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PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF SUNFLOWER HYBRIDS INOCULATED WITH BROOMRAPE

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ABSTRACT. Phenolic compounds such as polyphenols, tannins and flavonoids as well as antioxidant activity, play an important role in the plant defense mechanisms. The above mentioned parameters were measured in four sunflower hybrids (NORH-34, HOB-2, IMI-3-911 and NS-H-111) inoculated with broomrape (sunflower root parasitic plant). The synthesis and accumulation of these compounds depended on genotype and broomrape inoculation. Total phenolic content in healthy sunflower root tissue ranged from 2.87 up to 10.53 mg, while in infected root tissue this parameter ranged from 6.06 to 12.66 mg gallic acid equivalents/g dry root weight. Total tannins content in healthy root ranged from 2.01 to 6.74 and in infected root from 4.30 to 8.17 mg gallic acid equivalents/g dry root weight. Total flavonoids in healthy root tissue were within the range 3.36-44.94 and in infected root tissue 35.35-83.89 mg of rutin equivalents/g dry root weight. Antioxidant activity in sunflower roots correlated with the total phenolic and total tannins and flavonoids content.

Keywords: *broomrape, sunflower, polyphenol compounds, antioxidant activity*

INTRODUCTION

Plants encounter parasitic attacks by pathogenic microorganisms, herbivores and insects. Parasites have developed different strategies for colonizing plants, since they need regulation mechanisms to effectively adapt to changes in their environment. Plants have developed effective defense mechanisms against pathogenic infections. Following penetration by pathogens induced structural and chemical barriers are activated in order to prevent the pathogen's progression [1]. Under the stress conditions plants produce higher content of reactive oxygen species (hydroxyl and superoxide radicals). Plant

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defense mechanism includes activation of enzymatic and non-enzymatic system. Enzymatic mechanism is activation of different enzymes such as catalase, superoxide dismutase and different types of peroxidases. Non-enzymatic defense system includes higher synthesis of some biomolecules such as glutathione, vitamins C and E, and other scavengers of free radicals [2].

Plant phenolics are secondary metabolites that encompass several structurally diverse classes of natural products. The phenolic composition varies considerably both qualitatively and quantitatively between species and between individuals of the same species. Secondary metabolites have specific significance in plants ecology and their adaptation to different biotic and abiotic factors. Phenolic compounds involved in plant defense are either constitutive or synthesized *de novo* (postinfectional). Phenolic compounds synthesized *de novo* accumulate in response to plant infection by a pathogen [3]. These compounds have significant scavenging activity of reactive oxygen species [4].

Broomrapes (*Orobanche* spp.) belong to the family of obligate parasitic flowering plants-*Orobanchaceae*. Broomrapes are native primarily to the Mediterranean region (i.e. North Africa, the Middle East, and southern Europe), and western Asia [5]. With anticipated climatic changes *Orobanche* species could pose greater threats to agriculture by expanding their ranges farther north in Europe [6]. *O. cumana* (Wallr.) is the cause of many economic losses in sunflower production on a worldwide scale [7]. In addition to this pest's huge infectious potential, another great problem is caused by the heterogeneity of its population i.e. by the existence of multiple physiological races.

Definition of phenolic compounds defense role and the control of their synthesis could be important in the crop protection primarily through breeding programs [8]. Several authors indicated the involvement of lignification and cell wall phenolics deposition in the sunflower tissue as a defense response to broomrape penetration [9, 10, 11]. Resistant or tolerant sunflower hybrids are the most efficient way of suppression of this parasitic plant [7].

The objectives of the present study were to investigate the content of phenolic compounds and antioxidant response in root tissue of different sunflower hybrids infected by broomrape.

RESULTS AND DISCUSSION

The evaluation of broomrape attack on sunflower plants was done in the period of full flowering (Table 1). The highest attack was observed on hybrid NS-H-111 and on high-oleic hybrid HOB-2. Other two hybrids IMI-3-911 and NORH-34 had lower attack, below 10 broomrape plants per sunflower plant. These data showed the differences between hybrids in the resistance

to broomrape in the conditions of field infection. The reaction of the tested hybrids was expected and similar to the results obtained in our previous investigations [12].

Table 1. Broomrape attack on sunflower plants

Hybrid	Number of broomrape plants per sunflower plant
NORH-34	8
HOB-2	27
IMI-3-911	7
NS-H-111	29

The content of total phenolics (TP) and total tannins (TT) in the root of four tested sunflower hybrids in the period of full flowering are given in the Table 2. Sunflower hybrid, broomrape attack and interaction of these factors had effect on production of TP and TT in root. TP and TT contents were highest in hybrid NORH-34 (12.66 and 8.15 mg gallic acid equivalents (GAE)/g dry weight (DW) respectively). Production of these compounds in NORH-34 was approximately four times higher in infected sunflower roots at the contact point with broomrape (CP) than in healthy sunflower roots (HSR). Besides the resistance based on physical barriers; resistance could be based on the chemical response such as the production and secretion of the toxic phenolic compounds [13]. Resistant vetch defense mechanism involves elevated induction of the phenyl proponoid pathway upon *Orobanche* infection, conferring mechanical and chemical barriers confronting the invading parasite [14]. Reaction in the sunflower-broomrape pathosystem is in the correlation with increasing of phenolic level, peroxidase activity [15] and accumulation of substances on the inner part of host-plant xylem vessels [16]. Sunflower roots after the treatment with 1,2,3-benzothiadiazole-7-carbothioic acid S-methyl-ester (BTH) resistance-inducing agents (had higher bound phenolic content in cell walls and that might indicated that the plant formed structural barriers as defense to broomrape [17]. Accumulation of phenolic compounds could help in order to test resistance to broomrape in different selection phase [18]. Coumarins such as scopoletin, scopolin and ajapin (phenolic compounds) in sunflower inhibit germination and attaching of broomrape [19]. Hybrid IMI-3-911 also had high synthesis of TP and TT (11.74 and 8.17mg GAE/g DW respectively) and lower level of infestation. However there is no significant difference in this hybrid in content of TP and TT related to infection. In root of hybrids NS-H-111 and HBO-2 content of TP and TT was significantly lower in comparison to NORH-34 and IMI-3-911. However significant differences in the content of TP, in NS-H-111 and HBO-2

between HSR and CP indicate that these hybrids responded to infection by synthesis of phenolic compounds, but it was not enough to suppress the high intensity of broomrape attack in the period of full flowering. Production of secondary metabolites is caused by stress and they are produced in damaged tissue [20].

Table 2. Total polyphenols and tannins content in different sunflower hybrids

Hybrid	Infection	Total polyphenols[1]	Total tannins[1]
NORH-34	CP	12.66 ± 0.23^{a}	8.15 ±0.05^{a}
	INF+	4.59 ±0.18^{g}	2.89 ± 0.59^{de}
	HSR	2.87± 0.18^{i}	2.01± 0.25^{e}
HOB-2	CP	7.25 ± 0.22^{d}	5.38 ±0.06^{bc}
	INF+	6.80 ± 0.18^{de}	5.22 ± 0.20^{bc}
	HSR	6.54 ±0.25^{ef}	4.95 ± 0.09^{c}
IMI-3-911	CP	11.74 ±0.21^{b}	8.17 ± 0.18^{a}
	INF+	10.66 ± 0.49^{c}	7.33 ± 0.04^{a}
	HSR	10.53 ± 0.09^{c}	6.74 ± 1.71^{ab}
NS-H-111	CP	6.06 ± 0.20^{f}	4.30 ±0.04^{cd}
	INF+	6.16 ± 0.13^{ef}	4.14 ± 0.09^{cd}
	HSR	3.93 ±0.09^{h}	2.94 ± 0.13^{de}
p		Hybrid 0,00** Infection 0,00** Infection x Hybrid 0,00**	Hybrid 0,00** Infection 0,00** Infection x Hybrid 0,00**

Data are mean ± SE values
[1] Expressed as mg of gallic acid equivalents/g of dry root.
[a-i] the values without the same superscript within each column differ significantly (p< 0.05).
**- significant at probability level (p< 0.01).
CP - at the contact point of broomrape tubercle and sunflower root.
INF+ - outside of contact point from the parasite and host plant.
HSR - healthy sunflower roots.

There are strong evidences for a role of flavonoids in plant resistance and their induced formation after injury by pathogens or pests [21].Total flavonoids content (TF) in sunflower root was influenced by hybrid, intensity of infection and interaction of these two factors. The highest TF content had been measured in hybrid IMI-3-911 at CP and it was about two times higher than in HSR as well as in hybrid NS-H-111. Hybrid NORH-34 had 20 times higher TF content in attacked roots than in healthy ones. Accumulation of phenolic compounds, such as flavonoids, in the sunflower-broomrape resistance

mechanisms is well-known phenomenon [22, 23]. Recent investigations also showed increased levels of phenolics and flavonoids in the broomrape infected tomato roots [24]. Level of flavonoids and tannins and DPPH values was relatively high in high-oleic hybrid HOB-2 compared to other tested hybrids, but there are no significant differences between healthy and infected tissues (Table 3). According to these results it could be concluded that the broomrape infection did not trigger the synthesis of TT, TF and antioxidant activity in the root of HOB-2. However antioxidant activity in HOB-2 had the highest value in heathy root tissue. High values of these compounds in healthy tissue could be explained by genetic characteristics of this hybrid.

Table 3. Total flavonoids content and DPPH-values in different sunflower hybrids

Hybrid	Infection	Total flavonoids[1]	DPPH-values
NORH-34	CP	68.67 ± 2.34^{b}	63.40 ± 1.58^{a}
	INF+	27.32± 0.75^{e}	30.44 ±0.97^{d}
	HSR	3.36 ±0.48^{g}	17.14 ± 1.75^{f}
HOB-2	CP	35.35 ±1.02^{d}	53.61 ± 0.41^{b}
	INF+	32.84 ± 0.31^{d}	52.54 ±3.10^{bc}
	HSR	33.91± 0.78^{d}	62.74 ±0.26^{a}
IMI-3-911	CP	83.89 ± 2.29^{a}	66.25 ± 0.71^{a}
	INF+	64.47 ± 1.86^{b}	65.43 ±0.82^{a}
	HSR	44.94 ± 0.75^{c}	67.57 ± 0.66^{a}
NS-H-111	CP	41.58 ± 1.02^{c}	50.81 ± 1.02^{bc}
	INF+	32.48 ± 2.67^{d}	47.62 ± 1.50^{c}
	HSR	19.89 ± 0.67^{f}	24.58 ± 0.94^{e}
p		Hybrid 0,00** Infection 0,00** Infection x Hybrid 0,00**	Hybrid 0,00** Infection 0,00** Infection x Hybrid 0,00**

Data are mean ± SE values
[1] Expressed as mg of rutin/g of dry plant material.
[2] Expressed as % neutralized free DPPH radicals.
[a-g] the values without the same superscript within each column differ significantly (p< 0.05).
**- significant at probability level (p< 0.01).
CP - at the contact point of broomrape tubercle and sunflower root.
INF+ - outside of contact point from the parasite and host plant.
HSR - healthy sunflower roots.

DPPH values showed the differences between hybrids and level of infection. The IC_{50} DPPH-values for tested extracts varied from 17.14 to 67.57. The highest differences in this parameter between infected and healthy root were noticed in NORH-34. Antioxidant activity was also high in IMI-3-911, but with no significant difference depending on intensity of infection (Table 3).

Beside the antioxidant enzymes in the plants there are defense systems which are involved in eliminating of reactive oxygen species (ROS) or prevention of their production. ROS plays an important role in plant response to pathogen attack, but they are also extremely reactive. Production and accumulation of ROS at the point of infection has an important role in sunflower and broomrape relation [25, 26]. The correlation between the content of all phenolic compounds and DPPH activity was significant in NORH-34 and in NS-H-111 (Table 4). These results indicate that increase of all measured phenolic compounds leads to enhancement of antioxidant activity in roots. Higher level of antioxidant activity indicates that sunflower plants were defending from the broomrape attack by biosynthesis of phenolic compounds. The hybrid NORH-34 belongs to the group of resistant hybrids, while NS-H-111 is known as one of the widespread hybrids in the agroecological conditions of Serbia and Southeastern Europe.

Table 4. Correlation between DPPH-assay and investigated phenolic compounds in sunflower roots

	Correlation coefficient (r)			Coefficient of determination (r^2)		
DPPH	TP	TT	TF	TP	TT	TF
NORH-34	0.98*	0.96*	0.98*	0.97*	0.92*	0.97*
HOB-2	-0.61	-0.82*	0.10	0.37	0.68	0.01
IMI-3-911	-0.27	0.12	-0.34	0.07	0.01	0.11
NS-H-111	0.94*	0.97*	0.91	0.88*	0.94*	0.83*
* Values marked with astray are statistically significant (p>0.05)						

CONCLUSION

To conclude, this investigation showed variability between sunflower genotypes in measured chemical attributes in the healthy as well as in the broomrape infected tissue. Significantly higher production of polyphenols, tannins and flavonoids and antioxidant activity was observed in roots of hybrid NORH-34 at the contact point between host plant and broomrape, compared to the healthy roots. Hybrid IMI-3-911 showed also high levels of the measured compounds apart from inoculation. These two hybrids had the lowest level of

broomrape infection in the flowering period. In other tested hybrids there were no significant changes in measured parameters related to the infestation of broomrape.

Results of this study showed that the content of TP, TT, TF and DPPH value could be used as additional parameters in the evaluation of sunflower resistance to broomrape. However, genotypes with significantly higher production of TP, TT, TF and DPPH activity should be further evaluated in the inoculation tests.

EXPERIMENTAL SECTION

Plant material (Sunflower plants)

Resistance of sunflower hybrids to broomrape in the conditions of artificial infection was evaluated in the field trial in Svetozar Miletic locality (Northern part of Serbia). Broomrape seeds were put in seedbed together with sunflower seeds during the sowing. Four sunflower hybrids from different groups: resistant to broomrape NORH-34; high-oleic HOB-2; resistant to imidazolinone herbicides IMI-3-911 and standard hybrid NS-H-111 were included in the study of biochemical parameters. Four plants of the each hybrid were inoculated in three replicates.

The first broomrape attack on sunflower plants was observed in the full flowering. Infested sunflower plants were uprooted and taken to the laboratory where their roots were washed and the number of broomrape plants was counted.

Two types of sunflower root tissue samples were taken from the infected roots: 1. at the contact point of broomrape tubercle and sunflower root (CP); 2. outside of contact point from the parasite and host plant (INF+). Healthy sunflower roots (HSR) of the same hybrids were used as a control. Sunflower roots were dried in dark place at room-temperature to the constant weight.

Extraction and determination of total polyphenols, and tannins

Plant material (200 mg) was extracted with 70% aqueous acetone solution (50 mL) by sonication for 20 minutes in an ultrasonic bath at ambient temperature. The extracts were rapidly vacuum-filtered through a sintered glass funnel and kept refrigerated before assay. All extractions were done in triplicate.

Total polyphenols in the acetone extracts were determined colorimetrically (Jenway 6505, UK) at 720 nm using Folin-Ciocalteu reagent [27]. Gallic acid (GAE) was used as a standard (covering the concentration range between 0.1 and 1.0 mg/mL) and results were expressed as milligrams of GAE per gram of dry root (DW).

Total tannins content was determined by the Folin-Ciocalteu procedure, after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone) [28]. Calculated values were subtracted from total polyphenol contents, and total tannin contents were expressed as milligrams of GAE per gram of DW.

Extraction and determination of flavonoids

Total flavonoids were determined after extraction of 1 g of dry plant material with 20 mL of extracting solvent methanol water-acetic acid (140:50:10 by volume), for 60 minutes, according to the procedure of [29]. The amount of flavonoids was calculated as a rutin equivalent from the calibration curve of rutin standard solutions and expressed as milligrams of rutin per gram of DW.

Measurment of antioxidant activity

The potential antioxidant activity of the test samples havebeen assessed based on scavenging activity of the 10%aqueous acetone sunflower root extracts of the stable DPPH free radicals [30]. DPPH-radical scavenging activity was expressed as percentage of neutralized free radicals, assuming that the sample with the higher percentage has higher scavenging capacity. All measurements were done in triplicate.

Statistical analysis

Results were expressed as mean of determinations of three independent samples made in triplicates. Statistical significance was tested by analysis of variance followed by comparison of means by Duncan's multiple range test ($P<0.05$) calculated using STATISTICA (StatSoft, 9.0). Stepwise multiple regression analyses were used to determine correlation among variables.

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