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STRAWBERRY (*FRAGARIA X ANANASSA* DUCH) LEAF ANTIOXIDATIVE RESPONSE TO BIOSTIMULATORS AND REDUCED FERTILIZATION WITH N AND K

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SUMMARY

Strawberry cultivar Elsanta was grown in peat based substrate in a green house. Full dose and 50% reduced nitrogen and potassium fertilization were applied during fruit bearing period in spring, along with biostimulators Viva®, Megafol® and their combination. The specific activities of guaiacol peroxidase (GPXs; EC 1.11.1.7), catalase (CATs; EC 1.11.1.6), ascorbate peroxidase (APXs; EC 1.11.1.11) and glutathione reductase (GRs; EC 1.6.4.2) in strawberry leaf were stimulated by biostimulators and reduced fertilization. The strongest link seen here was between the enzymes of ascorbate-glutathione cycle (APXs and GRs), which were positively related to trifoliate leaf fresh mass (TLFM). The highest TLFM was observed in Megafol® treated plants.

Key-words: antioxidative activity, ascorbate peroxidase, biostimulators, catalase, fertilization, Fragaria x ananassa Duch, glutathione reductase, guaiacol peroxidase, leaf, strawberry

INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch) is known by its atractive and delicious fruit, which has an important role in human diet because of high antioxidans content.

Strawberry is commonly grown in the field, in glasshouses, other types of green houses and plastic tunnels.

After Takeda (2000), protected cultivation systems provide opportunities to extend strawberry production to areas traditionally considered unsuitable for open-field strawberry culture. However, growth conditions in protected areas can be stressful to plants as well, particularly due higher temperature and air moisture, salt accumulation in root zone, nutrient disorders etc. All these can result with the increased production of reactive oxygen species (ROS) that are highly reactive and their reactivity dictates that they are highly energetic compounds, able to undertake catalytic functions in the absence of enzymes (Turhan et al., 2008). Tolerant plants and genotypes can alleviate environmental stress and ROS-mediated damage through the activation of antioxidative mechanisms, among which enzymes such as different peroxidases, catalase and glutathione reductase have an important role.

Biostimulators or biostimulants are commercial liquid mixtures of different natural compounds extracted from plants, such as aminoacids, glucosides, vitamines, microelements, growth hormones and/or humic acids. There are many reports on stress-protective effects of biostimulators, followed by growth, yield and quality enhancement in different plant species (Zhang and Schmidt, 1999; Muralidharan et al., 2000; Vernieri et al., 2002; Csizinszky, 2003; Maini, 2006; Kauffman

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et al., 2007; Redžepović et al., 2008; Parađiković et al., 2008, 2009). Roussos et al. (2009) observed significant positive influence of the applied mixtures of plant growth stimulators, containing seaweed extract, on strawberry yield and some fruit quality indices. However, the understanding of the physiological effects of biostimulators at the molecular level is poor. Among many different abiotic stresses that were the subject in a plethora of reports so far, nutrient supply disorders and their triggering of an oxidative stress in plants were rarely investigated, especially in the case of strawberry.

Based on the above, the aim of this research was to investigate the strawberry leaf antioxidative response to different N and K supply as well as biostimulators application in fruitbearing period. Antioxidative activity in leaf was expressed by the specific activities of guaiacol peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2), while the evaluated vegetative growth parameters were plant total vegetative aboveground mass, as well as fresh and dry mass of fully developed trifoliate leaf.

MATERIAL AND METHODS

The cold stored frigo plantlets of strawberry (*Fragaria x ananassa* Duch, cultivar Elsanta) were planted in pots of 4.5 L volume, filled with commercial Stender B 400 substrate. A greenhouse was installed in the locality Dalj in the eastern Croatia. Between planting and treatment application, the plants have received 1.760 g N, 0.938 g P, 3.255 g K, 0.669 g Ca, 0.07144 g Mg and 0.189 g S per plant, as well as 6808 μg Fe, 3481 μg Mn, 1346 μg B, 1209 μg Zn, 774 μg Cu and 452 μg Mo. The experiment was set according to split plot method in 4 repetitions with 10 pots (40 plants per repetition). Reduced fertilization treatments and biostimulators were applied in the fruit bearing stage during three weeks (May 12 – June 2 2009). In fertilization variant 100% (F100) 0.540 g N, 0.973 g K, 0.453 g Ca per plant was applied, and in the reduced fertilization variant (F50) plants received 50% N and K less. Biostimulator treatments included the application of 0.25% v/v biostimulator solution of 250 mL per pot in volume. In one treatment biostimulator Viva® (B: V) was used and applied by hosing, in second Megafol® (B: M) applied by spraying, and in the third a combination of the two (B: V+M). Control plants (B: 0), received 250 mL of tap water per pot. The applied biostimulators Viva® and Megafol® contain polysaccharides, glycosides, proteins, aminoacids, vitamins and essential microelements.

Leaf samples for analyses of specific enzymatic activity consisted of ten middle leaflets from the most developed leaves (triplets) per repetition, which were kept in freezer at -20°C until the analyses. Guaiacol peroxidase activity was determined after Siegel and Galston (1967). Catalase activity was determined according to Aebi (1984), and the activity of ascorbate peroxidase was determined according to Nakano and Asada (1981). Activity of glutathione reductase was determined as described in Glutathione reductase assay kit from Oxford Biomedical Research (2001). Specific activity of analyzed enzymes is expressed in U mg^{-1} protein, where the leaf protein concentration was determined after Bradford (1976), using bovine serum albumine (BSA) as standard.

Fresh and dry trifoliate leaf mass (without petioles) was determined based on the ten most developed leaves taken from every repetition, before applying treatments and the week after the last application of biostimulators. Total vegetative over ground mass of plant was determined at the end of the experiment. Fresh trifoliate leaf mass was weighed immediately, while dry mass was measured after drying at 70°C for 24 h and 2 h at 105°C.

The results were analyzed using SAS software (SAS Institute, Cary, N.C.). Analyses of variance were performed using PROC GLM with LSD test at $P \leq 0.05$. Significance of linear correlations among the tested parameters was estimated with t- test.

RESULTS AND DISCUSSION

The antioxidative enzymatic activities in strawberry leaf were significantly higher after treatments with biostimulators and reduced fertilization as compared to the same plants before treatments, except in the case of CATs activity (Table 1). As for the fertilization level influence, GPXs and CATs activities were enhanced by lower N and K supply (Table 1; $P \leq 0.05$), regarding both sampling terms (before and after the treatments) as well as if only plants after receiving treatments are considered (Table 2). In the research of Kandlbinder et al. (2004), N deprivation caused a severe drop in CAT and APX activities in photosynthesizing leaves of *Arabidopsis thaliana.* After Cakmak (2005), the need for protection against photooxidative damage in N-deficient plants can be more marked when the N deficiency stress is combined with an environmental stress. This could explain higher activities of GPXs and CATs in the leaves of plants supplied with reduced fertilization level, implying less than adequate N and K supply in that treatment. In the research of Kim et al. (2010), a member of the type III peroxidase family, RCI3, was found to be up-regulated upon K deprivation and the authors concluded that this peroxidase appears to be another component of the low-K signal transduction pathway in *Arabidopsis* roots. Shin and Schachtman (2004) reported a significant increase in ROS accumulation in plants grown under K^+-deficient conditions and stated that the specificity of the ROS-induced responses to K deprivation are not known.

Table 1. Mean values of guaiacol peroxidase (GPX_s), catalase (CAT_s), ascorbate peroxidase (APX_s) and glutathione reductase (GR_s) specific activity in strawberry leaf under influence of term of sampling (A), fertilization level (B) and biostimulator treatment (C). (ANOVA, F test; means designated with the same letter (^{A,B,C}) are not statistically different per protected least significant difference procedure LSD; *P*=0.05)

Tablica 1. Prosječne specifične aktivnosti gvajakol peroksidaze (GPX_s), katalaze (CAT_s), askorbat peroksidaze (APX_s) i glutation reduktaze (GR_s) u listu jagoda pod utjecajem termina analize (A), razine gnojidbe (B) i tretmana biostimulatorima (C). (ANOVA, F test; prosjeci označeni istim slovom (^{A,B,C}) se ne razlikuju prema LSD testu; P=0,05)

		GPX_s	CAT_s	APX_s	GR_s
		ΔA min^{-1}mg^{-1} prot.			
Term of analyses (A) *Termin analize (A)*					
Before treatments (A1) *Prije tretmana (A1)*		1.098^B	5.093^A	1.325^B	1.335^B
After treatments (A2) *Poslije tretmana (A2)*		1.473^A	4.702^A	2.450^A	3.319^A
F test		47.36	2.25	88.81	190.36
P		<0.0001	0.1679	<0.0001	<0.0001
Fertilization variant (B) *Varijanta gnojidbe (B)*					
100% (B1)		1.199^B	4.489^B	1.796^A	2.203^A
50% (B2)		1.372^A	5.306^A	1.979^A	2.451^A
F test		10.11	9.81	2.35	2.98
P		0.0112	0.0121	0.1600	0.1186
Biostimulator treatment (C) *Tretman biostimulatorima (C)*					
Control (C1)		1.209^A	4.706^A	1.569^B	1.721^C
Viva® (C2)		1.292^A	4.811^A	1.856^B	2.388^B
Megafol® (C3)		1.291^A	4.87^A	2.407^A	2.953^A
Viva®+Megafol® (C4)		1.350^A	5.203^A	1.719^B	2.246^B
F test		1.13	0.68	9.36	12.41
P		0.3889	0.5883	0.0040	0.0015
Interactions *Interakcije*					
(AxB)	F test	10.15	6.07	7.02	4.02
P		0.0111	0.0359	0.0265	0.0759
(AxC)	F test	2.00	2.94	10.23	15.73
P		0.1848	0.0916	0.0029	0.0006
(BxC)	F test	2.91	2.22	3.51	1.38
P		0.0931	0.1547	0.0626	0.3091
(AxBxC)	F test	2.58	1.64	0.98	0.48
P		0.1187	0.2477	0.4448	0.7014

Upon changes in tissue K concentrations, it is likely that the activity of certain enzymes requiring K may be reduced (Schachtman and Shin, 2007); hence, biostimulators containing K, such as Megafol, might stimulate plant metabolism and growth through the activation of particular enzymes. Biostimulators effect on the antioxidative activity was significant in the case of APXs and GRs, considering both terms of analyses (Table 1).

Table 2. Mean values of guaiacol peroxidase (GPX_s), catalase (CAT_s), ascorbate peroxidase (APX_s) and glutathione reductase (GR_s) activity in strawberry leaf after the application of different fertilization level (A) and biostimulator treatment (B). (ANOVA, F test; means designated with the same letter (^{A,B,C}) are not statistically different per a protected least significant difference procedure LSD; *P*=0.05)

Tablica 2. Prosječne vrijednosti specifične aktivnosti gvajakol peroksidaze (GPX_s), katalaze (CAT_s), askorbat peroksidaze (APX_s) i glutation reduktaze (GR_s) u listu jagoda pod utjecajem varijante gnojidbe (A) i tretmana biostimulatorima (B) nakon primjene tretmana. (ANOVA, F test; prosjeci označeni istim slovom (^{A,B,C}) se ne razlikuju prema LSD testu; P=0,05)

	GPX_s	CAT_s	APX_s	GR_s
	ΔA min^{-1}mg^{-1} prot.			
Fertilization variant (A) *Varijanta gnojidbe (A)*				
100% (A1)	1.300^B	3.972^B	2.201^A	3.051^A
50% (A2)	1.647^A	5.432^A	2.700^A	3.588^A
F test	19.04	35.69	4.17	3.69
P	0.0018	0.0002	0.0715	0.0869
Biostimulator treatment (B) *Tretman biostimulatorima (B)*				
Control (B1)	1.325^A	4.161^B	1.706^B	1.973^C
Viva® (B2)	$1.456^{A,B}$	4.451^B	2.338^B	3.436^B
Megafol® (B3)	$1.465^{A,B}$	4.535^B	3.469^A	4.597^A
Viva®+Megafol® (B4)	1.647^A	5.663^A	2.288^B	3.271^B
F test	2.76	7.30	9.10	14.79
P	0.1039	0.0088	0.0044	0.0008
Interaction (AxB) *Interakcija (AxB)*				
F test	4.70	8.17	1.78	0.83
P	0.0308	0.0062	0.2203	0.5092

In the case of GPXs and CATs, there was a significant influence of the interaction fertilization level X biostimulator treatment. These enzymes were stimulated with lower nutrient supply (N and K; F50%) and biostimulators application (Figures 1 and

2) as well, with significant positive linear correlation established in plants after treatment (r=0.785*; Table 3).

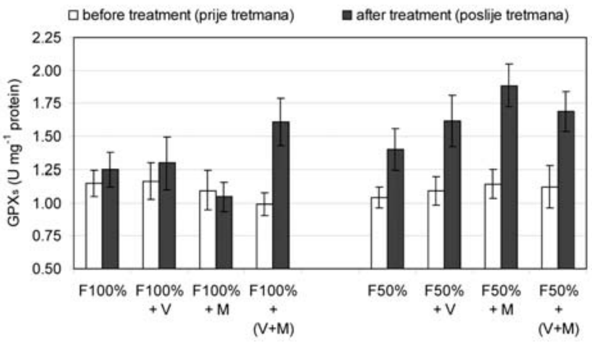

Figure 1. Guaiacol peroxidase specific activity (GPX_s) in leaf of soilless grown strawberry in green house, before and after the treatments with two- levels fertilization with N and K (F100% and F50%) and biostimulators Viva® (V) and Megafol® (M) (untreated control, Viva®, Megafol®, Viva® + Megafol®). Bars are means of four replicates with 40 plants, with standard errors (P≤0.05)

Grafikon 1. Specifična aktivnost gvajakol peroksidaze u listu jagode pri uzgoju bez tla u plasteniku, prije i nakon tretmana s dvije razine gnojidbe s N i K (F100% i F50%) te biostimulatorima Viva® (V) i Megafol® (M) (kontrola, Viva®, Megafol®, kombinacija Viva® + Megafol®). Stupci su prosječne vrijednosti četiri ponavljanja s 40 biljaka, uz standardne pogreške (P≤0,05)

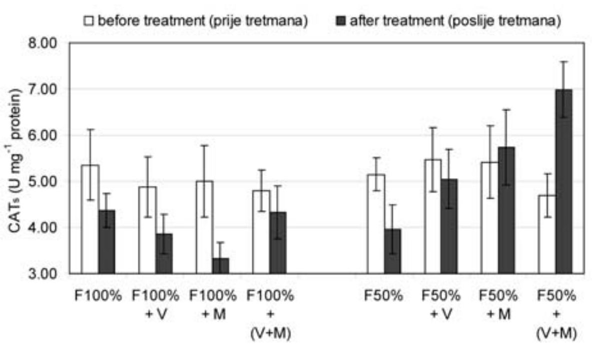

Figure 2. Catalase specific activity (CAT_s) in leaf of soilless grown strawberry in green house, before and after the treatments with two- levels fertilization with N and K (F100% and F50%) and biostimulators Viva® (V) and Megafol® (M) (untreated control, Viva®, Megafol®, Viva® + Megafol®). Bars are means of four replicates with 40 plants, with standard errors (P≤0.05)

Grafikon 2. Specifična aktivnost katalaze u listu jagode pri uzgoju bez tla u plasteniku, prije i nakon tretmana s dvije razine gnojidbe s N i K (F100% i F50%) te biostimulatorima Viva® (V) i Megafol® (M) (kontrola, Viva®, Megafo®l, kombinacija Viva® + Megafol®). Stupci su prosječne vrijednosti četiri ponavljanja s 40 biljaka, uz standardne pogreške (P≤0,05)

APXs and GRs showed the highest activities in plants treated with Megafol. Taking into account only data obtained with plants after treatments, there was a significant stimulative effect of biostimulators on CATs, APXs and GRs activities. In plants before treatments, GPXs and APXs were significantly correlated to GRs (Table 3). The strongest link seen here was between the enzymes of ascorbate-glutathione cycle (APXs and GRs), which were in positive relationship regardless of term of sampling.

Table 3. Coefficients of linear correlations among the antioxidative activity parameters in strawberry leaf (guaiacol peroxidase GPX_s, catalase CAT_s, ascorbate peroxidase APX_s and glutathione reductase GR_s specific activities; * P≤0,05; ** P≤0,01)

*Tablica 3. Koeficijenti linearnih korelacija između pokazatelja antioksidativne aktivnosti u listu jagoda (specifične aktivnosti gvajakol peroksidaze GPX_s, katalaze CAT_s, askorbat peroksidaze APX_s i glutation reduktaze GR_s; * P≤0,05; ** P≤0,01)*

Analiza prije tretmana (n=8) *Analyses before treatments (n=8)*		Analiza poslije tretmana (n=8) *Analyses after treatments (n=8)*		Oba termina analize (n=16) *Both terms of analysis (n=16)*	
x:y	r	x:y	r	x:y	r
GPXs:GRs	0.738*	GPXs:CATs	0.785*	GPXs:APXs	0.720**
APXs:GRs	0.789*	APXs:GRs	0.943**	GPXs:GRs	0.741**
				APXs:GRs	0.963**

While CATs activity was the highest in plants treated with the combination of two biostimulators (B4:V+M), especially in reduced fertilization treatment (Figure 2), APXs and GRs showed the highest activities in Megafol-treated plants (Figures 3 and 4), with stronger effect seen in F 50 variant. Here, the vegetative growth parameters (mean fresh trifoliate leaf mass - TLFM; mean dry trifoliate leaf mass - TLDM; total vegetative over ground mass per plant - TVFM) were not influenced by the applied fertilization level (Table 4). However, biostimulators effect was highly significant for TLFM (*P*=0.047; Table 4). Megafol treatment resulted in the highest values of leaf fresh and dry mass, as well as the greatest total vegetative over ground mass per plant. In the case of leaf, this growth promoting effect of Megafol was statisticaly significant after LSD test.

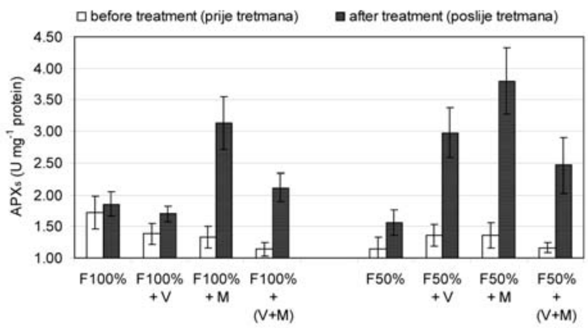

Figure 3. Ascorbate peroxidase specific activity (APX_s) in leaf of soilless grown strawberry in green house, before and after the treatments with two- levels fertilization with N and K (F100% and F50%) and biostimulators Viva® (V) and Megafol® (M) (untreated control, Viva®, Megafol®, Viva® + Megafol®). Bars are means of four replicates with 40 plants, with standard errors ($P \leq 0.05$)

Grafikon 3. Specifična aktivnost askorbat peroksidaze (APX_s) u listu jagode pri uzgoju bez tla u plasteniku, prije i nakon tretmana s dvije razine gnojidbe s N i K (F100% i F50%) te biostimulatorima Viva® (V) i Megafol® (M) (kontrola, Viva®, Megafol®, kombinacija Viva® + Megafol®). Stupci su prosječne vrijednosti četiri ponavljanja s 40 biljaka, uz standardne pogreške ($P \leq 0,05$)

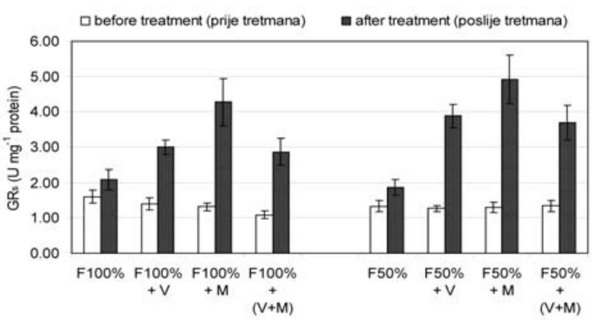

Figure 4. Glutathione reductase specific activity (GR_s) in the leaf of soilless grown strawberry in green house, before and after the treatments with two-levels fertilization with N and K (F100% and F50%) and biostimulators Viva® (V) and Megafol® (M) (untreated control, Viva®, Megafol®, Viva® + Megafol®). Bars are means of four replicates with 40 plants, with standard errors ($P \leq 0.05$)

Grafikon 4. Specifična aktivnost glutation reduktaze (GR_s) u listu jagode pri uzgoju bez tla u plasteniku, prije i nakon tretmana s dvije razine gnojidbe s N i K (F100% i F50%) te biostimulatorima Viva® (V) i Megafol® (M) (kontrola, Viva®, Megafol®, kombinacija Viva® + Megafol®). Stupci su prosječne vrijednosti četiri ponavljanja s 40 biljaka, uz standardne pogreške ($P \leq 0,05$)

Considering that the same biostimulator showed the strongest stimulative effect on APXs and GRs activities in leaf (Table 2, Figures 3 and 4), it seems that its components (high content of amino acids and K) have certain role in ascorbate-glutathione cycle in strawberry leaf. TLFM was also influenced by the interaction fertilization level X biostimulator treatment (Table 4). The analyzed biostimulators induced statistically highly significant differences in plant height, number of branches and fresh plant mass of rosemary (*Rosmarinus officinalis* L) seedlings in the research of Jelačić et al. (2007).

Table 4. Strawberry vegetative growth parameters (mean fresh trifoliate leaf mass - TLFM; mean dry trifoliate leaf mass - TLDM; total vegetative over ground mass per plant - TVFM) after the application of different fertilization level (A) and biostimulator treatment (B). (ANOVA, F test; means designated with the same letter (^{A,B,C}) are not statistically different per a protected least significant difference procedure LSD; P=0.05)

Tablica 4. Pokazatelji vegetativnog rasta jagoda (prosječna svježa masa troliske - TLFM; prosječna suha masa troliske - TLDM; ukupna vegetativna masa po biljci - TLFM) nakon primjene različite gnojidbe (A) i tretmana biostimulatorima (B). (ANOVA, F test; prosjeci označeni istim slovom (^{A,B,C}) se ne razlikuju prema LSD testu; P=0,05)

	TLFM	TLDM	TVFM
	g	g	g plant^{-1}
Fertilization variant (A) *Varijanta gnojidbe (A)*			
100 % (A1)	2.406^{A}	0.843^{A}	45.544^{A}
50 % (A2)	2.450^{A}	0.868^{A}	48.925^{A}
F test	0.31	0.43	4.02
P	0.5916	0.5276	0.0758
Biostimulator treatment (B) *Tretman biostimulatorima (B)*			
Control (B1)	$2.338^{B,C}$	$0.806^{A,B}$	46.525^{A}
Viva® (B2)	$2.500^{A,B}$	$0.904^{A,B}$	46.575^{A}
Megafol® (B3)	2.713^{A}	0.926^{A}	49.600^{A}
Viva®+Megafol® (B4)	2.163^{C}	0.784^{B}	46.238^{A}
F test	8.88	3.43	0.88
P	0.0047	0.0656	0.4858
Interaction (AxB) *Interakcija (AxB)*			
F test	6.52	1.48	3.20
P	0.0123	0.2858	0.0766

Considering the similar effect of Megafol on mentioned enzymes in 100% N and K fertilization level as observed here (Figures 3 and 4), it can be assumed that an explanation of positive effect of Megafol on enzyme activities and leaf fresh mass is not based on simple additional nutrient supply (N and K) by this biostimulator to plants. However, the usage of aminoacids in biostimulators as an easy available, low molecular, organic N source for plant can not be neglected.

CONCLUSION

GPXs and CATs activities were enhanced by lower N and K supply regarding both sampling terms as well as if only plants after treatments are considered and there was a significant influence of the interaction fertilization level X biostimulator treatment. These enzymes were stimulated with lower nutrient supply and biostimulators application. Megafol treatment resulted in the highest values of leaf fresh and dry mass, as well as the greatest total vegetative over ground mass per plant. The strongest link seen here was between the enzymes of ascorbate-glutathione cycle (APXs and GRs), which were in positive relationship regardless the term of sampling. In conclusion, the understanding of feasibly complex effects of various biostimulators on plants growth, stress tolerance and productivity, imply further and more comprehensive research.

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ANTIOKSIDATIVNI ODGOVOR U LISTU JAGODA (*Fragaria x ananassa* Duch) NA TRETMANE BIOSTIMULATORIMA UZ REDUCIRANU GNOJIDBU DUŠIKOM I KALIJEM

SAŽETAK

Sorta jagoda Elsanta uzgajana je u tresetnom supstratu u plasteniku. Puna doza i 50% smanjena doza dušika i kalija primjenjene su tijekom proljetnoga plodonošenja, uz primjenu biostimulatora Viva®, Megafol® i njihove kombinacije. Specifične aktivnosti gvajakol peroksidaze (GPXs; EC 1.11.1.7), katalaze (CATs; EC 1.11.1.6), askorbat peroksidaze (APXs; EC 1.11.1.11) i glutation reduktaze (GRs; EC 1.6.4.2) u listu jagode bile su značajno više poslije tretiranja biostimulatorima i reduciranom gnojidbom. Najjača povezanost uočena u ovom istraživanju, bila je između enzima askorbat-glutation ciklusa (APXs i GRs), koji su bili u pozitivnoj korelaciji s masom svježega lista (TLFM). Najveća masa svježega lista (TLFM) uočena je na biljkama tretiranim Megafolom®.

Ključne riječi: antioksidativna aktivnost, askorbat peroksidaza, biostimulatori, katalaza, gnojidba, Fragaria x ananassa Duch, glutation reduktaza, gvajakol peroksidaza, list, jagoda

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