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AGROCHEMICALS AFFECT THE ANTIOXIDATIVE DEFENSE POTENTIAL of COTTON PLANTS

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ABSTRACT

Application of insecticides used in cotton fields is often associated with secondary biotic stresses. One of possible reasons of such phenomenon is explained by decreased contents of plants' defense components. As peroxidase (POD) and polyphenoloxidase (PPO) are typical oxidoreductase enzymes scavenging cell oxidative damage, we studied their change levels in cotton leaves in response to the application of three insecticides field experiment. Moreover, the concentration of proline (Pro), methionine (Met) and cysteine (Cys) was studied. The plants were treated with Carbophos, Lannate and Sumi-alfa in early blooming stage at commonly used doses in. Leaf samples were taken on the 10thand 13th days of the treatment. A pyrethroid insecticide Sumi-alfa appeared to negatively impact activities of both POD and PPO ($P \leq 0.05$), contrasting the other two insecticides examined. On the other hand, levels of amino acids with antioxidative properties increased after application of all three insecticides at the end of experiment. Our results show that the oxidative balance of treated plants was interrupted by insecticides (especially Sumi-alfa) with potential impact on vulnerability to secondary stresses. Effects of these insecticides on cotton should be considered and/or studied in more detail for efficient application in agriculture.

Keywords: Cotton plant, Insecticides, Peroxidase, Polyphenoloxidase, Proline, Methionine, Cysteine

INTRODUCTION

Application of insecticides is often reported to be associated with secondary biotic stress. For example increased number of aphids in cotton fields, treated with pyrethroid λ-cyhalothrin against bollworm, has been observed **(Kerns and Gaylor, 1993; Leser, 1994; Ravindhran and Xavier, 1997; Asrorov *et al.* 2014)**. Further, rapid increase in aphid populations after sulprofos and cypermethrin was determined that is unlikely due to direct stimulation of aphid reproduction **(Kerns and Gaylor, 1993)**. Rapid increases in aphid numbers were associated also with applications of the pyrethroid insecticides cypermethrin, and deltamethrin **(Ravindhran and Xavier, 1997)**. Similar observations have been made for different agrochemicals applied on rice **(Wu *et al.* 2001)**. It has been suggested that some agrochemicals might alter the biochemistry of the treated plants **(Leroy *et al.* 2011)** and/or might disrupt the oxidative balance of cells leading to weakening of plant defense. The latter is under normal physiological conditions crucial for optimal functioning of biological processes and in plants is controlled by various antioxidative compounds (e.g. ascorbic acid, glutathione) **(Noori, 2012)** and/or by induction of antioxidative enzymes such as peroxidases, catalases or dismutases **(Zhang** and **Kirkham, 1994)**.

Interruption of oxidative balance results in accumulation of toxic reactive oxygenous species resulting in so called oxidative stress that can seriously injure the tissues. Several pesticides have previously been shown to alter the oxidative balance of cells in mammals and cause cell damages. To these pesticides belong an organochlorine insecticide dieldrin **(Bachowski *et al.* 1998)**, the pyrethroid insecticides Deltametrin **(El-Gohary *et al.* 1999)** and cypermethrin **(Kale *et al.* 1999)**. In plants pyrethroid insecticides were found to decrease catalase and phenylalanine ammonia lyase activities, and total ascorbate and cinnamic acid concentrations in tissues **(Ravindhran and Xavier, 1997; Bashir *et al.* 2007)**. On the other hand, some agrochemical have been suggested as potential plant immunization agents inducing systemic acquired resistance (SAR) in plants, leading to broad-based, long-lasting resistance to a wide range of pathogens **(Enyong Arrey Besong, 2008)**.

The present work was undertaken to study the effect of selected agrochemicals on oxidative balance in cotton plants that might be indicative for explaining the plant vulnerability to secondary stresses. As compounds of antioxidative defense systems we monitored the activity of antioxidative enzymes as well as accumulation of certain amino acids. Of these, peroxidase (POD) was found to have a defense role in eliminating oxidative burst in cotton upon abiotic stress **(Akhunov *et al.* 1999)** and also attack of bacterial blight **(Delannoy *et al.* 2003)**, the phytopathogen *Verticillium dahliae* **(Pshenichnov *et al.* 2011)** and herbivore feeding **(Stout *et al.* 1998)**. POD activity was often found greater in resistant variety than in susceptible **(Akhunov *et al.* 2010)**. On the other hand, polyphenoloxidase (PPO) has been shown to act upon attack of bacterial pathogen *Pseudomonas syringae pv.* **(Li and Steffens, 2002)** and arthropod herbivores **(Felton *et al.* 1989)**, common cutworm *Spodoptera litura* F., cotton bollworm *Helicoverpa armigera* (Hubner) and beet army worm *Spodoptera exigua* (Hubner) **(Thipyapong *et al.* 2007)**. Several previous investigations showed that the activities of these enzymes were affected after application of insecticide imidacloprid at recommended concentration (40 ml/acre) in three Bt cotton hybrids (RCH-134, JKCH-1947, NCEH-6R) **(Kaur *et al.* 2011)** and potato plants **(Chauhan *et al.* 2013)**.

Agrochemicals can also affect nitrogen metabolism **(Slosser *et al.* 2004)** including accumulation of proline (Pro), methionine (Met) and cysteine (Cys) with importance in plant defense. The free Pro has multifunctional role in plant immunity and development, while it is induced by reactive oxygen species signalling **(Ben Rejeb *et al.* 2014)**. Pro is also known to indirectly enhance plant antioxidant defense system **(Hoque *et al.*, 2007; Ozden *et al.* 2009)**. The sulphur containing amino acid Cys plays an important role as an extracellular reducing agent **(Atmaca 2004)**. It is a central metabolite serving as a sulphur donor for the synthesis of Met **(Zagorchev *et al.* 2013)**, which is an efficient scavenger of almost all oxidizing molecules under physiological condition, such as, hydrogen peroxidase, hydroxyl radicals **(Levin *et al.* 1996)**. Under water deficit in cotton plant leaves Met concentration fluctuated widely **(Marur *et al.* 1994)**. Under the effects of cypermethrin Met concentration increased in both organophosphorus insecticide resistant and susceptible plant varieties **(Saleem and Shakoori, 1993)**.

We studied the impact of three agrochemicals organophosphate Carbophos, pyrethroid Sumi-alfa and carbamate Lannate on antioxidative defense components in cotton plants. All three chemicals are being widely used in Uzbekistan for treatment against pests, but their possible (negative) impact on treated plant antioxidative system has not been studied in detail.

MATERIAL AND METHODS

Experimental Design

Field experiments were conducted on cotton variety S 26 (*G. hirsutum*) growing at the pre-bloom stage in the cotton field of the Institute for Plant Protection of the Ministry of Agriculture and Water Resources of Uzbekistan (Tashkent Region, Kibray District, Salar Township). The concentration of each insecticide was prepared according to the consumption norm recommended by producers against cotton pests (Table 1). The solutions were sprayed once in the early morning at 6:00 to 6:30AM. Treatment of plants with three insecticides was a randomized block with 4 replications. Sixteen plants were treated by each insecticide and water was used as control (a total of 4 treatments). The solutions were sprayed once in early morning. A total of 14-16 different developed stages of leaves were taken from the upper, middle, and lower parts of 60-65 plants on the10^{th} and 13^{th} days after treatment that our former results showed the highest changes in the quantity of soluble proteins and sugars were observed on these days **(Asrorov *et al*. 2014)**. They were averaged and lyophilized. During the experiments no effect of insects in the treated fields was observed.

Table 1 Treatment, chemical class and application rates

Treatment	Class	Application rate l/ha
Control		Water
Carbophos, Aerosoyuz, Russia	Organophosphate	0,6
Lannate, Du-Pont, France	Carbamate	0,25
Sumi-alfa, Sumimoto chemical, Japan	Pyrethroid	0,5

Protein Extractions and Analysis

Lyophilized cotton leaves were ground with liquid nitrogen using a mortar and pestle. After grinding, the proteins of control and treated leaves were extracted with Tris-HCl buffer (0.5 M Tris-HCl pH 6.8, 20 mM EDTA, 2 mM PMSF, 1% Triton X-100, and 150 mM DTT) for two hours with stirring. The mixture was filtered and in supernatant proteins were precipitated with cold absolute acetone (1:4 v/v) and centrifuged 30 min (8 000 r/min, at 4^0C). The residue was dissolved in water and freeze-dried. The quantity of soluble proteins was determined according to **Lowry et al. (1951)**. For calibration, albumin bovine (from bovine serum; Sigma A7030) in appropriate amounts was weighed and dissolved in Na_2HPO_4 buffer pH 7 to provide concentrations of 10-100 µg/ml.

POD Assay

The total POD activity of the proteins isolated was determined using benzidine as the chromogenic substrate. Thus, 1.9 ml 0.1 M pH 4.7 acetate buffer (11.5 ml glacial acetic acid + 27.25 g sodium acetate and the volume was adjusted to 1000 ml) was placed in a test tube, and 50 µl of benzidine (40 µg benzidine dissolved in 25 ml 70% ethanol) was added. The solution was stirred thoroughly then 100 µl of protein solution (containing the enzyme) was added. After the addition of 50 µl of hydrogen peroxide and 30 sec passed, the optical density of the solution was measured at 620 nm.

PPO Assay

Total PPO activity of proteins was studied using pyrogallol as a chromogenic substrate. Thus, to 2 ml of phosphate buffer pH 8, having protein solutions (61 g $Na_2HPO_4{\cdot}H_2O$ and 39 g $NaH_2PO_4{\cdot}H_2O$ were dissolved in 200 ml of water) was added 50 µl 0.15 M substrate solution. The intensity of colored solution was determined at 460 nm.

Extraction of amino acids and their modification

Dried samples of treated and control leaves were ground and pooled in liquid nitrogen. 200 mg of each sample was weighed and extracted in 5 ml of water-acetonitrile (9:1) for 15 minutes with homogenizer. The supernatant was isolated after centrifugation for 10 minutes (3000 rpm). Higher molecular compounds were precipitated adding 10% trichloroacetic acid (1:1) for 15 minutes (8000 rpm). 200 µl aliquot was taken and lyophilized. Dry extract was dissolved in 200 µl water-acetonitrile-triethylamine (1:7:1) then the extract was dried. The process was twice repeated. In order to obtain phenylthiocarbamoyl (PTC) derivatives purified samples and standard amino acid were modified with water -acetonitrile-thriethylamine + phenylisotiocyonate (1:7:1:1) for 30 minutes.

HPLC analysis

Quantity analyses of PTC derivatives were conducted in Agilent technologies 1200 (Column: Supelco Discovery HS C18, Cat 56925/-U 7.5 cm x 4.6 mm, 3 µm), with buffer: B – 0.14 M CH_3COONa + 0.05% tetraethyl ammonium, pH 6.4, A – MeCN, gradient % B/min, 0 – 6%, 5 min, 6 – 30%, 30 min, 30 – 60% 5 min, 60 – 100% 5 min. Amino acid derivatives were detected with a flow rate 1.2 ml/min at 269 nm.

RESULTS AND DISCUSSION

The three agrochemicals were once applied on cotton plants for 2 different time periods at doses commonly used in agriculture. Their sound effect on plant metabolism was obvious from data of protein yields isolated from the experimental plants. Sumi-alfa enhanced protein synthesis at both time periods (Fig. 1A). Similar effects were observed for imidacloprid on Bt cotton total protein **(Kaur *et al*. 2011)**. In contrast Carbophos revealed an opposite (negative) effect ($P \leq 0.05$) (Fig. 1 A). Effects of Lannate were significant after 10 days but disappeared at later time (Fig. 1A).

The agrochemicals influenced the activity of antioxidative enzymes investigated. Sumi-alfa significantly inhibited the activity of both POD as well as PPO, after both application times (Fig. 1 B, C). The other two insecticides, in contrast, evoked a multiple increase of PPO activity at both sampling points (Fig. 1 B, C). Their effect on POD, however, was ambiguous since slight decrease ($P \leq 0.05$) after 13 days was only observed for Carbophos and contrasting, time-dependent activity was measured for Lannate (Fig. 1B, C). Previously, imidacloprid insecticide at recommended concentration (40. ml/acre) caused increased POD activity in three Bt cotton hybrids (RCH-134, JKCH-1947, NCEH-6R) **(Kaur *et al*. 2011)**. Moreover, imidacloprid increased the activities of catalase, POD, PPO and the quantity of total protein in potato plants **(Chauhan *et al*. 2013)**. Increased POD activities in imidacloprid applied plants partly coincide with the effects of carbamate lannate and enhanced PPO activity matches with the effects of lannate and carbophos. Decreased POD and PPO activities in Sumi-alfa applied leaves do not correspond with increased levels of POD and PPO activities under treatment of deltamethrin, cypermethrin and fenvalerate either **(Ravindhran and Xavier, 1997)**.

Fluctuating changes were observed with Pro. After treatment with Lannate and Sumi-alfa lower than the control enzyme activities were found in samples taken on 10^{th} day after spray. However, later-on they caused higher enzyme activities than control. Twice higher Pro quantity was calculated after carbamate Lannate. Insignificant slight increases on the 10^{th} day samples and significant increases were studied on 13^{th} day samples after treatment with carbophos ($P \leq 0.05$) (Fig. 1 D). Similar changes were observed with sulphur containing amino acids in all treated samples. All three chemicals significantly increased the quantity of Met and Cys (Fig E, F) ($P \leq 0.05$). The highest increase was observed with samples treated with Carbophos that almost twice higher quantity of Met was gathered (10^{th} day) and mildest effects belonged to Sumi-alfa. ($P \leq 0.05$) (Fig. 1 E). Samples treated with Carbophos on the 10^{th} day contained more Met and Cys than samples taken three days later, whereas, in comparison to control, linear increases were observed with Met and Cys after Lannate and Sumi-alfa treatment. The increased levels of Met in all treated samples match with the effects sulprofos on total essential amino acids **(Kerns and Gaylor, 1993)**.

CONCLUSION

Our results show that application of the tested agrochemicals affects cotton plant antioxidative homeostasis that in turn is likely to have impact on defense potential. Among studied three insecticides pyrethroid sumi-alfa was found to negatively affect the activity of both enzymes. On the other hand, application of the tested insecticides evoked the accumulation of amino acids involved in antioxidative (and perhaps other) defense. Nevertheless, impact of these insecticides on defense potential of cotton should be considered and/or studied in more detail for efficient application in agriculture.

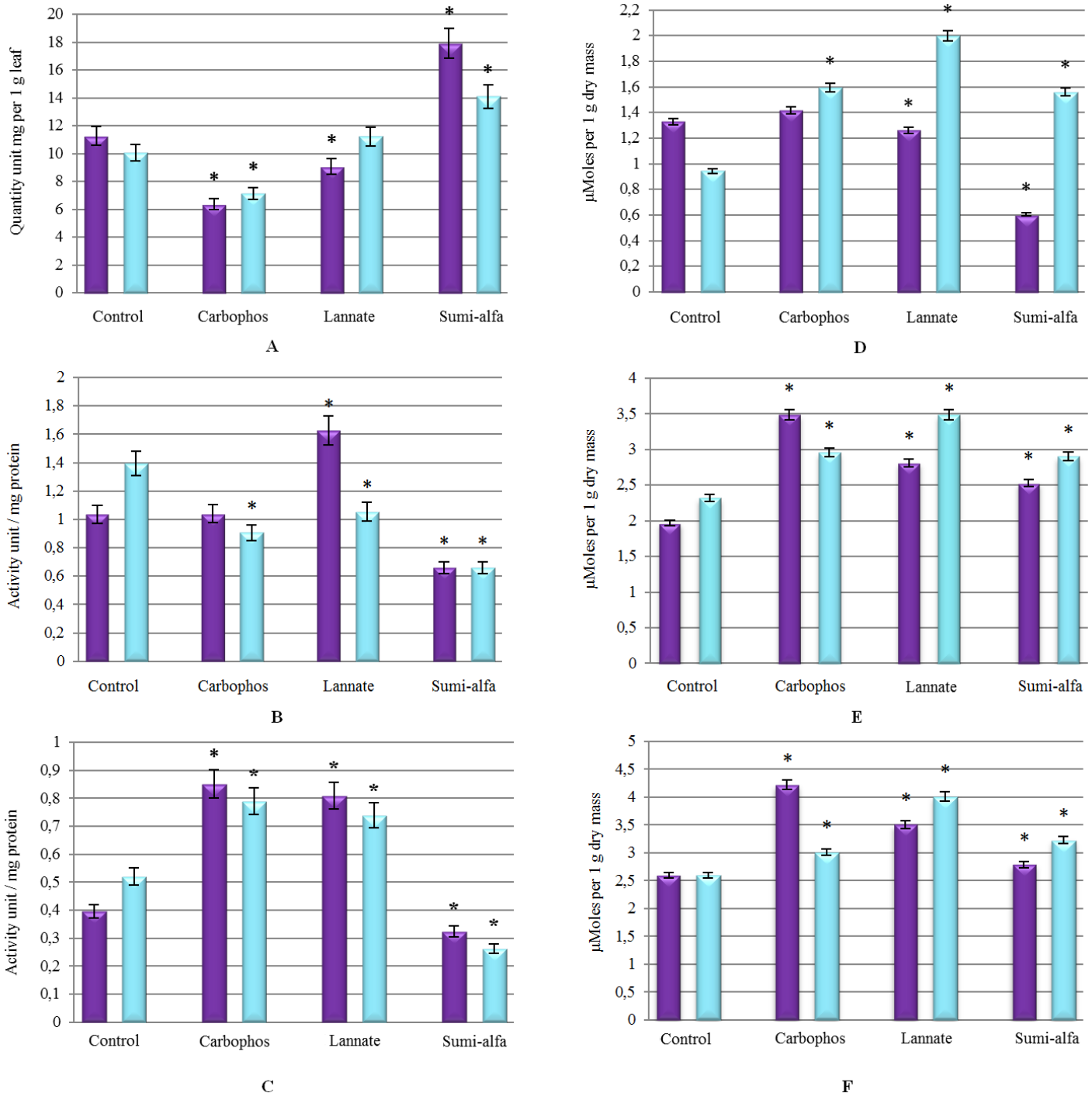

Figure 1 Changes in cotton leaves: **A** – Soluble proteins; **B** – POD activity; **C** – PPO activity; **D** – Pro content; **E** – Met content; **F** – Cys content. Leaves taken for the analysis were statistically different. The SE is less than 2%, data represent ±SEM (m3). Dark colours represent the 10th day and light colors represent the 13th day that samples taken after insecticides spray

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