirotetramat (Table 4). This may be related to increasing uptake and transport of nutrients from the environment [20]. Amino acids are building blocks for proteins, which are involved in enzyme activity and redox homeostasis [21]. They can also be modified in plants exposed to stress to produce specific secondary metabolites [22]. Cucumber seedlings exposed to spirotetramat showed changes in the contents of amino acids (Table 5). It is well-documented that amino acids have a central role in the detoxification of xenobiotics in plants by conjugation reactions which results in a conjugate with a higher molecular weight that is more water-soluble and is usually more susceptible to further processing in the plants [23].

In conclusion, this study focuses on observed physiological changes in plants exposed to hazardous substances present in the environment, such as insecticides. The results of this study can add to our understanding of the underlying consequences associated with the toxic state of xenobiotics on plants. There were metabolic changes in cucumber plants in response to spirotetramat application. Investigation in metabolic pathways of treated plants with insecticides could be targeted for future insecticide synthesis and their application in pest control tactics.

5. Figures and Tables

Table 1. Mean (±SE) specific activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and phenylalanine ammonia-lyase (PAL) (μmol min^{-1} mg^{-1} protein) in cucumber seedlings after exposure to recommended dose of spirotetramat (Movento®). Asterisks were used to show statistically significant difference between treated and non-treated plants.

Enzymes	Control	Treated	*t*-Value	*p*-Value
SOD	0.359 ± 0.026	0.578 ± 0.027 *	4.656	0.014
CAT	0.046 ± 0.002	0.056 ± 0.003 *	3.753	0.039
GPX	0.266 ± 0.018	0.354 ± 0.024 *	2.509	0.045
APX	0.359 ± 0.025	0.516 ± 0.041 *	2.107	0.032
GR	0.561 ± 0.021	0.724 ± 0.032 *	3.076	0.013
PAL	0.207 ± 0.003	0.299 ± 0.004 *	4.082	0.026

Table 2. Mean (±SE) contents of malondialdehyde (MDA), total chlorophyll (Chl), hydrogen peroxide (H_2O_2), salicylic acid (SA) (μg g^{-1} fresh weight) and electrolyte leakage index (ELI) (%) in cucumber seedlings after exposure to recommended dose of spirotetramat (Movento®). Asterisks were used to show statistically significant difference between treated and non-treated plants.

Parameters	Control	Treated	*t*-Value	*p*-Value
MDA	0.469 ± 0.026	0.458 ± 0.037	0.656	0.095
Chl	45.14 ± 5.021	49.56 ± 6.013	0.753	0.088
H_2O_2	9.376 ± 0.518	8.964 ± 0.424	0.819	0.076
SA	0.357 ± 0.029	0.491 ± 0.041 *	4.852	0.049
ELI	15.31 ± 0.413	14.78 ± 0.524	0.922	0.066

Table 3. Mean (±SE) concentration of sucrose, glucose, and fructose (mg g^{-1} fresh weight) in cucumber seedlings after exposure to recommended dose of spirotetramat (Movento®). Asterisks were used to show statistically significant difference between treated and non-treated plants.

Parameters	Control	Treated	*t*-Value	*p*-Value
Sucrose	1.22 ± 0.261	3.11 ± 0.581 *	5.513	0.039
Glucose	0.91 ± 0.101	2.29 ± 0.011 *	4.413	0.024
Fructose	0.32 ± 0.008	0.83 ± 0.003 *	3.844	0.016

Table 4. Mean (±SE) contents of minerlas (mg g^{-1} fresh weight) in cucumber seedlings after exposure to recommended dose of spirotetramat (Movento®). Asterisks were used to show statistically significant difference between treated and non-treated plants.

Parameters	Control	Treated	*t*-Value	*p*-Value
Calcium	23.73 ± 0.161	25.11 ± 0.181 *	5.623	0.045
Manganese	0.058 ± 0.015	0.095 ± 0.011 *	4.523	0.035
Copper	0.012 ± 0.008	0.019 ± 0.003 *	3.954	0.027
Zinc	0.007 ± 0.001	0.012 ± 0.002 *	2.921	0.017
Iron	0.138 ± 0.003	0.205 ± 0.005 *	5.489	0.048
Magnesium	13.97 ± 0.19	14.68 ± 0.11 *	4.952	0.031
Nitrogen	0.031 ± 0.002	0.037 ± 0.003 *	3.101	0.025

Table 5. Mean (±SE) contents of amino acids (mg g^{-1} fresh weight) in cucumber seedlings after exposure to recommended dose of spirotetramat (Movento®). Asterisks were used to show statistically significant difference between treated and non-treated plants.

Amino Acids	Control	Treated	*t*-Value	*p*-Value
Alanine	15.91 ± 0.64	15.74 ± 0.68	0.946	0.870
Arginine	2.76 ± 0.44	6.82 ± 0.29 *	2.302	0.015
Asparagine	5.17 ± 0.45	5.66 ± 0.81	0.529	0.623
Aspartic acid	44.92 ± 2.77	43.92 ± 2.14	0.662	0.789
Cysteine	10.96 ± 0.30	24.41 ± 0.33 *	3.569	0.019
GABA	0.34 ± 0.02	0.65 ± 0.06 *	4.629	0.025
Glutamic acid	68.68 ± 4.85	163.6 ± 4.67 *	5.364	0.037
Glutamine	19.20 ± 0.35	67.13 ± 4.81 *	2.159	0.047
Glycine	4.02 ± 0.26	9.94 ± 0.84 *	3.179	0.011
Histidine	6.09 ± 1.12	9.42 ± 3.13	0.715	0.373
Isoleucine	2.28 ± 0.07	3.73 ± 0.21 *	4.801	0.026
Leucine	1.36 ± 0.06	1.18 ± 0.05	0.805	0.101
Lysine	3.00 ± 0.14	9.18 ± 0.28 *	5.521	0.036
Methionine	1.35 ± 0.25	2.80 ± 0.34 *	4.582	0.026
Ornithine	0.05 ± 0.01	0.021 ± 0.03 *	2.360	0.019
Phenylalanine	2.21 ± 0.08	6.39 ± 0.24 *	3.892	0.0402

Serine	22.10 ± 0.36	21.94 ± 1.03	0.922	0.820
Threonine	14.07 ± 0.36	14.19 ± 0.52	0.452	0.866
Tryptophan	3.92 ± 0.96	10.11 ± 1.75 *	5.520	0.036
Tyrosine	2.65 ± 0.09	5.83 ± 0.02 *	4.852	0.015
Valine	2.38 ± 0.08	2.66 ± 0.07	0.850	0.752

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