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COMPARISON OF OXIDATIVE STRESS PARAMETERS IN SOYBEAN SEEDLINGS INOCULATED WITH *MACROPHOMINA PHASEOLINA* FROM DIFFERENT ISOLATES*

ĐORĐE MALENČIĆ, BILJANA KIPROVSKI, VERA STOJŠIN, DRAGANA BUDAKOV, GORANA POGANČEV[1]

SUMMARY: The research was focused on biotic stress caused by Macrophomina phaseolina (Tassi) Goid., a plant pathogen that causes charcoal rot by infecting the root and lower stem of soybean. Differences in pathogenicity of fungi cultures from five isolates (two from sugar beet, one from maize, one from soybean and one from sunflower) have been investigated in seedlings of two soybean cultivars (Meli and Balkan). Comparison of antioxidant systems of soybean seedlings showed different reactions of plants infected with M. phaseolina isolates of different origin. The highest pathogenicity showed isolate from sugar beet ŠR62/4 (2.80), while no significant difference was found for isolates ŠR55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26). The isolate from maize, MphK, showed lowest pathogenicity (1.94). For the most of the biochemical parameters tested (lipid peroxidation, phenylalanine ammonium-lyase activity, total polyphenols, tannins and proanthocyanidins contents) the isolate from sugar beet (ŠR55(3)/09) induced the most significant reaction in soybean seedlings among all isolates investigated. Presence of this pathogen in the field conditions should be further monitored and the crop rotation should be seriously taken into account.

***Key words**: isolates, Macrophomina phaseolina, oxidative stress, pathogenicity, soybean.*

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

Development and life cycle of plants are affected by various external factors. Among these, two groups can be differentiated – abiotic factors such as the climate, or soil composition, and biotic factors such as influence of various pathogens and predators. A typical feature of resistance in plants is the induction of cell death at the site of attempted attack by the pathogen: the hypersensitive response (HR). This response occurs when a plant can specifically recognize the pathogen, during an incompatible interaction. One of the earliest biochemical changes and resistance responses observed after pathogen recognition is the oxidative burst, that is, the controlled release of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) at the site of the pathogen ingress. The oxidative burst is thought to be required for several subsequent defense responses and is expressed in most if not all plant species (Malenčić et al., 2010). As a reaction to stress, antioxidant system is activated in plant cells. Antioxidant defence systems have to be part of aerobic metabolism to counteract oxidative damage from ROS. This includes antioxidant enzymes such as superoxide-dismutase (SOD, EC 1.15.1.1), catalase (Cat, EC 1.11.1.6) and different kinds of plant peroxidases, as well as some non-enzymatic antioxidants such as glutathione, tocopherols, carotenoids, ascorbate, flavonoids and other phenolic compounds (Malenčić et al., 2008).

This research focused on biotic stress caused by necrotrophic fungus *Macrophomina phaseolina* (Tassi) Goid., a plant pathogen that causes charcoal rot by infecting the root and lower stem of over 500 plant species. Although being described as typical pest of dry climate, it appears regularly in Serbia. Moreover, due to its ability to cause infection during all phenophases, it is considered as one of the economically most important pests on soybean, maize, sugar beet and sunflower.

The aim of this study was to compare biochemical parameters of oxidative stress induced by different *M. phaseolina* isolates and to examine relationship between the origin of isolates, their pathogenicity and soybean seedlings response to infection.

MATERIALS AND METHODS

In order to compare parameters of oxidative stress, both phytopathological and biochemical tests were applied. Morphological and breeding characteristics, as well as the pathogenicity test, were performed for five *M. phaseolina* isolates of different origin (host species). These included two isolates from sugar beet (ŠR55(3)/09 and ŠR62/4), one from maize (MphK), one from soybean (MphSo) and one from sunflower (MphSu). Seeds of two different soybean cultivars (Meli and Balkan) were obtained from the Institute of Field and Vegetable crops, from Novi Sad, Serbia. Seeds were inoculated using the method of artificial inoculation on filter paper in Petri dishes.

Seven-day-old seedlings were collected for the biochemical assays. One g of fresh plant material was homogenized with 10 ml 0.1 M K_2HPO_4 at pH 7.0. After centrifugation for 10 min at 4°C and 15 000 *g* the supernatant was used for enzyme activity measurements.

Superoxide-dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm, according to method by Panda (2012), and expressed as U g^{-1} fresh weight. Phenylalanine-ammonium lyase

(EC 4.3.1.5) activity was determined according to Gerasimova et al. (2005) and expressed in U g^{-1} fresh material (fr.m.). The lipid peroxidation intensity (LP) was measured as malondialdehyde (MDA) production at 532 nm with thiobarbituric acid (TBA). The total amount of TBARS (TBA-reactive substance) is given as nmol MDA g^{-1} fresh weight, as described by Moon and Shibamoto (2009).

Total phenols were extracted and determined from air-dried and pulverized plant material. Dry extracts have been made by mixing 0.2 g of previously dried and minced seedlings with 70% acetone (1/50, w/v).Their content was determined spectrophotometrically at 720 nm after reaction with Folin-Ciocalteu reagent using (+)-catechine for the standard curve. The total amount of soluble phenols was expressed as mg catechine g^{-1} dry weight (d.w.) (Hagerman et al., 2000). Total tannins were determined by method of Makkar (2003), with addition of polyvinylpolypirolidone (PVPP), and the results are expressed as mg catechine g^{-1} dry weight. Proanthocyanidins were determined according to the same method and expressed in mg leucoanthocyanidin g^{-1} dry weight.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) can produce stable free radicals in ethanolic (EtOH) solution. Due to its odd electron, DPPH strongly absorbs at 517 nm. In order to evaluate the antioxidant potency through free radical scavenging by test samples, the change of optical density between test samples containing plant extracts and blank (without plant extracts) was assessed to evaluate the antioxidant potential of test samples (Lee et al., 1998).

Results were expressed as mean ± standard error of mean (SEM) (the average of three experiments) and tested by ANOVA followed by comparison of the means by Duncan's multiple range test (P<0.05). Data were analyzed using STATISTICA for Windows version 11.0.

RESULTS AND DISCUSSION

The highest pathogenicity in cultivar Meli showed isolate ŠR62/4 (2.80). Pathogenicity of ŠR 55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26) isolates showed no significant difference. The isolate MphK showed statistically lowest pathogenicity (1.94). Rather similarly, the isolate ŠR62/4 proved to have the highest pathogenicity in cultivar Balkan, as well (2.47). Patogenicity of ŠR55(3)/09 (2.31), MphSu (2.28), MphSo (2.24) and MphK (2.03) isolates did not differ in Balkan cultivar.

In addition to this, comparison of biochemical parameters showed differences between cultivars (Figure 1). Results obtained for intensity of lipid peroxidation (LP) proved lower MDA concentration in Meli cultivar. Control plants (uninfected plants) of cultivar Meli had higher values of MDA content than infected ones, however, Balkan plants infected with ŠR55(3)/09 and MphK isolates had MDA values higher than the control. These high concentrations indicate plants' inability to protect its cell membranes from ROS damage.

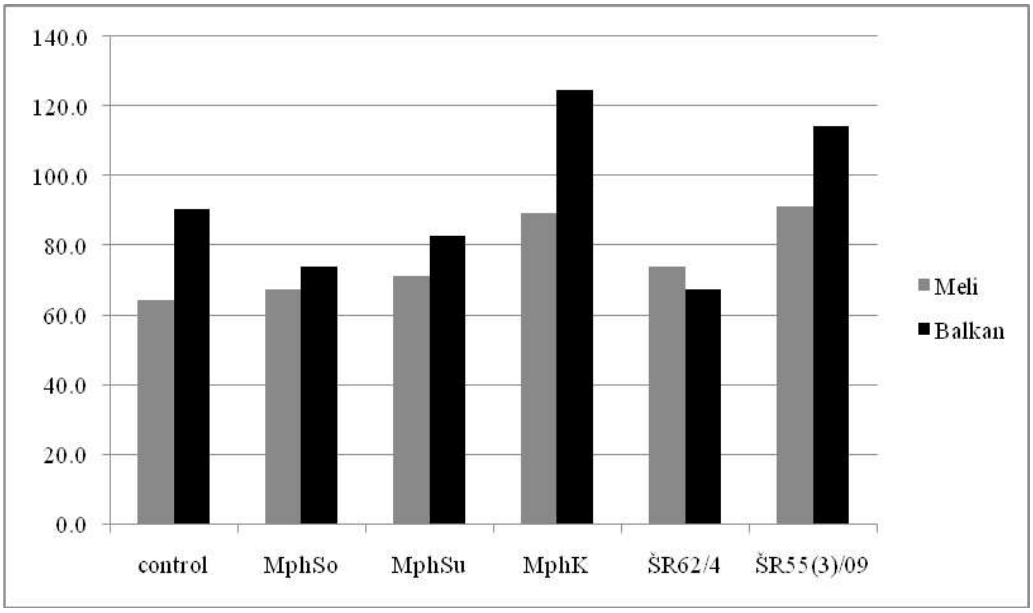

Figure 1. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on lipid peroxidation (nmol MDA g^{-1} fr.w.)

The results for the superoxide-dismutase (SOD) activity showed no significant differences between control and infected plants after pathogen invasion (Figure 2). Isolate MphSo from soybean showed invariable values for SOD activity in infected and uninfected plants of both investigated cultivars, while isolate MphSu from sunflower caused an increase in SOD activity in cultivar Meli.

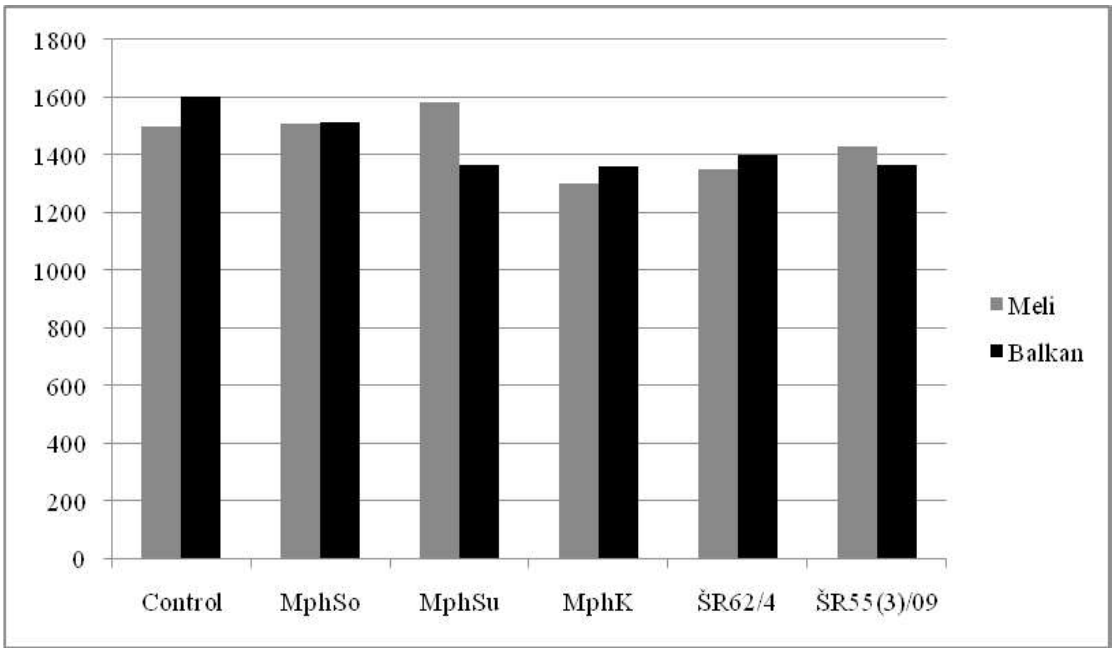

Figure 2. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on superoxide-dismutase activity (U g^{-1} fr.w.)

Since one of the first responses observed in plants after pathogen recognition is the oxidative burst, it has been expected for infected plants to produce large amounts of $O_2^{\cdot-}$ at the site of the attack. SOD is an inducible enzyme and its activity usually correlates with the $O_2^{\cdot-}$ quantity. These results are not in agreement with our previous results which proved increase in SOD values after inoculation with *Sclerotinia sclerotiorum* as a response to biotic stress (Malenčić et al., 2010). Still, the scavenging system against reactive oxygen species seems to be less effective in soybean, and the activity of SOD is not enough to prevent oxidative damage, which is in agreement with some other findings (Malenčić et al., 2008; Baisak et al., 1994).

Similarly to our previous experiments on impact of necrotrophic fungi on phenylalanine ammonium-lyase (PAL) activity (Kiprovski et al., 2012), seedlings of Balkan cultivar infected with isolates MphSu, MphK and ŠR 55(3)/09, had markedly increased PAL activity in comparison to uninfected plants. The higher activity of the enzyme was detected in plants inoculated with isolates of lower pathogenicity. Contrary to that, all infected plants of cultivar Meli had decreased PAL activity in comparison to control (Figure 3).

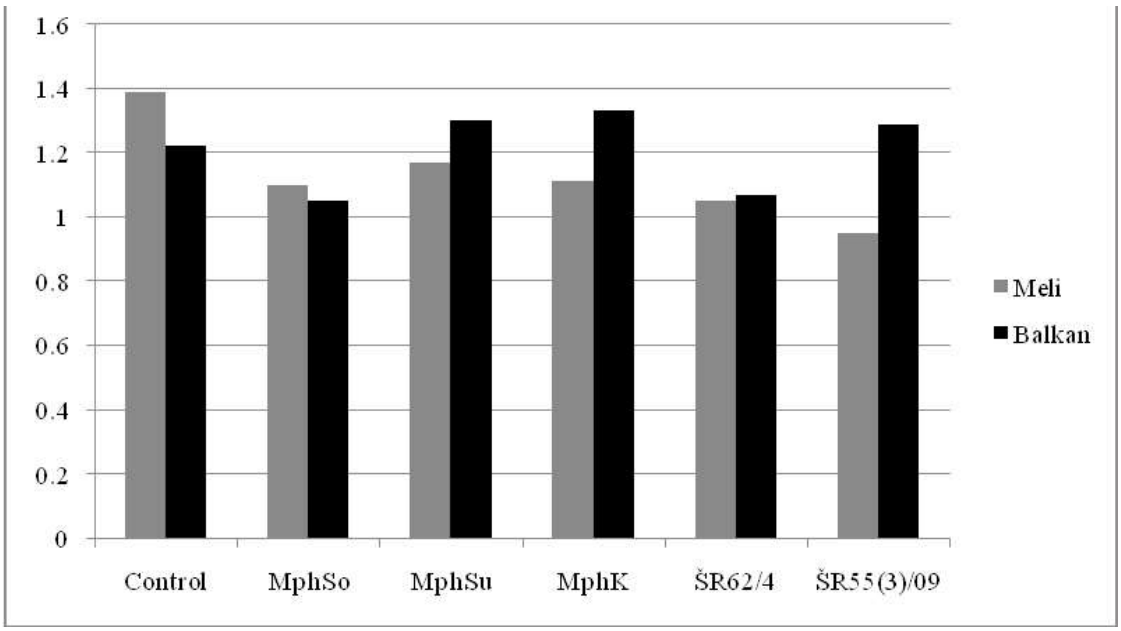

Figure 3. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on phenylalanine ammonium-lyase activity (U g^{-1} fr.m.)

It seems that both enzymes assayed, SOD and PAL, were heavily affected with the pathogen infection and similarly to some other diseases, such as *Sclerotinia sclerotiorum* or *Rhizoctonia solani*, soybean plants have no mechanisms to cope with the *Macrophomina phaseolina*, either. Plant specimens were harvested when infection occurred on 50% of treated plants, and it seems now that timing was too prolonged, i.e. the seedlings were already too affected. For this reason, we suggest in the future, in enzymatic studies, that seedlings/young plants of intolerant plant species should be collected earlier, not later than period when 30% of samples are showing full symptoms of the disease.

Taking into account that polyphenols play important role in plant immune system,

and results from previous research (Boudet et al. 1998; Kiprovski et al., 2014) we also focused on different polyphenol classes present in the plant tissues. The first parameter, describing total polyphenol content, showed effect of accumulation of these compounds in both cultivars investigated, with all isolates applied. Especially cv. Meli showed an increase in phenolics accumulation, ranging from 100% (for isolate MphSo) to 150% (for isolate ŠR55(3)/09. In cv. Balkan, same as in cv. Meli, the isolate ŠR 55(3)/09 induced the most pronounced plant response (Figure 4).

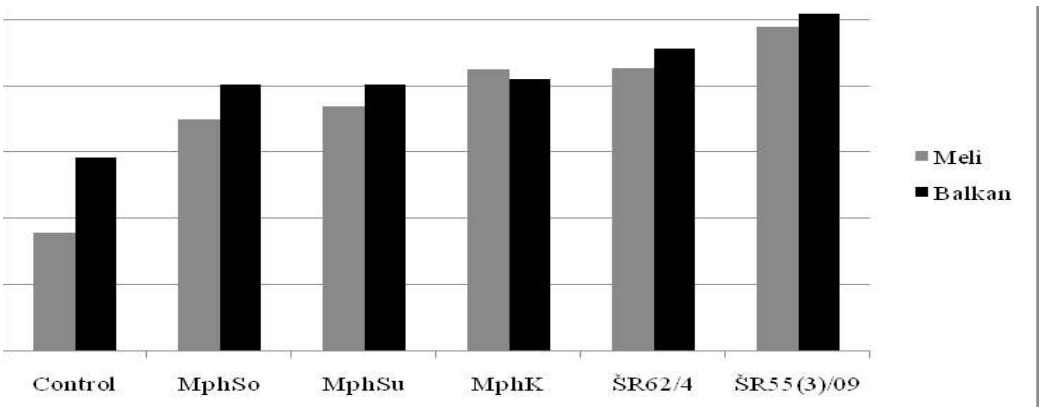

Figure 4. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on total polyphenols content (mg catechine g^{-1} d.w.)

Total tannin content proved to be significantly higher in infected plants compared to control which was expected due to higher polyphenols content (Figure 5). Tannins comprise large amount of total phenols in plant cell and are known as good reactive oxygen scavengers. Thus, when attacked, plants produce additional tannins as a response to pathogen presence. Similarly to results for total polyphenols, the isolate ŠR55(3)/09 caused the highest tannin accumulation in both cultivars. The isolate with the lowest tannin accumulation induction, in both cultivars, was the isolate MphSu.

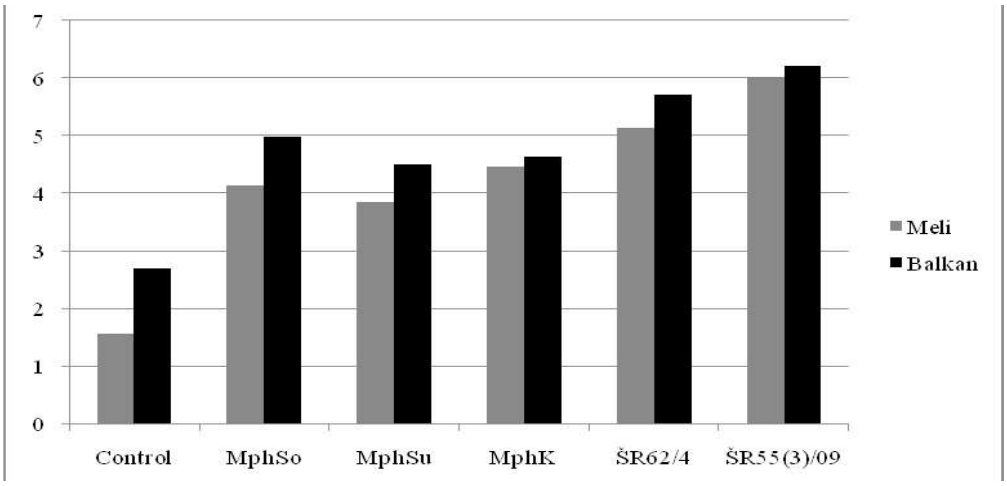

Figure 5. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on total tannins content (mg catechine g^{-1} d.w.)

Proanthocyanidins content showed statistically significant differences between the cultivars. The constitutive level of these compounds in cv. Balkan was more than two-fold higher compared to cv. Meli. Still, cv. Meli responded to pathogen attack in uniform manner, i.e. all isolates used in the experiment provoked proanthocyanidins accumulation significantly higher than in uninfected plants. In cv. Balkan only isolates MphSo and ŠR55(3)/09 triggered elevated biosynthesis of these valuable antioxidant substances. Once again, isolate ŠR55(3)/09 induced the highest accumulation of proanthocyanidins in both cultivars. Comparison of proanthocyanidin contents among cultivars is presented in Figure 6.

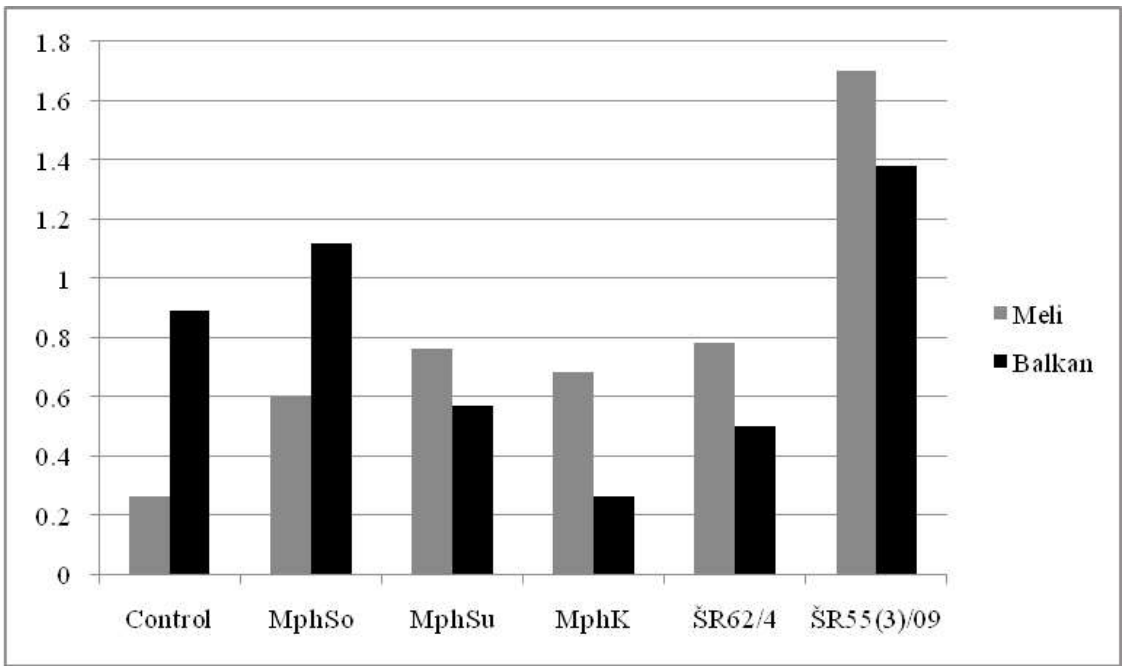

Figure 6. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on proanthocyanidins content (mg leucoanthocyanidin g^{-1} d.w.)

DPPH radical scavenging activity is a measure of non-enzymatic antioxidant activity. Higher levels of DPPH activity have been correlated with tolerance to different stress conditions (Kang and Saltveit, 2002), but they also point out to the presence of biologically active biomolecules with pronounced antioxidant activity. In both cultivars the results showed that isolates MphSu, MphK and ŠR55(3)/09 had higher DPPH-scavenging values than the control and are in agreement with the results obtained for total polyphenols, tannin and proanthocyanidins contents. Lower values compared to uninfected plants obtained for isolates MphSo and ŠR62/4 imply that the infected plant lack natural plants antioxidants in concentration high enough to neutralize DPPH-free radicals. Results are expressed in % of neutralized DPPH-free radicals and presented in Figure 7.

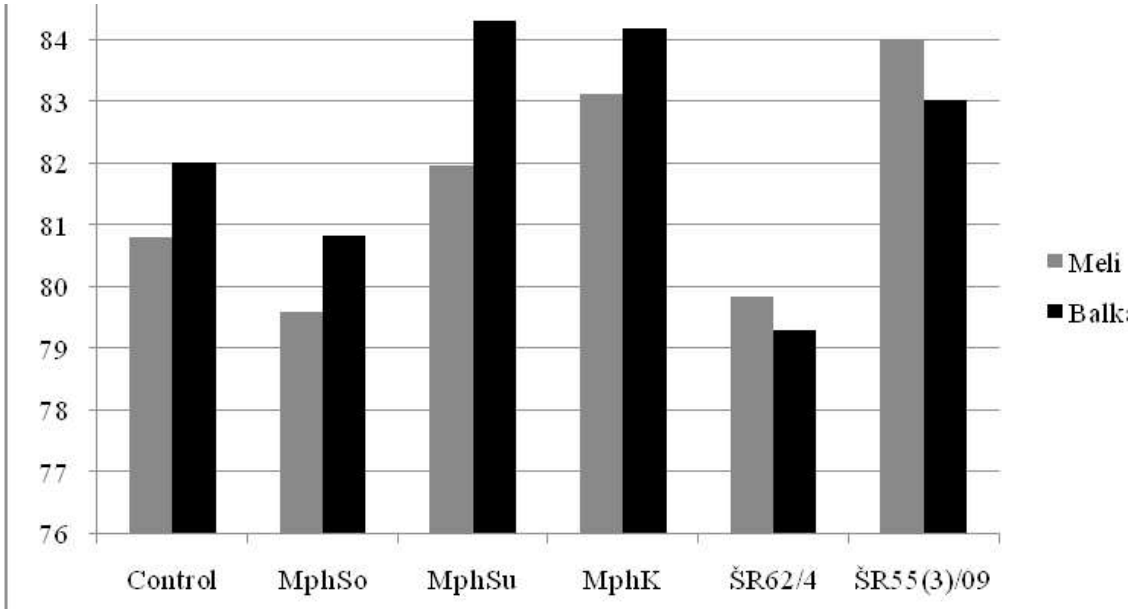

Figure 7. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on DPPH-radical scavenging activity (%)

CONCLUSION

Comparison of antioxidant systems in soybean plants showed different reactions between plants infected with *Macrophomina phaseolina* isolates of different origin. Overall pathogenicity was proven to be rather similar in both cultivars - Meli (2.07) and Balkan (2.00). Isolates provoked an intense reaction in infected plants, compared to each other; yet the highest pathogenicity showed isolate from sugar beet ŠR62/4 (2.80). Patogenicity of ŠR55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26) did not differ significantly in both investigated cultivars. The isolate from maize, MphK, showed lowest pathogenicity (1.94).

In Meli cultivar all of the isolates have induced increased lipid peroxidation intensity, whereas in Balkan cultivar the increase in MDA concentration was only recorded in plants infected with MpK and ŠR55(3)/09 isolates. Regarding activity of SOD it seems that scavenging system against reactive oxygen species was not effective in soybean seedlings, and the activity of SOD could not prevent oxidative damage. After inoculation with isolates of lower pathogenicity (MphSu, MphK and ŠR55(3)/09), PAL activity in cultivar Balkan increased, while in cultivar Meli the activity of this enzyme was invariable. Pathogen presence caused accumulation of plant total polyphenols and tannins in both cultivars. Accumulation of proanthocyanidins was the highest in both infected cultivars when isolate ŠR55(3)/09 was applied. Total non-enzymatic antioxidant capacity, presented as DPPH-test, was high in both cultivars, being around 80% of neutralized radicals. Overall, the *M. phaseolina* isolate coming from sugar beet - ŠR55(3)/09 showed the most significant changes for the most of the parameters tested in both cultivars and could be considered as the most detrimental to soybean cultivars. Its presence in the field conditions should be further monitored and the crop rotation should be seriously taken into account.

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POREĐENJE PARAMETARA OKSIDATIVNOG STRESA U KLIJANCIMA SOJE INOKULISANIM SA RAZLIČITIM IZOLATIMA *MACROPHOMINA PHASEOLINA*

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Izvod

U radu je ispitivan biotički stres u klijancima soje izazvan gljivom *Macrophomina phaseolina* (Tassi Goid.), patogenom biljaka i uzročnikom ugljenaste truleži korena i donjeg dela stabla. Različite karakteristike izolata gljive (dobijenih sa šećerne repe (2), kukuruza (1), soje (1) i suncokreta (1)), ispitane su u dve sorte soje (Meli i Balkan). Poređenje antioksidantnih sistema u biljkama soje ukazalo je na različite odgovore između sorti soje inficiranih izolatima *M. phaseolina* različitog porekla. Izolati su izazvali intenzivnu reakciju u inficiranim klijancima: najveća patogenost zabeležena je kod izolata sa šećerne repe ŠR62/4 (2.80). Izolati ŠR55(3)/09 (2.58), MphSo (2.32) i MphSu (2.26) nisu pokazali statistički značajnu razliku u patogenosti. Izolat sa kukuruza, MphK, pokazao je najnižu patogenost (1.94). Za većinu ispitivanih biohemijskih parametara (lipidna peroksidacija, aktivnost fenilalanin amonijum-liaze, sadržaji ukupnih polifenola, tanina i proantocijanidina), izolat ŠR55(3)/09 sa šećerne repe, izazvao je najintenzivnije i najznačajnije reakcije u klijancima soje u poređenju sa svim ostalim ispitanim izolatima. Njegovo prisustvo u poljskim uslovima se mora podvrgnuti monitoringu, a o rotaciji useva na takvim oranicama se mora povesti više pažnje.

Ključne reči: izolati, *Macrophomina phaseolina*, oksidativni stres, patogenost, soja.

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