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RESEARCH ARTICLE

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Biostimulant prevents yield loss and reduces oxidative damage in tomato plants grown on reduced NPK nutrition*

Ivana Koleška[a‡], Dino Hasanagić[b‡], Vida Todorović[a], Senad Murtić[c], Izudin Klokić[d], Nada Parađiković[e] and Biljana Kukavica[b]

[a]Faculty of Agriculture, University of Banja Luka, Banja Luka, Bosnia and Herzegovina; [b]Faculty of Natural Sciences and Mathematics, University of Banja Luka, Banja Luka, Bosnia and Herzegovina; [c]Faculty of Agricultural and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina; [d]Syngenta Agro AG Company, Gradiška, Bosnia and Herzegovina; [e]Faculty of Agriculture in Osijek, University of J.J. Strossmayer in Osijek, Osijek, Croatia

ABSTRACT

Plant biostimulants are substances which have the capacity to modify physiological processes in plants in a way that provides potential benefits to growth, development or stress response. Effects of biostimulant application on two tomato hybrids (Ombeline F1 and Bostina F1) submitted to reduced nitrogen, phosphorus and potassium (NPK) nutrition aiming at prevention of oxidative stress generation as well as yield and fruit quality loss were investigated in this study. According to obtained results, foliar applied Viva® biostimulant decreased superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7) activity in tomato leaves even when recommended NPK nutrition was reduced at 40%. Fruit quality parameters (total soluble solids, total acidity, ascorbic acid and lycopene content) and yield were also maintained in reduced macronutrient fertilization when biostimulant was added. Combination of biostimulant with reduced NPK fertilizer enabled stability of cell homeostasis in tomato plants and their better adaptation to stress conditions. The possibility of biostimulant being used as environmental friendly tool in the reduction of mineral fertilizers without negative consequences regarding yield and fruit quality was discussed.

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KEYWORDS
Biostimulant; tomato; reduced nutrition; oxidative stress

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of economically most important vegetable crops with over 163 million tons of greenhouse production in the European countries (FAO 2013) and its cultivation is highly dependent on mineral nutrition. Well-applied mineral fertilization maintains and raises soil fertility, increases crop yields, and improves the feeding value of agricultural products (Voisin 1965). Reduced fertilization leads to many negative consequences in plant metabolism where a major problem is the deficit of nitrogen (N), phosphorus (P) and potassium (K) (Raven & Smith 1976; Tan et al. 2005). Lack of these elements leads to change in intracellular pH, ionic disbalance, protein content, organic acids and carbohydrates (Marshner 1995; Raab & Terry 1995). Disturbed osmotic and ionic balance leads to hyperproduction of reactive oxygen species (ROS), whereby hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are most often increasingly generated (Tewari et al. 2007). ROS can lead to oxidative damages of all cell structures, starting from DNA to proteins, lipids, sugars and other biomolecules, endangering in that way the vital functions of plant cell (Arora et al. 2002; Gill & Tuteja 2010; Ahmad et al. 2011). In the strategy of stress overcoming, plants adapt their metabolic pathways, which are primarily related to synthesis of enzymatic and low molecular weight non-enzymatic antioxidants (Gill et al. 2013; Al Hassan et al. 2015). Primary antioxidative defense consists of several enzymatic systems, the most important being superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and plant peroxidases (POD, EC 1.11.1.7) that use different phenolic compounds for substrate (Sharma et al. 2012). It has been proven that K deficiency increases plant sensitivity to oxidative stress whereby chloroplast degradation and enzyme antioxidative activity increase (Tang et al. 2015; Zhao et al. 2016). There are numerous data in the literature indicating that N deficiency adversely affects the chlorophyll concentration and photosynthetic activity which automatically results in the growth and yield reduction (Ciompi et al. 1996; Huang et al. 2004; Zhao et al. 2005). In addition, some researches have shown that there was a correlation between plant antioxidative enzymes and N deficiency (Polesskaya et al. 2004). Phosphorus deficiency in plants often leads to increase of hydrogen peroxide concentration which automatically changes cell redox balance by increasing the concentration of antioxidative enzymes (Yao et al. 2007). On the other hand, intensive mineral fertilization in greenhouses can be a problem since there is no natural soil leaching and salt stress often occurs in those conditions (Kastori et al. 2013). Also, ROS hyperproduction occurs in plant cells because of disturbed osmotic balance caused by increased salinity, which is one of serious limiting factors in plant production, with harmful effects on germination, vitality and ultimately total yield (Munns & Tester 2008; Taiz & Zaiger 2010; Zhai et al. 2015). In addition, excessive mineral fertilization apart from adverse financial effect represents environmental burden also (Halpern et al. 2015).

CONTACT Ivana Koleška ivana.koleska@agro.unibl.org
*This research was conducted at the Faculty of Agriculture, University of Banja Luka.
‡Ivana Koleška and Dino Hasanagić equally contributed to this work.

Table 1. The composition and physical properties of biostimulant VIVA® (www.valagro.com).

Organic matter (DW 33%)	Polysaccharides	Proteins, polypeptides, amino acids	Vitamin complexes	Humic acids	pH (1% aqueous solution)	Conductivity 1‰ (mS/cm) 18°C	Density (g/cm^3) 20°C
12.0%	2.0%	12.5%	0.18%	2.9%	8.6	0.195	1.21

Recent studies in plant cultivation increasingly include research of biostimulants, preparations based on different extract sources, which at low concentrations enhance growth and metabolic status of plants (Tkalec et al. 2010; Bulgari et al. 2015). Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality (www.biostimulants.eu). It is possible to alleviate the symptoms of oxidative stress by applying different biostimulants containing amino acids, humic acids, vitamins and minerals (Polesskaya et al. 2004). Humic substances (HS) are natural constituents of the soil organic matter, resulting from decomposition of plant, animal and microbial residues. According to their molecular weight and solubility, HS are categorized into humins, humic acids and fulvic acids. Humic substances enhance phosphorus availability and interfere with calcium phosphate precipitation, which immediately increases uptake of macro and micronutrients. It has been proven that HS indirectly enhance nutrient import via stimulation of plasma membrane H^+-ATP-ases, which convert the free energy released by ATP hydrolysis into a transmembrane electrochemical potential (Jindo et al. 2012). In addition, HS from biostimulants enhance activity of key enzymes involved in phenylpropanoid metabolism, suggesting their role in stress response (Olivares et al. 2015). Biostimulants usually consist of amino acids and peptide mixtures obtained by chemical hydrolysis from plant sources and animal wastes (Halpern et al. 2015). These protein hydrolysates modulate N uptake and assimilation, regulate enzyme activities of the Krebs cycle, and also contribute to the cross talk between C and N metabolisms (du Jardin 2015). Novel investigations have increasingly focused on biostimulants application aiming at more rational use of mineral fertilizers, and have very important role in environmental protection (Vernieri et al. 2006; Le Mire et al. 2016). Therefore, the aim of this research was to examine biostimulant influence on the alleviation of oxidative stress and yield in conditions of reduced nitrogen, phosphorus and potassium (NPK)nutrition at 40% in two different tomato hybrids.

2. Material and methods

Two indeterminate tomato hybrids were selected for the research, Bostina F1 and Ombeline F1 (Syngenta AG, Switzerland). Sixty-day-old seedlings with five to six fully developed leaves were placed in plastic containers and filled with 10 L of substrate (Klasmann-Deilmann TS 3 GmbH, Geeste, Germany). The containers were placed in the greenhouse with controlled conditions: average temperature 26–28°C, relative air humidity 50–60%, photoperiod 12/12 h. After planting, the seedlings were divided into four groups and acclimatized for seven days. After acclimatization, each group of plants received different nutritive regimens for 16 weeks (April–July) once a week. The first group (standard nutrition – S) received a complete mineral nutrition (100% NPK) through drip irrigation system recommended by the manufacturer (YARA International Company, Norway). In the second group, mineral nutrition was reduced to 40% NPK (reduced nutrition – R). The third group of plants with 100% NPK nutrition received twice a month biostimulant Viva® (Valagro SpA Italy, www.valagro.com) by foliar application in the quantity of 10.5 mL/plant (dissolved in 0.5 L H_2O in the ratio 1:50 v/v) (standard nutrition with biostimulant – SV). The fourth group with reduced mineral nutrition (40% NPK) received Viva® biostimulant in the same way as the third group (reduced nutrition with biostimulant – RV). Chemical composition and physical properties of biostimulant Viva® are presented in Table 1. The exact quantity of NPK for each plant group is presented in Table 2.

Table 2. Applied mineral nutrition (NPK) per plant (g/plant).

Period	Fertilizer type	Standard nutrition (100%) group S (g/plant)	Reduced nutrition (40%) group R (g/plant)
1 week	STARTER (15:30:15)	2.00	0.80
2–4 Week	YARA I (14:11:25)	3.25	1.30
5–8 Week	YARA II (24:08:16)	2.50	1.00
9–12 week	YARA III (10:05:26)	17.50	7.00
13–16 week	YARA (18:18:18)	7.25	2.90

2.1. Sampling of leaves and fruits

Composite leaves (leaf plate without the petiole) were sampled under the second, third and fourth fruit-bearing branch in order to get homogenous sample. Samples were divided into two groups: the first part was powdered in liquid nitrogen and was used for enzymatic extraction and photosynthetic pigments analysis. The second part of the leaves were dried at normal room temperature and used for analysis of total phenols and total antioxidative capacity (TAC).

Fruits were sampled from the first, second, third and fourth fruit-bearing branch, homogenized in blender and the resulting mash was used for analysis of total soluble solids, total acidity, lycopene and ascorbic acid.

2.2. Determination of total yield

For total fruit yield determination, all fruits were sampled from fruit-bearing branches and their mass was measured using technical balance KERN 440, and results are presented as gram/plant.

2.3. Determination of ascorbic acid content in the fruits

Determination of ascorbic acid content was performed by titrimetric method using standardized analytical procedure (AOAC 1990). Fruit mash (25 g) was homogenized in mortar with 20 mL of 1% HCl (w/v). After filtration, the extract was dissolved in 100 mL of 1% oxalic acid and 10 mL of aliquots was titrated with 2,6-dichlorophenol-indophenol (Tillman's

reagent). The end point of the titration was defined as a pink color that persists through at least 15 s of swirling. Commercial L-ascorbic acid was used as a standard and calculated values were expressed as $\mu g \times g^{-1}$ FW.

2.4. Determination of total soluble solids in the fruits

Fresh tomato juice from the fruit mash was taken for the total soluble solids content determination using digital refractometer. Juice from the sample was squeezed directly onto a refractometer and values were expressed in °Brix units against refractive index.

2.5. Determination of total acidity of the fruits

Fruit mash (25 g) was extracted in mortar with dH_2O and homogenate was incubated in water bath at 80°C for 30 min. After filtration, the extract was dissolved in 250 mL of dH_2O. The content of titratable acids was determined by potentiometric titration using 0.1 M sodium hydroxide and phenolphthalein as indicator (Caretto et al. 2008). The values are expressed as $mg \times g^{-1}$ FW.

2.6. Determination of lycopene content in the fruits

The extraction method was performed according to Fish et al. (2002). Fruit mash (0.5 g) was homogenized in 5 mL of 0.05% butylated hydroxy toluene (BHT) dissolved in acetone (w/v), and 15 mL of ethanol:hexane mixture (1:2) was added. Samples were then stirred on a magnetic stirring plate for 15 min and after shaking 3 mL of dH_2O was added. After phases separation at room temperature for 5 min, the hexane layer was used for absorbance measuring at 503 nm using hexane as blank. Commercial lycopene mixture was used as standard compound and the content of lycopene in tomato fruits is expressed as $\mu g \times g^{-1}$ FW.

2.7. Photosynthetic pigment concentration determination

Plant material (0.5 g) was taken (leaf plate without petiole) and homogenized with pestle in mortar using 100% acetone. After filtration and diluting 25 mL volume, absorbance was measured at 662, 644 and 440 nm with acetone as blank. Molar absorption coefficients of Holm (1954) and Wettstein (1957) were used for estimation and results are expressed as $mg \times g^{-1}$ FW.

2.8. Determination of total phenols concentration (TP) and TAC in the leaves

Dry leaves were powdered and homogenized in mortar with 30% ethanol (1:40 w/v). The homogenate was incubated in water bath for one hour at 60°C using reflux condenser. After filtration, the extract was dissolved in 30% ethanol and used for further analysis. Total phenols content was determined spectrophotometrically, based on phenols reaction with Folin–Ciocalteu reagent (Ough & Amerine 1988). Gallic acid (GA) was used as standard and phenol concentration was expressed as mg GA $\times g^{-1}$ DW. TAC was determined by FRAP (Ferric Reducing/Antioxidative power) method (Benzie & Strain 1996), which is based on the ability of extract to reduce Fe^{3+} ions to Fe^{2+} ions in the solution 2, 4, 6- tripiridyl-s-triazine (TPTZ) and TAC is expressed as $Fe^{2+} \times g^{-1}$ DW.

2.9. Determination of POD and SOD activity in the leaves

Plant material was extracted in 100 mM Na-Pi buffer pH 6.4 containing 0.2% TWEEN and 1 mM phenylmethanesulfonyl fluoride. Homogenate was centrifuged for 10 min at 10,000 rpm at +4°C and supernatant was used for soluble protein analysis. Total protein content was determined by Lowry et al. (1951). Native electrophoresis was performed on 10% polyacrylamide gel with electrophoresis buffer 0.025 M Tris and 0.192 M glycine (pH 8.3) and electric current intensity of 120 and 160 V. Before loading on the gel, samples were mixed with loading buffer (50 mM Tris pH 6.8, 10% glycerol and 0.1% bromophenol blue) at a ratio of 2:1 and 15 µg of protein was applied.

For SOD, visualization gels were incubated 30 min in specific dying solution (100 mM Tris buffer pH 7.8, 0.1 M EDTA, 0.245 mM nitroblue tetrazolium, 0.133 mM riboflavine, 1.72 mM TEMED). After incubation, the gels were illuminated under the UV light and SOD isoforms were detected as white bands on the violet gel.

POD isoforms were detected as violet bands after gel incubation in specific dying solution (0.01% 4-chloro-α-naphtol and 0.03% hydrogen peroxide in 0.1 M Na-Pi pH 6.4. All gels were scanned, and then Rf values of isoforms and enzymatic activities were determined by densitometry method using Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA).

2.10. Statistical data processing

Data were analyzed using SPSS Statistics 23 (2013). Analysis of variance (ANOVA) was conducted and significance of differences among treatment was tested using the least significant difference (LSD). Differences were declared significant at $p < .05$ probability level.

3. Results

3.1. Total yield

The obtained results showed that nutrition variant and biostimulant application significantly affect the total yield in examined tomato hybrids (Table 3). So, in the variant of reduced NPK nutrition, total yield decreased in both hybrids in comparison with standard nutrition (in Ombeline F1 by 8% and in Bostina F1 by 13%). On the other side, with biostimulant application in standard NPK nutrition, yield increased in both hybrids (in Ombeline F1 by 14% and in Bostina F1 by 4%). In the variant of reduced NPK nutrition, applied biostimulant increased yield by 14% in Ombeline F1 and by 13% in Bostina F1.

3.2. Fruit quality parameters

Reduced NPK nutrition led to statistically significant decrease in soluble solids and total acidity in both hybrids in comparison with control (Figure 1(a) and (b)). It is important to emphasize that content of total acidity decreased more than the content of soluble solids. So, in Ombeline F1, reduced

Table 3. Total fruit yield (g) per plant ± SE at Ombeline F1 and Bostina F1 tomato hybrids at different nutrition variants.

NPK nutrition and biostimulant variant	S	R	SV	RV
Ombeline F1	2276 ± 339	2096 ± 278*	2628 ± 348**	2431 ± 312***
Bostina F1	2631 ± 316	2299 ± 265*	2727 ± 279**	2619 ± 324***

Notes: S: standard nutrition (100% NPK); R: reduced nutrition (40% NPK); SV: standard nutrition (100% NPK) with biostimulant; RV: reduced nutrition (40% NPK) with biostimulant.
*significantly different ($p < .05$) using the least significant difference (LSD) test and indicates differences of S group from R group.
**significantly different ($p < .05$) using the least significant difference (LSD) test and indicates differences of S group from SV group.
***significantly different ($p < .05$) using the least significant difference (LSD) test and indicates differences of R group from RV group.

nutrition caused the reduction of soluble solids content by 20% and total acidity by 30%, while in Bostina F1, soluble solids decreased by 21% and total acidity by 32%. These results may indicate that NPK deficiency strongly inhibited the synthesis of acids than soluble solids in fruits of both hybrids. The slight decrease in soluble solids content was noticed in tomato fruits with standard NPK nutrition with biostimulant (in Ombeline F1 by 12% and in Bostina F1 by 10%). Biostimulant application in standard NPK nutrition decreased total fruit acidity only in hybrid Ombeline F1, by 11%. In the plants growing on a reduced NPK nutrition with biostimulant, statistically significant decrease of soluble solids but increased total fruit acidity in hybrid Bostina F1 were observed. In hybrid Ombeline F1, no significant changes of those two parameters were noticed.

The obtained results for ascorbic acid and lycopene content in tomato fruits showed that nutrition variants have very important role in the metabolism of these antioxidants. Reduced NPK nutrition in both hybrids induced statistically significant increase in ascorbic acid concentration with simultaneous decrease in lycopene content in comparison with standard nutrition (Figure 1(c) and (d)). In both examined hybrids, due to NPK reduction, ascorbic acid content increased by 16% while lycopene in Ombeline F1 decreased by 41%, and in Bostina F1, by 35%. Addition of biostimulant to standard nutrition induced statistically significant increase in ascorbic acid and lycopene in both hybrids. If we compare the variants of reduced nutrition with and without biostimulant, it can be concluded that application of Viva® prevented ascorbic acid increase and lycopene decrease.

3.3. Leaf protein and photosynthetic pigment concentration

Tomato plants grown on standard nutrition had significantly lower ($p < .001$) protein level in comparison to plants under reduced nutrition (Table 4). Plants with reduced nutrition significantly differed ($p = .002$) regarding whether the biostimulant was added or not, that is, plants with biostimulant had lower protein levels in comparison with plants without biostimulant. In plants with standard nutrition, biostimulant significantly ($p < .001$) increased the protein level. Reduced NPK nutrition in both examined hybrids led to statistically significant decrease ($p < .001$) in concentration of all photosynthetic pigments (Table 4). In Ombeline F1, total chlorophyll in reduced nutrition decreased by 42% and total carotenoids by 40%. However, reduced nutrition in hybrid Bostina F1 caused smaller decrease of total chlorophyll (12%) and carotenoid (16%) in comparison with standard nutrition. Addition of biostimulant to standard nutrition induced the increase in total chlorophyll content in both hybrids, which is statistically significant only in Ombeline F1, but carotenoid concentration has not changed in any of the examined hybrids. Addition of biostimulant to reduced nutrition prevented the decrease in total chlorophyll and carotenoid content in hybrid Ombeline F1. In Bostina F1, no statistically significant difference was noticed in photosynthetic pigments content in reduced nutrition with and without biostimulant.

3.4. Leaf antioxidative capacity and phenol content

Obtained results showed statistically significant increase in total phenol content (TPC) in plants grown on reduced nutrition in comparison with standard nutrition in both examined hybrids (in Ombeline F1 28% and in Bostina F1 71%) (Figure 2(a)). In addition, reduced nutrition resulted in increase of TAC, in Ombeline F1 for 43% and in Bostina F1 for 80% (Figure 2(b)). Biostimulant in combination with standard nutrition increased TP and TAC with statistical significance in both examined hybrids, while biostimulant with reduced nutrition led to significant decrease in TC and TAC in tomato leaves. In Ombeline F1, biostimulant addition to reduced nutrition decreased TP by 57% and TAC by 59%, while in Bostina F1, the specified treatment decreased TP by 14% and TAC by 24%.

3.5. Leaf enzyme antioxidative activity

Native electrophoresis resolved the presence of four POD isoforms in the leaves of both hybrids with all nutrition

Table 4. Total soluble protein and photosynthetic pigment (total chlorophyll and carotenoids) concentration ± SE in the leaves of Ombeline F1 and Bostina F1 tomato hybrids at different nutrition variants.

		Nutrition variant			
Parameters	Hybrid	S	R	SV	RV
Total chlorophyll (mg × g^{-1} FW)	Ombeline F1	1.67 ± 0.01	0.98 ± 0.00*	1.93 ± 0.08**	1.33 ± 0.05***
	Bostina F1	1.34 ± 0.01	1.19 ± 0.08*	1.39 ± 0.03	0.96 ± 0.01
Carotenoids (mg × g^{-1} FW)	Ombeline F1	0.41 ± 0.03	0.25 ± 0.02*	0.40 ± 0.02	0.38 ± 0.01***
	Bostina F1	0.33 ± 0.01	0.28 ± 0.01*	0.34 ± 0.01	0.24 ± 0.01
Total soluble proteins (mg × g^{-1} FW)	Ombeline F1	2.47 ± 0.20	9.16 ± 0.08*	6.71 ± 0.64**	8.04 ± 0.58***
	Bostina F1	1.40 ± 0.08	7.43 ± 0.05*	6.20 ± 0.40**	6.92 ± 0.44***

Notes: S: standard nutrition (100% NPK); R: reduced nutrition (40% NPK); SV: standard nutrition (100% NPK) with addition of biostimulant; RV: reduced nutrition (40% NPK) with addition of biostimulant.
*significantly different ($p < .05$) using the LSD test and indicates differences of S group from R group.
**significantly different ($p < .05$) using the LSD test and indicates differences of S group from SV group.
***significantly different ($p < .05$) using the LSD test and indicates differences of R group from RV group.

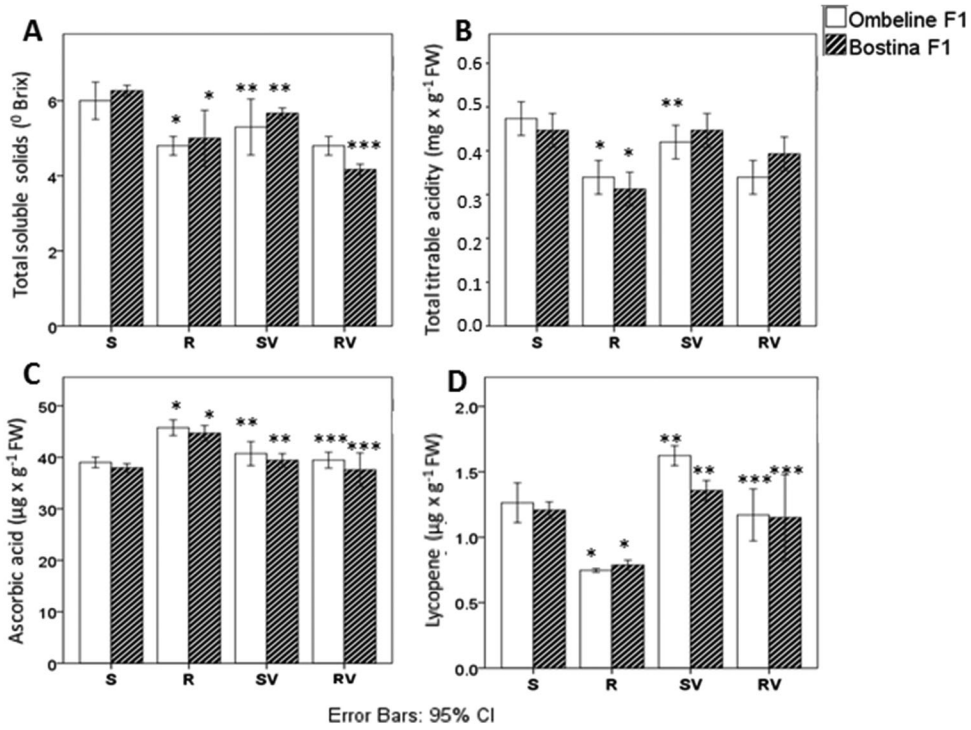

Figure 1. Content of total soluble solids (A), total titrable acidity (B), ascorbic acid (C) and lycopene (D) in the fruits of Ombeline F1 and Bostina F1 tomato hybrids at different nutrition variants: S: standard nutrition (100% NPK); R: reduced nutrition (40% NPK); SV: standard nutrition (100% NPK) with addition of biostimulants; RV: reduced nutrition (40% NPK) with addition of biostimulants.*: significantly different ($p < .05$) using the LSD test and indicates differences of S group from R group.**: significantly different ($p < .05$) using the LSD test and indicates differences of S group from SV group.***: significantly different ($p < .05$) using the LSD test and indicates differences of R group from RV group.

variants (Rf POD1 = 0.28; Rf POD2 = 0.37; Rf POD3 = 0.52; Rf POD4 = 0.58) (Figure 3(a)). Reduced nutrition in both hybrids increased POD activity with statistical significance ($p < .001$) when compared with standard nutrition, namely by 41% in Ombeline F1 and by 60% in Bostina F1 (Figure 3(c)). Addition of biostimulants to standard nutrition did not have influence on statistically significant change of POD activity in either of hybrids ($p > .105$). Application of biostimulant with reduced nutrition decreased POD activity with statistical significance ($p < .001$) in both hybrids: by 35% in Ombeline F1 and 46% in Bostina F1 (Figure 3(c)). Two SOD isoforms were detected by native electrophoresis in the leaves of both hybrids at all nutrition variants (Rf SOD1 = 0.49, Rf SOD2 = 0.58) (Figure 3(b)). Due to reduced NPK nutrition, statistically significant increase of SOD activity occurred in comparison to standard nutrition, in both hybrids: Ombeline F1 for 41% and Bostina F1 for 60% (Figure 3(d)). Addition of biostimulant did not significantly change the SOD activity in standard nutrition ($p > .191$). At reduced nutrition, addition of biostimulant

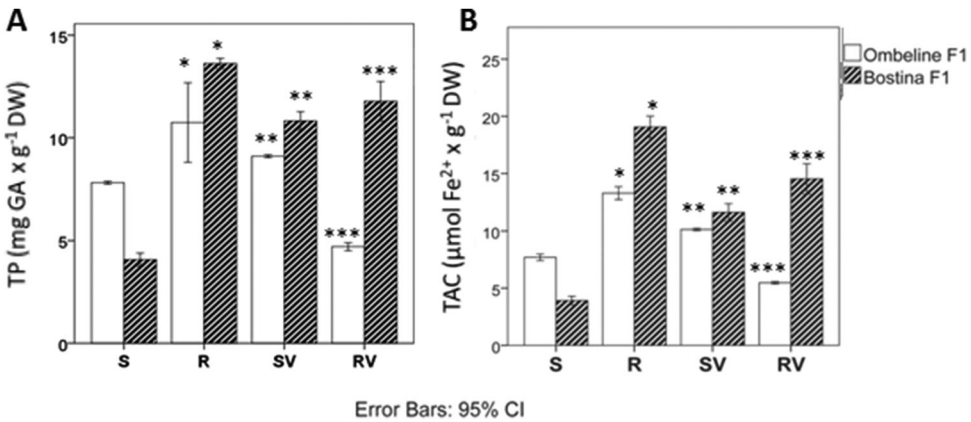

Figure 2. Concentration of total phenols, TP (A) and total antioxidative capacity, TAC (B) in the leaves of Ombeline F1 and Bostina F1 tomato hybrids at different nutrition variants. S: standard nutrition (100% NPK); R: reduced nutrition (40% NPK); SV: standard nutrition (100% NPK) with addition of biostimulants; RV: reduced nutrition (40% NPK) with addition of biostimulants.*: significantly different ($p < .05$) using the LSD test and indicates differences of S group from R group.**: significantly different ($p < .05$) using the LSD test and indicates differences of S group from SV group.***: significantly different ($p < .05$) using the LSD test and indicates differences of R group from RV group.

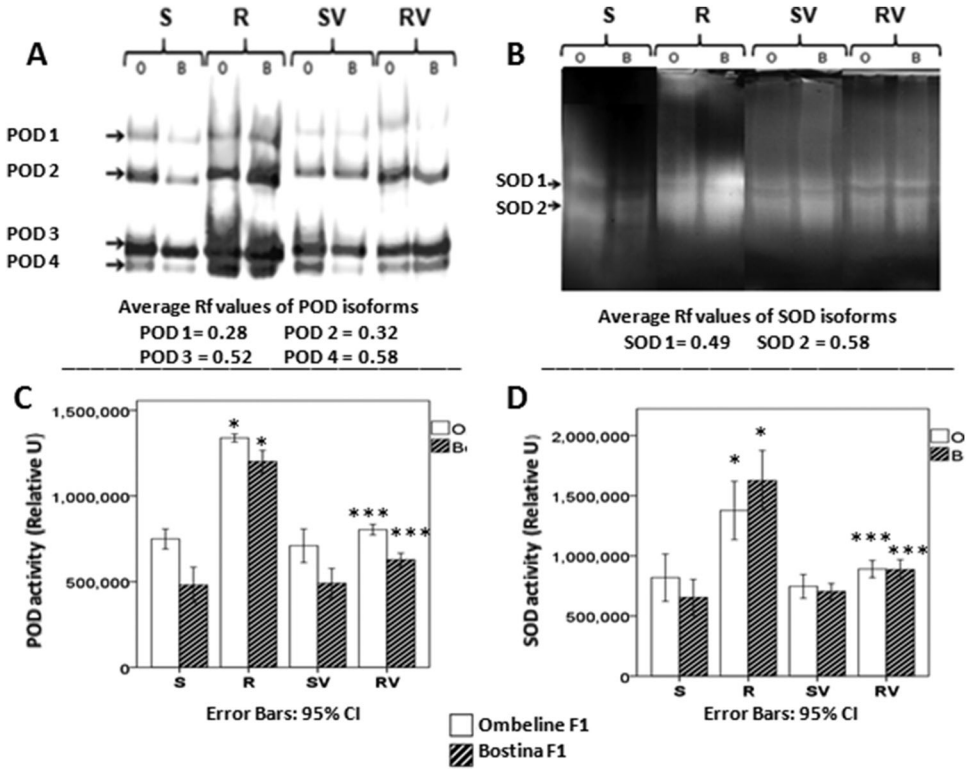

Figure 3. Isoenzyme profile and total activity of peroxidases and superoxide dismutases: POD isoenzyme profile with Rf values (A), POD enzyme activity (C), SOD isoenzyme profile with Rf values (B) and SOD enzyme activity (C) in the leaves of Ombeline F1 (O) and Bostina F1 (B) tomato hybrids at different nutrition variants: S: standard nutrition (100% NPK); R: reduced nutrition (40% NPK); SV: standard nutrition (100% NPK) with addition of biostimulant; RV: reduced nutrition (40% NPK) with addition of biostimulant. POD and SOD activity was calculated by densiometry method in Image Master Total Lab TL 120 program.*: significantly different ($p < .05$) using the LSD test and indicates differences of S group from R group.**: significantly different ($p < .05$) using the LSD test and indicates differences of S group from SV group.***: significantly different ($p < .05$) using the LSD test and indicates differences of R group from RV group.

decreased the SOD activity in both hybrids with statistical significance compared to reduced nutrition without biostimulant ($p < .001$): 36% in Ombeline F1 and by 46% in Bostina F1 (Figure 3(d)).

4. Discussion

The biostimulants application has been projected to reach $2241 million by 2018 and to have an annual growth rate of 12.5% from 2013 to 2018 (Calvo et al. 2014). Possibility of commercial biostimulant application aiming at mineral fertilizers reduction is becoming a global trend in agricultural production (Vernieri et al. 2006). It is known that plants do not uptake minerals from the soil in full capacity, and biostimulants addition can improve the nutrient absorption. Anjum et al. (2014) showed that biostimulant application with half the recommended NPK nutrition for garlic effectively increased the bulb yield. Csizinszky (2003) proved that biostimulant has achieved a positive effect on tomato yield, if nitrogen and potassium were added. Those results are in correlation with ours, but we also proved that biostimulant enhances the tomato yield even when NPK fertilizer is reduced. The aim of modern agriculture is to reduce inputs without reducing the yield and fruit quality, and biostimulants application through different pathways may provide that balance. Biostimulants are considered as substances which enhance growth and yield since they improve nutrient uptake and participate in antioxidative defense. Some authors believe that humic acids are physiologically the most significant components of natural biostimulants, because of their oxygenated functional groups (CO_2H_2, OH phenols and C=O) interacting with metal ions which improve nutrients adoption (Schiavon et al. 2010; Berbara & García 2014). According to numerous studies, different biostimulants which contain of amino acids, humic and fulvic acids even in a small amount increase quantitative yield components, such as fruit set, mean weight, length and diameter, and the number of fruit per plant (Karakurt et al. 2009; El-Nemr et al. 2012; Befrozfar et al. 2013).

4.1. Fruit quality parameters

The soluble sugars glucose and fructose are the largest contributors to the total soluble solids expressed commonly in °Brix. Both soluble sugars and acidity determine the sensorial quality of tomato fruits (Anthon et al. 2011). NPK nutrition is very important for metabolism of carbohydrates and organic acids, because these macronutrients participate in enzymes activation which regulates photosynthetic pathways and transport of metabolites. Potassium has the most important role in carbohydrates transport via phloem, whereby it participates in their translocation from leaves to the fruit during early stages of ripening (Kafkafi et al. 2001). We showed that biostimulant application lowered total soluble solids and total acidity that correlated with each other, regardless of the genotype. Some other authors confirmed that biostimulants decrease sugar and total acids content in tomato fruits, and also change their ratio (Manna et al. 2012). The results of our experiment showed that biostimulant application in standard NPK nutrition slightly decreases total sugars but at the same time increases total phenols (Figures 1 and 2).The possible explanation for this trend of changes of mentioned metabolites is that sugars are used in the phenol biosynthesis. Some other

authors have indicated the positive correlation between phenol synthesis and activities of glucose 6-phosphate dehydrogenase which stimulates mobilization of sugars to phenylpropanoid pathway (Randhir & Shetty 2007). Our results showed that biostimulant in conditions of reduced NPK nutrition can affect the ratio of total sugars and acids, but the change of these two parameters is specific for particular genotype (Figure 1).

Ascorbic acid is an exceptionally important water-soluble antioxidant in plant cells, which, in cooperation with other antioxidative system components, protects the plants from oxidative damages (Smirnoff 1996). Reducing properties of ascorbic acid originate from reactive endiol group owing to which it can directly remove different ROS forms (singlet oxygen, superoxide anion and hydroxyl radical) (Bodannes & Chan 1979). On the other hand, as a substrate for ascorbate peroxidase, ascorbate also contributed to the removal of ROS and protects plant cells from oxidative stress (Apel & Hirt 2004). The main source of ascorbic acid in the plants is leaves and its transport to fruits goes via phloem (Franceschi & Tarlyn 2002). Moreover, increased ascorbic acid synthesis in terms of the oxidative stress is most often associated with its role in redox processes of ascorbate – glutathione cycle (Foyer & Noctor 2005). According to our results, the highest ascorbate content was noticed in fruits of plants treated with reduced NPK, which indicates their higher need for antioxidative defense. On the other hand, biostimulant with standard nutrition caused a slight increase in fruit ascorbate content. Some authors state that humic acids and sugars present in biostimulants improve the biosynthesis of low molecular weight antioxidative compounds such as ascorbate and phenols (Ertani et al. 2015). Our obtained results indicate that biostimulant added with reduced NPK significantly reduced oxidative stress resulting in a lower ascorbate concentration.

A very important water-insoluble antioxidant in the tomato fruits is lycopene. Its antioxidative activity is manifested in ROS neutralization and stabilization of membrane structures due to properties of its β-ionone ring and the presence of eleven conjugated double bonds (Thompson et al. 2000). Lycopene biosynthesis takes place in photosynthetically active tissues in special branch of isoprenoid pathway, whereby it represents a central molecule in carotenoid biosynthesis (Fraser et al. 2001). It has been demonstrated that the availability of macronutrients in the substrate, primarily N, P and K significantly affects the lycopene concentration in tomato fruits (Trudel & Ozbun 1970; Bruulsema et al. 2004). These macronutrients have an important role as enzyme cofactors that participate in metabolism of phytoene and phytofluene, which are essential precursors in the lycopene biosynthesis (Brandt & Molgaard 2001). In connection therewith, decreased content of lycopene in tomato fruits which were subjected to reduced NPK nutrition in our research, confirms the essential role of specified macronutrients in carotenoid biosynthesis. On the other side, exogenous application of humic substances contained in biostimulants can significantly increase the lycopene content in tomato fruits, since it affects the biosynthesis of proteins important in the metabolism of photosynthetic pigments (Grabowska et al. 2015). Results obtained in our research have shown that exogenous application of biostimulant in the reduced nutrition variant prevents the loss of lycopene in fruits (Figure 1).

4.2. Leaf protein and photosynthetic pigment concentration

Since nitrogen is one of the key elements of amino acids and protein structure, its deficiency is often correlated with their increased biosynthesis (Jiang et al. 2011). Potassium is an essential element for nitrate reductase activation, and deficiency of potassium is often associated with decreases in the protein content (Lavres Junior et al. 2010). Phosphorus deficit indirectly decreases protein synthesis due to photosynthetic apparatus damages and lower assimilation as well (Terry & Ulrich 1973). Our results indicate an increase in total protein content with addition of biostimulant, which was expected considering that the plants received additional source of amino acids in this way (Table 4). Supplying of plants with nitrogen directly affects the growth and development, primarily due to essential role of this macroelement in photosynthetic processes and its connection with carboxylation enzymes (Pandey et al. 2000). Insufficient supply of nitrogen, but also potassium and phosphorus, almost always leads to decrease in chlorophyll concentration and rate of photosynthetic processes (Zhao et al. 2001; Bown et al. 2009). The humic acids in biostimulants' preparations contribute the most to preservation of chlorophyll content in plants under abiotic stress conditions (Selim et al. 2012). It has been shown that exogenous application of humic acids activates specific genes included in transcription of proteins important for photosynthetic processes (Trevisan et al. 2011). Our results confirmed that biostimulant prevented photosynthetic pigments loss caused by NPK reduction only in hybrid Ombeline F1, while Bostina F1 was more sensitive since Viva® application did not help in pigments preservation (Table 4).

4.3. Leaf antioxidative capacity and phenol content

Increased phenol content caused by NPK deficiency has been proven in many researches (Chishaki & Horiguchi 1997; Delgado et al. 2006; Fauriel et al. 2007). In addition, it was found that nitrogen deficiency leads to increased activity of phenylalanine ammonia lyase (PAL) whereby cinnamic acid was formed and used for flavonoids and amino groups biosynthesis (Kovačik & Bačkor 2007). In a large number of plants due to increased phenol content, TAC often increases also, since functional groups of phenolic compounds are sequesters of free radicals (Pantelidis et al. 2007; Du et al. 2009). On the other side, addition of humic substances through application of different biostimulants significantly affects the phenylpropanoid pathway. In that case, different classes of phenolic compounds, especially gallic acid, flavanols and stilbenes increase (Pardo-Garcia et al. 2014). According to our results, NPK deficiency significantly enhanced TP and TAC in tomato leaves, but applied biostimulant with standard nutrition caused the same trend (Figure 2). Also, biostimulant in reduced nutrition caused less generation of TP and TAC which refers to its role in oxidative stress preventing. The obtained results in our research showed positive correlation between increase of fruit ascorbic acid content, TPC in the leaves, as well as TAC (Figure 1(c) and Figure 2) and a similar trend was obtained in some other research studies (Parađiković et al. 2011; Zodape et al. 2011). According to literature data, increased content of ascorbic acid is closely associated with phenol concentration due to stimulation of

phenylpropanoid pathway, whereby antioxidative capacity also increases (Randhir & Shetty 2007).

4.4. Leaf enzyme antioxidative activity

Macronutrient status has a significant influence on the chloroplast membrane integrity, and their deficiency can enhance photooxidation processes and superoxide and peroxide generation (Waraich et al. 2011). Superoxide radicals are scavenged by superoxide dismutase (SOD), while the resulting H_2O_2 is reduced to H_2O by peroxidase (POD) and phenolic compounds as co-substrates (Apel & Hirt 2004). Many researches confirmed that N and P deficiency in plant nutrition, led to ROS hyperproduction, which automatically trigger expression of gene for intracellular peroxidase synthesis (Shin et al. 2005; Kovačik & Bačkor 2007). Presence of peroxidase gene *TPX1* whose activation is related to deficiency of P (Quiroga et al. 2000) was found in tomato root. Nitrogen deficiency increases electron excitation in photosystem II reaction centers and automatically intensifies Mehler reaction causing increased generation of H_2O_2 and superoxide which enhance peroxidase and superoxide dismutase response (De Groot & Rauen 1998). Plant peroxidases have very important role in H_2O_2 detoxification which is increasingly generated at deficiency of potassium and leads to necrotic leaf changes (Cakmak 1994). Biostimulants have the capability to decrease oxidative stress, since its active components (humic acids and amino acids) significantly contribute to improving the regulation of hormone activity and antioxidative defense (Zhang et al. 2005; Canellas et al. 2008). It has been demonstrated that humic acids alleviate the oxidative stress symptoms by increasing peroxidase activity, which is automatically reflected in decrease in hydrogen peroxide concentration and membrane permeability improvement (García et al. 2012). According to literature data, in addition to humic substances, plants can adopt amino acids foliar as well (Maini 2006; Stiegler et al. 2013). Exogenous application of structural and non-protein amino acids, including glutamate, histidine, proline and glycine betaine, can provide protection from oxidative stress or (Sharma & Dietz 2006; Forde & Lea 2007; Vranova et al. 2011; Liang et al. 2013). Takahama and Oniki (1997), in their researches, proved that oxidative stress peroxidases can use phenols as substrate for electron transfer. The authors pointed to the role of ascorbic acid in the system POD/phenols/ascorbic acid where the ascorbic acid regenerates oxidized phenolic compounds. Our results showed that reduced NPK nutrition in both hybrids led to increased peroxidase and superoxid dismutase activities (Figure 3). In addition, Viva® biostimulant diminished the activity of SOD and POD in the leaves of plants grown at a reduced NPK, which confirmed its role in oxidative stress prevention (Figure 3). Also, our research showed that biostimulant in combination with reduced NPK led to positive correlation between antioxidative enzyme activity, phenol content and TAC in the leaves, as well as fruit ascorbic acid content in both examined hybrids. This positive correlation is resulting in maintenance of cell homeostasis and plants adaptation to stress conditions.

5. Conclusion

Applied Viva® biostimulant to tomato plants growing under reduced NPK nutrition prevented the occurrence of oxidative stress in the leaves of both examined hybrids without affecting the fruit yield and quality. Taking into consideration all obtained results in this research, we can conclude that the application of biostimulant can decrease non-rational and environmentally harmful use of mineral fertilizers.

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Disclosure statement

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