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ENZYMATIC ANTIOXIDANT RESPONSES TO BIOSTIMULANTS IN CHERRY TOMATO SUBJECTED TO DROUGHT

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ABSTRACT

This study was conducted to evaluate the effects of biostimulants on antioxidant enzymes activity in leaves of cherry tomato seedlings (*Lycopersicon esculentum* Mill. cv. Sakura) under drought conditions. The pot experiment was set in a greenhouse environment at public communal company 'Park' in Sarajevo. Three biostimulants used in this research were as follows: Bio-algeen S92 (biostimulant made by extracting of seaweed *Ascophyllum nodosum* (L.) Le Jol.), Slavol (microbial-based stimulant), and Ergonfill (biostimulant obtained by the hydrolysis of proteins of animal origin). Drought induced increases in ascorbate peroxidase (APOD), superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOD) and pyrogallol peroxidase (PPOD) activities in leaves od seedlings, regardless of biostimulant treatment. The increase in the activities of SOD, APOD, PPOD and GPOD were recorded already at the beginning stage of stress (first day after exposure to stress), while the increase in CAT activity was less pronounced at this phase of stress. The increase in activities of all examined enzymes was significantly slower in leaves of seedlings treated with biostimulants Bio-algeen S92, Ergonfill and Slavol compared to non-treated seedlings under drought conditions, suggesting that the application of these biostimulants contributes to better adaptation of the seedlings to drought.

Key words: antioxidant, biostimulant, drought, enzyme activity, leaves, stress.

INTRODUCTION

Drought is the most prevalent environmental factor limiting crop productivity. The lack of the water in soil reduces the plant-cell's water potential and turgor, leading to growth inhibition and reproductive failure (Bray, 1997). One of the inevitable consequences of drought stress is also oxidative stress caused by an imbalance of reactive oxygen species (ROS). Increased levels of ROS can cause damage to cellular components and, if sufficiently severe, can result in cell death (Mittler, 2002).

Plants, however, have evolved numerous mechanisms against oxidative stress including the enzymatic and non-enzymatic antioxidant systems (Carvalho, 2008). Purely enzymatic antioxidant systems include a variety of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) ascorbate peroxidase (APOD), guaiacol peroxidase (GPOD) and pyrogallol peroxidase (PPOD). Non-enzymatic defences include secondary metabolites such as ascorbate, glutathione, and phenolics. These compounds protect plant cells by directly scavenging superoxide radicals and hydrogen peroxide, hydroxyl radicals and other oxygen radicals, converting them to harmful or less reactive species. A first line of defence against ROS in plant cells is antioxidant enzymatic activity (Suzuki *et al.*, 2012).

To overcome the problems arising from oxidative stress caused by water scarcity, it is necessary to implement approaches that help crops to improve its defence mechanisms (Farooq *et al*., 2009). Many studies have shown that the application of biostimulants such as microbial inoculants, amino acids, humic acids, fulvic acids, seaweed extracts and more, improves antioxidant defence systems of plant and increases plant tolerance to drought (Mohamed, 2006; Hammad, 2008, Khan *et al*., 2009). Biostimulants application promotes and enhances agro-ecosystem health, and therefore it is expected to increase their use in plant production, especially in stressful conditions. Lately, the biostimulants that are often used in organic vegetable production in Sarajevo region are Bio-algeen S92, Ergonfill and Slavol.

Bio-algeen S-92 (Schulze &Hermsen GmbH) is an organic biostimulant, derived from seaweed *Ascophyllum nodosum* (L.) Le Jol (Battacharyya *et al*., 2015). According to the product specification, Bio-algeen S-92 contains 96% water, 0.02% N, 0.006% P, 0.096% K, 0.31% Ca, 0.08% S, 1.31% Na, a certain amount of essential amino acids (alanine, glycine, tryptophan, histidine, proline, glutamine) vitamins (B_1, B_3, B_6, B_9, E), and microelements (B, Fe, Zn, Mn, Cu). Ergonfill (Adriatica) is a biostimulant obtained by the hydrolysis of proteins of animal origin (Parađiković *et al*., 2018). It contains 3.4% N, 10% C, 2% MgO, 0.2% Fe and 0.003% Mo, vitamin B_1, B_2 and amino acids; phenylalanine,

tyrosine, tryptophan, glutamine, proline, leucine, lysine, asparagine and alanine as specified in the product information data. Slavol (Agrounik) is a liquid microbial formulation containing nitrogen-fixing and phosphate solubilizing bacteria (Miskoska-Milevska *et al*., 2018). To our knowledge, the effect of these biostimulants on antioxidant enzymes activity in cherry tomato under drought stress conditions has not been tested so far.

The aim of the present study was to evaluate the enzymatic activity (SOD, CAT, APOD, GPOD, PPOD) in leaves of cherry tomato seedlings under drought stress conditions after applying biostimulants Bio-algeen S92, Slavol and Ergonfill. Cherry tomato was selected primarily because the global production of this vegetable is consistently increasing and therefore any attempt to improve its production, especially in stressful conditions, is of great interest to producers and consumers.

MATERIALS AND METHODS

Plant material and experimental conditions: Cherry tomato (*Lycopersicon esculentum* Mill. cv. Sakura) seedlings were used as the plant material in this study. Before starting the experiment, cherry tomato seedlings were transplanted into individual pots (20 cm diameter × 13 cm height), containing commercial peat-based substrate Florahum-SP. There was no observed significant difference between seedlings in terms of vigor, size and appearance. The experiment was conducted under controlled conditions, in greenhouse of public communal company 'Park' in Sarajevo from 20 May to 16 June 2017. Over the course of the experiment, air temperature in greenhouse was maintained at 24 ± 3°C during the day and 18 ± 3°C during the night. Relative humidity (RH) was maintained between 60% and 70%, with combined high-pressure fog systems to increase RH, and venting to reduce RH. During warm days shade cloth was used to prevent excessive light intensity.

Experimental design: The experiment was set up in a randomized block design with four biostimulant treatment in three replications. Each treatment was applied to forty seedlings. Treatments were as follows: (T1) Bio-algeen S92 0.2%; (T2) Slavol 1%; (T3) Ergonfill 0.1%; (T4) untreated seedlings. Biostimulants were applied at the concentrations recommended by the manufacturers. Bio-algeen S92 and Ergonfill were applied foliarly (50 ml per plant), while Slavol application was performed through soil (also 50 ml per plant). The first application of biostimulants was carried out immediately after the transplanting of seedlings and the second 15 days later. Five days after the second application, one half of the cherry tomato seedlings from each treatment (20 plants) was exposed to drought, while the second half (also 20 plants) served as the controls (they were regularly watered). Exposure to drought lasted until the moment in which the first visible effects of drought in the form of falling leaves were observed; these symptoms appeared 48 h after the seedlings were exposed to drought and at this point the sampling of leaves for analysis was started. Each leaf sample consisted of three fully expanded leaves from base of each cherry tomato seedlings. Fresh leaves were cut and immediately frozen with liquid nitrogen. Leaf samples were collected daily during 5 days period and stored at -20 °C for further analysis. All analyses were carried out in laboratory of Faculty of Agriculture and Food Sciences, University of Sarajevo, Bosnia and Herzegovina.

Chemicals: Chemicals with analytical purity were used in the present study. Potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), bovine serum albumin, Bradford protein assay concentrate, xanthine, xanthine oxidase, cytochrome C oxidase from bovine heart, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals used in this research were obtained from Merck (Darmstadt, Germany).

Protein extraction and determination: The fresh leaves (0.5 g) were ground to a fine powder in liquid nitrogen with a mortar and pestle. Soluble proteins were extracted by homogenizing the powder in extraction buffer (50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM DTT and 0.05 % (w/v) polyvinylpyrrolidone-40 (PVP-40). The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant fraction was collected and stored at -20°C for protein and enzyme activity measurements. Protein content was determined by Bradford's method using Bovine serum albumin as standard (Bradford, 1976).

Superoxide dismutase (SOD) activity assay: SOD activity was assayed using the xanthine/xanthine oxidase/cytochrome c method according to McCord and Fridovich (1969). In this coupled reaction, SOD inhibits the reduction of cytochrome c by superoxide anions generated from xanthine. The assay mixture (860 - 1.000 µl) included 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 50 µM xanthine, 10µM cytochrome C, and xanthine oxidase (enough to cause an increase in absorbance at 550 nm of 0.025 min^{-1} at 25°C in the absence of SOD). Samples (10 - 50 µl) were added to the reaction mixture and the rate of reduction of cytochrome c was followed spectrophotometrically at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of cytochrome c by 50%. Thus, the activity of SOD is expressed as U per mg protein.

Catalase (CAT) activity assay: The enzymatic activity of CAT was assessed spectrophotometrically according to

the method of Aebi (1984). Reaction mixture (950 µl) consisted of 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0). The reaction was run at 25°C, after adding the enzyme extract (50 µl). Enzyme activity was assayed by monitoring the decrease in absorbance at 240 nm at an interval of 5 to 120 sec as a result of H_2O_2 consumption. Extinction coefficient of 40 mM^{-1} cm^{-1} was used to express the CAT activity as micromoles decomposed H_2O_2 per min per mg of protein.

Guaiacol peroxidase (GPOD) activity assay: The enzymatic activity of GPOD was assessed spectrophotometrically according to Chance and Maehly (1955). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 18 mM guaiacol, 5 mM H_2O_2 and extract (100 µl) in a total volume of 1 ml. GPOD activity was measured by the increase in absorbance at 470 nm at an interval of 15 sec up to 180 sec, due to the guaiacol oxidation. Extinction coefficient of 26.6 mM^{-1} cm^{-1} was used to express the GPOD activity as micromoles tetraguaiacol (product of guaiacol oxidation) per min per mg of protein.

Pyrogallol peroxidase (PPOD) activity assay: The enzymatic activity of PPOD was assessed spectrophotometrically according to Chance and Maehly (1955). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 20 mM pyrogallol, 1 mM H_2O_2 and extract (50 µl) in a total volume of 1 ml. The PPOD activity was estimated by monitoring the increase in absorbance at 430 nm, at an interval of 15 sec up to 180 sec due to the oxidation of pyrogallol. Extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to express the PPOD activity as micromoles of purpurogallin (product of pyrogallol oxidation) per min per mg of protein.

Ascorbate peroxidase (APOD) activity assay: The APOD activity was measured spectrophotometrically according to the method of Nakano and Asada (1981). Reaction mixture (2 ml) contained 1 ml 50 mM potassium phosphate buffer (pH 7.0), 500 µl of 0.2 mM ascorbic acid, 100 µl of 0.2 mM EDTA, 300 µl of 6% H_2O_2 and 100 µl of protein extract (last component that was added). Enzyme activity was assayed by monitoring the decrease in absorbance at 290 nm at an interval of 10 to 120 sec. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to express APOD activity as micromoles ascorbic acid oxidized per min per mg of protein.

Statistical analysis: All experimental measurements were done in triplicates and the results were presented as mean ± standard deviation. All statistical analyses were performed using Microsoft excel software for Windows 10. Differences in enzymes activity between treatments were analyzed with a one-way analysis of variance (ANOVA) and least significant difference (LSD) test at a significance level of $P \leq 0.05$.

RESULTS

SOD activity: The effects of biostimulant treatments on SOD activity in cherry tomato seedlings under drought stress and standard growth conditions (without stress) are shown in Table 1.

Table 1. SOD activity in leaves of cherry tomato seedlings.

Treatments[1]	SOD activity (U/mg protein)				
	Day 1	Day 2	Day 3	Day 4	Day 5
1. B (stress)	14.88 ± 3.63^{abc}	22.05 ± 7.84^{c}	28.39 ± 3.95^{c}	30.67 ± 3.69^{cd}	29.86 ± 5.40^{a}
1. B (control)	8.00 ± 2.61^{e}	10.49 ± 2.15^{e}	11.33 ± 2.47^{e}	12.00 ± 2.92^{e}	10.06 ± 5.18^{e}
2. S (stress)	15.22 ± 3.05^{ab}	30.71 ± 8.62^{a}	36.10 ± 7.99^{a}	36.52 ± 7.7^{ab}	14.13 ± 5.44^{cd}
2. S (control)	8.46 ± 3.11^{e}	8.36 ± 1.65^{e}	8.40 ± 1.44^{e}	9.04 ± 2.36^{e}	11.96 ± 6.14^{cde}
3. E (stress)	$13.21 \pm 2.05^{a-d}$	16.26 ± 2.01^{d}	18.44 ± 3.30^{d}	32.49 ± 6.65^{bc}	28.20 ± 6.84^{ab}
3. E (control)	7.68 ± 2.43^{e}	11.00 ± 1.60^{e}	12.28 ± 3.70^{e}	12.66 ± 6.34^{e}	12.78 ± 2.82^{cde}
4. N (stress)	15.49 ± 4.17^{a}	27.35 ± 4.08^{ab}	32.50 ± 6.51^{ab}	39.55 ± 7.32^{a}	15.84 ± 3.12^{c}
4. N(control)	6.84 ± 1.89^{e}	12.19 ± 2.21^{de}	9.68 ± 1.74^{e}	9.33 ± 3.11^{e}	8.49 ± 1.34^{e}
$LSD_{0.05}$	2.64	4.21	3.97	5.11	4.48

[1]Treatments: B = Bio-algeen S92; S = Slavol; E = Ergonfill; N = Non-treated

Values are means ± SD; the values marked with different letters in the same column indicate significantly differences ($P \leq 0.05$)

The SOD activity was significantly higher in leaves of cherry tomato seedlings exposed to drought compared to seedlings grown under standard growth conditions, regardless of biostimulant treatment. The increase in SOD activity was recorded already in the first day of measurement. With the progress of stress, the activity of this enzyme has continued to increase until the fourth day when the activity of SOD has generally reached its maximum. On the fifth day of exposure of seedlings to stress, the SOD activity in leaves of seedlings was decreased, especially in untreated seedlings and seedlings treated with the Slavol.

The SOD activity in seedlings treated by biostimulant Bio-algeen S92 and Ergonfill was found to

be significantly lower than those in non-treated seedlings or seedlings treated by Slavol under stressful conditions. In the present study there was no statistically significant difference in SOD activity in leaves of cherry tomato seedlings under standard growth conditions (without stress) regardless of biostimulant treatment.

CAT activity: The CAT activity in leaves of cherry tomato seedlings was significantly higher in stressful growth conditions as compared by seedlings grown under standard growth conditions, regardless of biostimulant treatment (Table 2).

Table 2. CAT activity in leaves of cherry tomato seedlings.

Treatments[1]	CAT activity (□mol/min/mg protein)				
	Day 1	Day 2	Day 3	Day 4	Day 5
1. B (stress)	0.009 ± 0.002	0.012 ± 0.007^{a-d}	0.018 ± 0.008^{cd}	0.024 ± 0.006^{cd}	0.045 ± 0.017^{b}
1. B (control)	0.008 ± 0.005	0.010 ± 0.005^{cd}	0.011 ± 0.005^{de}	0.012 ± 0.006^{e}	0.015 ± 0.010^{e}
2. S (stress)	0.016 ± 0.005	0.014 ± 0.003^{a-c}	0.061 ± 0.012^{a}	0.085 ± 0.014^{ab}	0.040 ± 0.010^{bc}
2. S (control)	0.011 ± 0.005	0.010 ± 0.004^{cd}	0.013 ± 0.006^{cde}	0.017 ± 0.005^{de}	0.016 ± 0.008^{e}
3. E (stress)	0.010 ± 0.007	0.015 ± 0.005^{ab}	0.019 ± 0.007^{c}	0.031 ± 0.012^{c}	0.072 ± 0.018^{a}
3. E (control)	0.007 ± 0.004	0.010 ± 0.003^{d}	0.012 ± 0.006^{cde}	0.010 ± 0.006^{e}	0.016 ± 0.013^{e}
4. N (stress)	0.014 ± 0.005	0.016 ± 0.004^{a}	0.045 ± 0.011^{b}	0.092 ± 0.013^{a}	0.033 ± 0.011^{cd}
4. N (control)	0.009 ± 0.003	0.010 ± 0.005^{d}	0.009 ± 0.002^{e}	0.012 ± 0.007^{e}	0.015 ± 0.009^{e}
$LSD_{0.05}$	-	0.0042	0.0072	0.0085	0.0112

[1]Treatments: B = Bio-algeen S92; S = Slavol; E = Ergonfill; N = Non-treated
Values are means ± SD; the values marked with different letters in the same column indicate significantly differences (P ≤ 0.05)

Results also showed that the increase of CAT activity in all stressed seedlings was less pronounced in the first days of stress, and that it was in the last days of exposure of seedlings to drought, the CAT activity significantly increased. An exception of this rule was observed in non-treated seedlings and seedlings treated with Slavol. On the last day of exposure of these seedlings to drought, the CAT activity was rapidly reduced.

The treatment with biostimulants has shown a positive influence on the reduction of the increase of CAT activity, suggesting that this treatment helps the antioxidant defence systems in plant cells under stress conditions. In control variants (i.e. in variants where seedlings were not exposed to stressful conditions), the CAT activity in leaves did not change significantly during the five days of measurement, regardless of biostimulant treatment.

GPOD activity: As shown in Table 3, the peroxidase activity (assayed with guaiacol) was higher in seedlings exposed to drought compared to non-stressed seedlings, regardless of biostimulant treatment.

Table 3. GPOD activity in leaves of cherry tomato seedlings.

Treatments[1]	GPOD activity (□mol/min/mg protein)				
	Day 1	Day 2	Day 3	Day 4	Dax 5
1. B (stress)	0.11 ± 0.05^{abc}	0.15 ± 0.05^{abc}	0.18 ± 0.06^{a-d}	0.22 ± 0.08^{ab}	0.23 ± 0.08^{abc}
1. B (control)	0.06 ± 0.05^{e}	0.07 ± 0.05^{e}	0.07 ± 0.05^{e}	0.10 ± 0.03^{e}	0.12 ± 0.06^{e}
2. S (stress)	0.11 ± 0.03^{a-d}	0.14 ± 0.04^{a-d}	0.20 ± 0.09^{ab}	0.22 ± 0.06^{abc}	0.24 ± 0.10^{ab}
2. S (control)	0.07 ± 0.05^{b-e}	0.09 ± 0.05^{e}	0.09 ± 0.08^{e}	0.11 ± 0.07^{e}	0.12 ± 0.08^{e}
3. E (stress)	0.11 ± 0.07^{ab}	0.16 ± 0.05^{ab}	0.18 ± 0.05^{abc}	0.20 ± 0.05^{a-d}	0.21 ± 0.07^{a-d}
3. E (control)	0.07 ± 0.05^{e}	0.10 ± 0.04^{cde}	0.13 ± 0.08^{cde}	0.12 ± 0.04^{e}	0.12 ± 0.06^{e}
4. N (stress)	0.14 ± 0.05^{a}	0.18 ± 0.05^{a}	0.21 ± 0.12^{a}	0.25 ± 0.11^{a}	0.28 ± 0.08^{a}
4. N (control)	0.08 ± 0.07^{b-e}	0.10 ± 0.05^{cde}	0.12 ± 0.03^{cde}	0.13 ± 0.05^{e}	0.12 ± 0.03^{e}
$LSD_{0.05}$	0.048	0.043	0.071	0.059	0.070

[1]Treatments: B = Bio-algeen S92; S = Slavol; E = Ergonfill; N = Non-treated
Values are means ± SD; the values marked with different letters in the same column indicate significantly differences (P ≤ 0.05)

The increase of GPOD activity in leaves of stressed seedlings was recorded already in the first day of measurement, and with the progress of stress, the activity of this enzyme was increasing continuously up to the last day of exposure of seedlings to drought. The biostimulant treatment caused reduction of the increase of GPOD activity in stressed seedlings in comparison with non-treated seedlings. Contrary, in standard growth

conditions, the statistically significant difference in GPOD activity between experimental treatments was not determined.

PPOD activity: The effects of biostimulants on PPOD activity in cherry tomato seedlings under drought stress and standard growth conditions (without stress) are shown in Table 4.

Table 4. PPOD activity in leaves of cherry tomato seedlings.

Treatments[1]	PPOD activity (□mol/min/mg protein)				
	Day 1	Day 2	Day 3	Day 4	Day 5
1. B (stress)	0.63 ± 0.30^{bcd}	1.21 ± 0.29^{c}	1.41 ± 0.20^{d}	2.06 ± 0.39^{cd}	2.22 ± 0.55^{cd}
1. B (control)	0.48 ± 0.12^{d}	0.67 ± 0.32^{d}	1.02 ± 0.30^{e}	1.19 ± 0.44^{e}	1.22 ± 0.35^{e}
2. S (stress)	0.97 ± 0.31^{a}	1.56 ± 0.23^{ab}	2.47 ± 0.38^{a}	3.01 ± 0.50^{a}	3.10 ± 0.20^{ab}
2. S (control)	$0.71 \pm 0.10^{a-d}$	0.83 ± 0.43^{d}	1.11 ± 0.21^{de}	1.18 ± 0.46^{e}	1.23 ± 0.41^{e}
3. E (stress)	0.77 ± 0.30^{abc}	1.32 ± 0.20^{bc}	2.01 ± 0.39^{c}	2.23 ± 0.47^{c}	2.55 ± 0.70^{c}
3. E (control)	0.60 ± 0.50^{bcd}	0.86 ± 0.20^{d}	1.21 ± 0.36^{de}	1.16 ± 0.26^{e}	1.33 ± 0.16^{e}
4. N (stress)	0.83 ± 0.27^{ab}	1.64 ± 0.36^{a}	2.43 ± 0.57^{ab}	2.99 ± 0.38^{ab}	3.31 ± 1.09^{a}
4. N (control)	0.55 ± 0.33^{cd}	0.81 ± 0.25^{d}	1.19 ± 0.13^{de}	1.25 ± 0.30^{e}	1.30 ± 0.40^{e}
$LSD_{0.05}$	0.27	0.29	0.33	0.37	0.51

[1]Treatments: B = Bio-algeen S92; S = Slavol; E = Ergonfill; N = Non-treated
Values are means ± SD; the values marked with different letters in the same column indicate significantly differences ($P \leq 0.05$)

PPOD activity (the peroxidase activity assayed with pyrogallol) was markedly increased in leaves of all seedlings exposed to drought. The activity of these enzymes was already expressed in the first days of stress and with the progress of stress, its activity was gradually increased. Addition of biostimulants has shown a positive influence on the reduction of the increase of PPOD activity, especially treatment with Bio-algeen S92. Conversely, the biostimulant treatment did not affect the PPOD activity under standard growth conditions.

APOD activity: The effects of biostimulants on APOD activity in cherry tomato seedlings under drought and standard growth conditions (without stress) are shown in Table 5.

Table 5. APOD activity in leaves of cherry tomato seedlings.

Treatments[1]	APOD activity (□mol/min/mg protein)				
	Day 1	Day 2	Day 3	Day 4	Day 5
1. B (stress)	0.09 ± 0.04^{cd}	0.16 ± 0.11^{bcd}	0.26 ± 0.08^{de}	0.65 ± 0.18^{bc}	0.46 ± 0.19^{ab}
1. B (control)	0.05 ± 0.03^{d}	$0.12 \pm 0.04^{b-e}$	0.11 ± 0.05^{f}	0.19 ± 0.07^{g}	0.19 ± 0.07^{e}
2. S (stress)	0.13 ± 0.07^{bc}	0.18 ± 0.10^{b}	0.70 ± 0.12^{bc}	0.67 ± 0.14^{b}	0.38 ± 0.14^{bc}
2. S (control)	0.07 ± 0.03^{d}	0.07 ± 0.03^{e}	0.08 ± 0.04^{f}	0.24 ± 0.10^{efg}	0.23 ± 0.09^{e}
3. E (stress)	0.18 ± 0.09^{ab}	0.18 ± 0.07^{bc}	0.83 ± 0.16^{a}	1.09 ± 0.37^{a}	0.51 ± 0.10^{a}
3. E (control)	0.04 ± 0.04^{d}	$0.12 \pm 0.05^{b-e}$	0.27 ± 0.18^{d}	0.39 ± 0.12^{de}	0.24 ± 0.10^{e}
4. N (stress)	0.22 ± 0.05^{a}	0.28 ± 0.13^{a}	0.74 ± 0.21^{b}	0.49 ± 0.11^{d}	0.37 ± 0.12^{bcd}
4. N (control)	0.09 ± 0.05^{cd}	$0.12 \pm 0.05^{b-e}$	0.12 ± 0.07^{f}	0.36 ± 0.12^{def}	0.27 ± 0.08^{de}
$LSD_{0.05}$	0.052	0.075	0.116	0.157	0.108

[1]Treatments: B = Bio-algeen S92; S = Slavol; E = Ergonfill; N = Non-treated
Values are means ± SD; the values marked with different letters in the same column indicate significantly differences ($P \leq 0.05$)

The APOD activity was higher in seedlings exposed to drought compared to non-stressed seedlings, which was expected for plants as a response to stress.

The increase in APOD activity in leaves of all seedlings under stress was also observed in the first day of measurement. With the progress of stress, the activity of this enzyme has continued to increase until the third or fourth day when the activity of APOD has generally reached its maximum. After that, the APOD activity decreased, regardless of biostimulant treatment. Application of biostimulants has reduced the increase of APOD activity in leaves of stressed seedlings. In control variants (i.e. in variants where seedlings were not exposed to stressful conditions), application of biostimulants had not shown significant influence on APOD activity, regardless of biostimulant treatment.

DISCUSSION

Efficient scavenging of ROS produced during drought stress requires the action of several enzymatic antioxidants present in the plant cells. SOD is one of the key enzymes of the cell protection system against ROS. It catalyzes the dismutation of two molecules of superoxide radical to less hazardous molecules: H_2O_2 and molecular oxygen (Banowetz *et al*., 2004). The increased activity of this enzyme in plants under water stress conditions has been confirmed by many scientists (Acar *et al*., 2001; Li *et al*., 2006; Zlatev *et al*., 2006; Lu *et al*., 2010). In cherry tomato, the increase in SOD activity by drought was clearly observed in this study. This activity in leaves of cherry tomato seedlings was recorded already in the initial stage of stress, suggesting that SOD acts as a component of first line defence system against reactive oxygen species. The SOD activity in seedlings treated by biostimulant Bio-algeen S92, Ergonfill and Slavol was significantly lower than those in non-treated seedlings under drought conditions. In view of fact that higher SOD activity is one of the top stress indicators in plant, it is obvious that the application of these biostimulants postpones drought stress and thereby reduces negative effects of drought in cherry tomato seedlings cultivation. The highest efficacy in increasing the tolerance of seedlings to drought has been demonstrated by biostimulants Bio-algeen S92 and Ergonfill. It is assumed that this effect is a result of its composition characterized by high content of osmotic active substances and essential amino acids, as well as the ability of cherry tomato seedlings to maximally utilize the active substances in these biostimulants for osmotic adjustment and plant metabolic efficiency. The positive influence of Bio-algeen S-92 and amino acid biostimulants on enzymatic antioxidant systems against stress has been confirmed by results of many studies (Sharma and Dietz, 2006; Vranova *et al*., 2011; Liang *et al*., 2013).

CAT is a tetrameric heme-containing enzyme that protects the cell from H_2O_2 by catalysing its decomposition into O_2 and H_2O (Foyer and Noctor, 2000). One of the properties of CAT that makes it so important in antioxidant defence system is that it can degrade H_2O_2 without using cellular reducing equivalents (Kurutas, 2016). Therefore, CAT provides the plant cells with energy-efficient mechanism to remove H_2O_2. In the present study, CAT activity was higher in leaves of cherry tomato seedlings exposed to drought in comparison with seedlings grown in standard, i.e., well-watered conditions, regardless of biostimulant treatment. Many studies have shown similar results about the effect of drought on the CAT activity in plant cells (Bailly *et al*., 2000; Tohidi-Moghadam *et al*., 2009). Results of this study also showed that the increase in CAT activity in cherry tomato seedlings was less pronounced at the beginning of stress (in the first and second days of stress), and that with the progress of stress, its activity was increasing continuously until the fourth or fifth, last day of exposure of seedlings to drought. Interestingly, the CAT activity in plant cells of seedlings treated with Slavol or non- treated seedlings (after reaching its maximum activity in fourth day) decreased rapidly in the last, fifth day of measurement, suggesting that plant cells at that moment had a very poor physiological activity. One of the probably reasons for lower efficiency of microbial biostimulant Slavol on the physiological activity of the plant cells and their tolerance to drought in comparison with biostimulant Bio-algeen S92 and Ergonfill is the low activity of applied microorganisms in soils. Namely, survival, activity and distribution of microorganisms in soil depend on many factors: plant-microbe interactions, soil water status, physical and chemical properties of soil, etc. (Hungria *et al*., 2013; Menge and Chazdon, 2016).

POD (peroxidase) protects cells against the negative effects of H_2O_2 by catalysing its decomposition through oxidation of phenolic and endiolic co-substrates (Lin and Kao, 2002). In the present study, POD activity, assayed with either guaiacol (GPOD) or pyrogallol (PPOD), was markedly increased in leaves of seedlings exposed to drought, regardless of biostimulant treatment. Higher PPOD and GPOD activity were also observed in the first day of measurement and with the progress of stress their activity gradually increased. Between PPOD and GPOD activity there was slight difference in this study, but it is evident that these enzymes followed a similar pattern during the same period of stress. The fastest increase in PPOD and GPOD activity was found in untreated seedlings as well as in seedlings treated with biostimulant Slavol. Seedlings treated with Bio-algeen S92 and Ergonfill exhibited a slower increase in PPOD and GPOD activity under drought conditions. Considering that faster increase in POD activity indicates high level of stress (Zoz *et al*., 2013), these results suggest that application of Bio-algeen S92 and Ergonfill can mitigate the negative effects of drought on cherry tomato seedlings.

If we compare the results between the CAT and POD activity in leaves of cherry tomato seedlings depending on the stress duration, significant differences have been observed in this study. POD activity was markedly increased in the initial phase of stress, while CAT activity was less pronounced during that period, although both enzymes act on the same substrate (H_2O_2). Jones (2008) reported that the affinity of CAT for hydrogen peroxide is surprisingly low, which is probably the main raison for its low activity in the initial phase of stress. Interestingly, the rate of degradation of hydrogen peroxide by CAT is extremely high (Jones and Suggett, 1968). Therefore, CAT is the key enzyme in protecting plant cells against oxidative stress characterised by high level of H_2O_2 (Mhamdi *et al*., 2010). In contrast, the

GPOD and PPOD exhibit binding affinities for peroxide in the low micromolar range (Heck *et al*., 2010). Hence, the higher activity of these enzymes in the initial stages of stress is expected and the results of this study support this hypothesis.

Apart from the above mentioned systems, the efficient hydrogen peroxide-detoxification system in plant cells is ascorbate-glutathione cycle in which APOD play a direct role (Asada, 1992). APOD functions as scavenger of hydrogen peroxide (H_2O_2) using ascorbate as the specific electron donor (Chew *et al*., 2003). In the present study, the APOD activity was also significantly higher in leaves of cherry tomato seedlings exposed to drought compared to non-stressed seedlings, regardless of biostimulant treatment. However, the APOD activity was increasing continuously until the third (seedlings treated by Slavol and non-treated seedlings) or fourth day of stress (seedlings treated by Bio-algeen S92 and Ergonfill), and then, its activity decreased. Early response of APOD isoenzymes in plant cells under drought stress has been observed in many studies (Yoshimura *et al*., 1998; Shigeoka *et al*., 2002; D'Arcy-Lameta *et al*., 2006; Bonifacio *et al*., 2011). Lee *et al*. (2009) reported that the APOD activity in white clover (*Trifolium repens* L.) leaves was most rapidly activated during the first week of water stress, then its activity decreased, which is comparable with the results of our study. Other scientists in their studies presented similar observations about this issue (Fu and Huang, 2001; Xu *et al*., 2011). There are many hypotheses that attempt to explain the association of APOD activity with stress duration and intensity. Zhang and Kirkham (1996) reported that APOD requires a reductant substrate (ascorbate) for efficient scavenging of H_2O_2, therefore a certain content of ascorbate in plant cells is always necessary for its activity. The regeneration of ascorbate depends of many factors, including the activity of several enzymes: monodehydroascorbate reductase (MR), dehydroascorbate reductase (DR) and glutathione reductase (GR) (Blokhina *et al*., 2003). It is assumed that the disorder in ascorbate regeneration is one of the reasons for rapid decrease of APOD activity in leaves of cherry tomato seedlings under drought conditions in the present study.

In the present study there was no statistically significant difference in SOD, CAT, POD and APOD activity in leaves of cherry tomato seedlings grown under standard growth conditions (without stress) regardless of biostimulant treatment. These data supported the hypothesis that drought as a stress factor lead to oxidative stress, and consequently to higher activity of antioxidant enzymes, and this hypothesis has, in fact, been confirmed by many scientists (Rizhsky *et al.*, 2002; Pandey *et al*., 2015; Jin *et al*., 2016).

In conclusion, the cherry tomato seedlings treated by Bio-algeen S92, Ergonfill and Slavol exhibited a slower increase in antioxidant enzyme activity in leaves under drought conditions compared to non-treated seedlings, suggesting that the application of these biostimulants contributes to better adaptation of the seedlings to drought. The highest efficacy in increasing the tolerance of cherry tomato seedlings to drought has been demonstrated by biostimulant Bio-algeen S92, obtained from extraction of seaweeds.

REFERENCES

Acar, O., I. Turkan, and F. Ozdemir (2001). Superoxide dismutase and peroxidase activities in drought sensitive and resistant barley (*Hordeum vulgare* L.) varieties. Acta Physiol. Plant. 23(3): 351-356.

Aebi, H. (1984). Catalase in vitro. Meth. Enzymol. 105: 121-126.

Asada, K. (1992). Ascorbate peroxidase - A hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85: 235-241.

Bailly, C., A. Benamar, F. Corbineau, and D. Come (2000). Antioxidant systems in sunflower seeds as affected by priming. Seed Sci. Res. 10: 35-42.

Banowetz, G.M., K.P. Dierksen, M.D. Azevedo, and R. Stout (2004). Microplate quantification of plant leaf superoxide dismutases. Anal. Biochem. 332(2): 314-320.

Battacharyya, D., M.Z. Babgohari, P. Rathor, and B. Prithiviraj (2015). Seaweed extracts as biostimulants in horticulture. Sci. Hortic. 196: 39-48.

Blokhina, O., E. Virolainen, and K.V. Fagerstedt (2003). Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann. Bot. 91: 179-194.

Bonifacio, A., M.O. Martins, C.W Ribeiro, A.V. Fontenele, F.E. Carvalho, M. Margis-Pinheiro, and J.A. Silveira (2011). Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant Cell Environ. 34(10): 1705-1722.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248-259.

Bray, E.A. (1997). Plant responses to water deficit. Trends. Plant Sci. 2(2): 48-54.

Carvalho, M.C. (2008). Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signal. Behav. 3(3): 156-165.

Chance, B., and A.C. Maehly (1955). Assay of catalases and peroxidases. Meth. Enzymol. 2: 764-775.

Chew, O., J. Whelan, and A.H. Millar (2003). Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting

of antioxidant defenses in plants. J. Biol. Chem. 278(47): 46869-46877.

D'Arcy-Lameta, A., R. Ferrari-Iliou, D. Contour-Ansel, A.T. Pham-Thi, and Y. Zuily-Fodil (2006). Isolation and characterization of four ascorbate peroxidase cDNAs responsive to water deficit in cowpea leaves. Ann. Bot. 97(1): 133-140.

Farooq, M., A. Wahid, N. Kobayashi, D. Fujita, and S.M.A. Basra (2009). Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. 29(1): 185-212.

Foyer, C.H., and G. Noctor (2000). Oxygen processing in photosynthesis: regulation and signalling. New Phytol. 146(3): 359 -388.

Fu, J.M. and B.R. Huang (2001). Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45(2): 105-114.

Hammad, S.A.R. (2008). Physiological and anatomical studies on drought tolerance of pea plants by application of some natural extracts. Ann. Agric. Sci. 53(2): 285-305.

Heck, D.E., M.P. Shakarjian, H.D. Kim, J.D. Laskin, and A.M. Vetrano (2010). Mechanisms of oxidant generation by catalase. Ann. N. Y. Acad. Sci. 1203: 120-125.

Hungria, M., M.A. Nogueira, and R.S. Araujo (2013). Co-inoculation of soybeans and common beans with rhizobia and azospirilla: Strategies to improve sustainability. Biol. Fertil. Soils 49: 791-801.

Jin, R., Y. Wang, R. Liu, J. Gou, and Z. Chan (2016). Physiological and metabolic changes of purslane (*Portulaca oleracea* L.) in response to drought, heat, and combined stresses. Front. Plant Sci. 6: 1123.

Jones, D.P. (2008). Radical-free biology of oxidative stress. Am. J. Physiol. Cell Physiol. 295(4): 849-868.

Jones, P., and A. Suggett (1968). The Catalase-Hydrogen Peroxide System: kinetics of catalytic action at high substrate concentrations. Biochem. J. 110(4): 617-620.

Khan, W., U.P. Rayirath, S. Subramanian, M.N. Jithesh, P. Rayorath, D.M. Hodges, A.T. Critchley, J.S. Craigie, J. Norrie, and B. Prithiviraj (2009). Seaweed extracts as biostimulants of plant growth and development. Plant Growth Regul. 28(4): 386-399.

Kurutas, E.B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr. J. 15(1): 71.

Lee, B.R., L.S. Li, W.J. Jung, Y.L. Jin, J.C. Avice, A. Ourry, and T.H. Kim (2009). Water deficit-induced oxidative stress and the activation of antioxidant enzymes in white clover leaves. Biol. Plant. 53(3): 505-510.

Li, Y., X.P. Deng, S.S. Kwak, and K. Tanaka (2006). Drought tolerance of transgenic sweet potato expressing both Cu/Zn superoxide dismutase and ascorbate peroxidase. J. Plant Physiol. Mol. Biol. 32(4): 451-457.

Liang, X.W., L. Zhang, S.K. Natarajan, and D.F. Beckker (2013). Proline mechanisms of stress survival. Antioxid. Redox Signal. 19(9): 998-1011.

Lin, C.L., and C.H. Kao (2002). Osmotic stress-induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. Plant Growth Regul. 37(2): 177-184.

Lu, Y.Y., X.P. Deng, and S.S. Kwak (2010). Over expression of CuZn superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) in transgenic sweet potato enhances tolerance and recovery from drought stress. Afr. J. Biotechnol. 9: 8378-8391.

McCord, J.M., and I. Fridovich (1969). Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244(22): 6049-6055.

Menge, D.N., and Chazdon R.L. (2016). Higher survival drives the success of nitrogen-fixing trees through succession in Costa Rican rainforests. New Phytol. 209: 965-977.

Mhamdi, A., G. Queval, S. Chaouch, S. Vanderauwera, F. Van Breusegem, and G. Noctor (2010). Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. J. Exp. Bot. 61: 4197-4220.

Miskoska-Milevska, E., O. Najdenovska, A. Z. Popovski, and D. Dimovska (2018). The influence of the microbiological fertilizer - Slavol on cauliflower growth. Rom. Biotech. Lett. 23(2): 13511-13516.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7(9): 405-410.

Mohamed, A.M. (2006). Effect of Some Bio-chemical Fertilization Regimes on Yield of Maize. M.Sc. Thesis, Fac. of Agric., Zagazig Univ., Egypt.

Nakano, Y., and K. Asada (1981). Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22(5): 867-880.

Pandey, P., V. Ramegowda, and M. Senthil-Kumar (2015). Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. Front. Plant Sci. 6: 723.

Parađiković, N., T. Teklić, S. Željković, M. Lisjak, M. Špoljarević (2018). Biostimulants research in

some horticultural plant species - A review. Food Energy Secur. e00162.

Rizhsky, L, H. Liang, and R. Mittler (2002). The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. 130(3): 1143-1151.

Sharma, S.S., and K.J. Dietz (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J. Exp. Bot. 57(4): 711-726.

Shigeoka, S., T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta, and K. Yoshimura (2002). Regulation and function of ascorbate peroxidase isoenzymes. J. Exp. Bot. 53(372): 1305-1319.

Suzuki, N., S. Koussevitzky, R. Mittler, and G. Miller (2012). ROS and redox signaling in the response of plants to abiotic stress. Plant Cell Environ. 35(2): 259-270.

Tohidi-Moghadam, H.R., A.H. Shirani-Rad, and G. Nour-Mohammadi (2009). Effect of super absorbent application on antioxidant enzyme activities in canola (*Brassica napus* L.) cultivars under water stress conditions. Asian J. Adv. Basic Sci.. 4: 215-223.

Vranova, V., K. Rejsek, K.R. Skene, and P. Formanck (2011). Non-protein amino acids: plant, soil and acosystem interactions. Plant Soil. 342: 31-48.

Xu, L., L. Han, and B. Huang (2011). Antioxidant Enzyme Activities and Gene Expression Patterns in Leaves of Kentucky Bluegrass in Response to Drought and Post-drought Recovery. J. Am. Soc. Hortic. Sci. 136(4): 247-255.

Yoshimura, K., T. Ishikawa, Y. Nakamura, M. Tamoi, T. Takeda, T. Tada, K. Nishimura, and S.Shigeoka (1998).Comparative study on recombinant chloroplastic and cytosolic ascorbate peroxidase isozymes of spinach. Arch. Biochem. Biophys. 353: 55-63.

Zhang, J., and M.B. Kirkham (1996). Enzymatic Responses of the Ascorbate-Glutathione Cycle to Drought in Sorghum and Sunflower Plants. Plant Sci. 113(2): 139-147.

Zlatev, Z.S., F.C. Lidon, J.C. Ramalho, and I.T. Yordanov (2006). Comparison of resistance to drought of three bean cultivars. Biol. Plant. 50(3): 389-394.

Zoz, T., F. Steiner, V.F. Guimarães, D.D. Castagnara, C.C. Meinerz, and R. Fey (2013). Peroxidase activity as an indicator of water deficit tolerance in soybean cultivars. Biosci. J. 29(1): 1664-1671.