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CHANGES IN THE CONCENTRATIONS OF PHENOLIC ACIDS IN CARROT PLANTS INOCULATED WITH *Alternaria radicina* Meier, Drechsler & Eddy

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Abstract. Similary to other fungal species of the genus Alternaria, *Alternaria radicina* is a major pathogen that infects both the aboveground and underground parts of carrot plants. Changes in the concentrations of phenolic compounds are observed in infected carrot plants. Carrot seedlings were inoculated with the most pathogenic isolates of *Alternaria radicina* selected in a laboratory test. A phytopathological analysis was performed to determine the health status of carrot plants. The concentrations of phenolic acids in petioles were determined four weeks after inoculation with *A. radicina* and at harvest. The results of a greenhouse experiment revealed more severe disease symptoms on carrot plants cv. Koral inoculated with *A. radicina* isolates, compared with cv. Bolero. The content of the predominant phenolic acid (chlorogenic acid) was found to decrease in the leaf stalks of carrots cv. Koral inoculated with *A. radicina.* A statistically non-significant increase in chlorogenic acid levels was noted in the leaf stalks of carrots cv. Bolero in the experimental and control groups.

Key words: *Daucus carota* L., plant health, pathogens, phenolic compounds

INTRODUCTION

Plants growing under natural conditions are constantly exposed to a variety of environmental abiotic and biotic stress factors. Their ability to thrive in a given environment, develop as an individual, produce offspring and generate yield depends on how they are adaptable to living in this environment. Plants have managed to develop complex mechanisms which help them to maintain homeostasis and to resist – in different

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degrees – stress factors which appear in nature. Phenolic compounds play an important role in this process [Weidner et al. 2009, 2011, Wróbel et al. 2005].

Phenolics are involved in the induction of defense mechanisms of plants exposed to biotic stress. Crops attacked by pathogens and pests synthesize phenolic compounds characterized by strong cytotoxic properties [Brandt and Molgaard 2001]. Under stress conditions, phenolic compounds are also accumulated in the peel of carrot roots [Lafuente et al. 1989]. Pigmented scales of colored onions resistant to *Colletotrichum circinas* were characterized by increased concentrations of catechol and protocatechuic acid. Freytag et al. [1994] pointed to the importance of phenolic compounds, in particular chlorogenic acid, in enhancing potato resistance to late blight caused by *Phytophthora infestans* and wilt caused by *Verticillium albo-atrum*. Low concentrations of chlorogenic acid in potato tubers stimulate the growth of *P. infestans* and *Fusarium solani* var. *coeruleum*. Barkai-Golan [2001] demonstrated that inoculation of potato tubers with the fungal pathogen *Fusarium sambucinum* induced the synthesis of phenolic acids. The cited author reported that phenolics, including chlorogenic acid and ferulic acid, inhibited the growth of *Fusarium oxysporum* and *Sclerotinia sclerotiorum in vitro* tests. The author also found that benzoic acid derivatives had an inhibitory effect on the development of carrot pathogens such as *Alternaria* spp., *Botrytis cinerea*, *Penicillium digitatum*, *S. sclerotiorum*, *F. oxysporum*, which are common in storage facilities.

The aim of this study was to evaluate the pathogenicity of *Alternaria radicina* isolates against the petioles and seedlings of selected carrot cultivars. The severity of *Alternaria* blight on carrot plants and roots (cv. Bolero and cv. Koral), inoculated with the above fungal pathogen, was estimated in a greenhouse experiment. Biochemical analyses were performed to determine changes in the concentrations of phenolic compounds in the petioles.

MATERIALS AND METHODS

Pathogenicity test. The pathogenicity of eight isolates of *Alternaria radicina* (microscopic identification according to the monograph [Ellis 1971] from carrots grown in north-eastern Poland was tested: isolates from seedlings of carrots: ”2” – cv. Bolero (experimental plots in Bałcyny), ”15” – cv. Perfekcja (experimental plots in Tomaszkowo) and ”41” – cv. Sukces (plantation under organic farming system in Godki); isolate from seeds: ”45” – cv. Koral (Bałcyny); isolates from leaves: ”228” – cv. Flakke (plantation under integrated farming system in Królikowo) and ”178” – cv. Bolero (Królikowo); isolates from roots: ”269” – cv. Sukces (Królikowo) and ”290” – cv. Nantes (plantation under integrated farming system in Mielno). The test was performed twice at the laboratory. The above isolates were used to inoculate petioles and seedlings (10 replications) of seven carrot cultivars: Bolero, Fayette F1, Flakke, Koral, Nantes, Perfekcja and Sukces. The fungal inoculum consisted of agar disks (5 mm in diameter) overgrown by five-day-old cultures of fungal isolates. In the control treatment, agar disks without the mycelium were used.

Petiole infection – petiole assay [Grzebelus et al. 2003]. Petiole segments (7–8 cm in length) were placed in Petri dishes lined with wet filter paper. Agar disks overgrown by the hyphae of the tested species were placed on the petioles, 1–3 cm from their base (inoculated of plant material were kept in thermostat; in darkness, at 22ºC). The susceptibility of carrot cultivars to the analyzed fungal isolates was determined after seven days. The results were expressed as the length of necrotic lesions on the petioles, in cm.

Seedling infection. Two-week-old seedlings grown from seeds disinfected with a 0.1% sodium hypochlorite solution for 10 min were inoculated with agar disks overgrown by the hyphae of the studied fungal isolates. Incubation was carried out in a controlled environment, in a phytotron chamber (a daily 12:12 h light: dark cycle at 21ºC and 80 to 85% RH). The severity of disease symptoms was evaluated seven days after inoculation, on a three-point scale: 0º – no symptoms, 3º – the most severe symptoms, and the results were expressed as average values.

Greenhouse experiment. The experiment was conducted twice, in 2011 and 2012 (in the second and third year of the study) in the greenhouse of the University of Warmia and Mazury in Olsztyn. The experimental materials comprised two carrot cultivars selected based on the results of a pathogenicity test: Koral – sensitive and Bolero – with increased resistance to *A. radicina*. Seeds disinfected with a 0.1% sodium hypochlorite solution for 10 min were sown at the beginning of May in 9 dm^3 pots filled with thermally disinfected garden soil (chemical properties: 0.096% total N, 0.91% C, 19.7 mg P_2O_5, 22.4 mg K_2O, 7.1 mg Mg and 31.5 mg CaO_3 in 100 mg – available forms, pH – 5.5). Before seed sowing, soil in all pots was thoroughly mixed with the granular garden fertilizer Azofoska (1/N/:0.5/P2O5/:1.4/K2O/, Mg, S, B, Cu, Fe, Mn, Mo, Zn) applied at equal doses. The watering regime was identical in all treatments, a daily 12:12-h light–dark cycle. The experiment involved the following treatments: control – non-inoculated plants, and experimental – plants inoculated with the fungal pathogen *A. radicina.* Each treatment consisted of 10 pots (10 replications), with five plants per pot. Inoculation – agar disks (5 mm in diameter) overgrown by five-day-old culture of the most pathogenic isolate of *A. radicina* "228" were placed on the hypocotyl of healthy 4-week-old seedlings (infected plants were removed). Inoculated plants were stored in plastic bags for 48 hours.

Evaluation of the severity of *Alternaria* blight. The severity of *Alternaria* blight was evaluated twice, at the stage of leaf development (stage I) and at the stage of root expansion (stage II), on a 9-point scale proposed by Pawelec et al. [2006]; 0º – no disease symptoms, 9º – severe defoliation. The rates of root infection by *Alternaria* spp. were estimated at harvest and after three- and six-month storage, on a four-point scale: 1º – black lesions without root narrowing, 4º – black lesions with root narrowing > 50% at discolored sites. The results were expressed as a percentage, in the form of infection index Ii. The infection rates determined after storage were given as mean values for two sampling dates.

Phenolic content of petioles. For each treatment, the concentrations of phenolic compounds in petioles were determined four weeks after inoculation with *A. radicina* and at harvest. Extraction – analytical samples (10 g) of carrots cv. Koral and Bolero were diced, immersed in 100 ml of an 80% (v/v) aqueous solution of methanol,

and homogenized for 3 minutes. The homogenate was quantitatively transferred to tightly sealed 250 ml bottles, which were placed in a shaking water bath (Julabo SW 22). Extraction was carried out for 15 minutes at 70ºC. The extract was cooled and filtered through Whatman 1 filter paper, and the residue was quantitatively transferred to a bottle. After the second extraction the procedure was repeated, and the third extraction was performed. The extracts were combined, methanol was distilled in a rotary evaporator (Büchi R-200) at 45ºC, and water was removed by lyophilization (Labconco freeze dryer). The lyophilizate was weighed to determine extraction efficiency. Phenolic compounds were identified by RP-HPLC using the Shimadzu system which comprised two LC-10AD pumps, an SPD-M10 photodiode array detector, and an SCL-10A controller. Samples (40 mg) were dissolved in 2 ml of 80% methanol and filtered through 0.45 μm filter paper. Samples were analyzed on a Luna C_{18} column (Phenomenex; 4 × 250 mm, 5 μm). A gradient of two solvents was generated: solvent A – a 5% (v/v) aqueous solution of acetonitrile + 0.1% trifluoroacetic acid (TFA), solvent B – a 60% (v/v) aqueous solution of acetonitrile + 0.1% TFA. The solvent B content of the mobile phase changed from 0 to 60% over 50 minutes, after which time columns were rinsed with solvent A for 10 minutes. Injection volume was 20 μl, mobile phase flow rate – 1 ml/min, detection at a wavelength of 320 nm. The standards used to determine phenolic compounds were chlorogenic acid, *p*-coumaric acid, and rutin (Sigma).

Statistical analysis. After ANOVA mean values were compared by Duncan's (biological assays) or Student's t-test (HPLC results) at significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Table 1 presents the results of a laboratory test which evaluated the pathogenicity of selected *Alternaria radicina* isolates against carrot seedlings and petioles. The fungus caused brown discoloration of plant tissue, followed by necrosis. The first disease symptoms, i.e. greenish-brown discoloration of petioles, were noted on carrot plants cv. Flakke after four days of the experiment. Well-visible brown spots with an average length of 5.3 cm were noted on the petioles of carrot plants cv. Koral inoculated with isolate "45". The pathogenicity of the analyzed isolates was at a similar level – isolate "45" was characterized by the highest pathogenicity, and isolate "2" by the lowest. Necrotic lesions on seedlings were first noted after five days, on carrots cv. Perfekcja inoculated with isolate "228" which showed the highest pathogenicity – the infection index determined for this isolate was significantly higher, compared with the other isolates.

Alternaria radicina isolate selected in a laboratory test was pathogenic against the aboveground parts and roots of carrot cultivars grown in a greenhouse. Disease symptoms, in the form of necrotic lesions on petioles, were more severe during the growing season, and the highest rates (above 20%) were noted on carrot plants cv. Koral in the second year of the study (tab. 2).

Table 1. Symptoms of infection of carrot seedlings and petioles by *Alternaria radicina* – laboratory analysis

A. radicina isolates		Cultivar						
		Bolero	Fayette F1	Flakke	Koral	Nantejska	Perfekcja	Sukces
Petioles (length of necrotic lesion, cm)	2	1.80 n	3.20 h–l	2.90 j–m	3.90 c–j	3.50 f–l	3.60 e–l	4.80abc
	15	2.20 mn	3.90 c–j	4.00 c–i	3.80 ck	4.00 c–i	4.10 l–h	4.60 a–e
	41	3.40 g–l	2.80 klm	3.40 g–l	3.90 c–j	4.50 a–f	4.70 a–d	4.80abc
	45	3.25 h–l	3.30 h–l	3.80 c–k	4.00 c–i	4.80 abc	3.70 d–l	5.30 a
	178	3.50 f–l	2.80 klm	3.20 h–l	3.70 d–l	2.90 j–m	3.90 c–j	4.70a–d
	228	3.80 c–k	2.70 lm	3.90 c–j	3.50 f–l	4.10 b–h	3.30 h–l	4.50 a–f
	269	3.00 i–l	4.00 c–i	3.80 c–k	3.50 f–l	4.70 a–d	4.00 c–i	5.00 ab
	290	3.60 e–l	3.10 h–m	4.00 c–i	3.70 d–l	3.30 h–l	4.40 a–g	3.80c–k
Seedlings (average infection rate)	2	2.50abc	2.17 abc	2.50abc	2.67abc	2.83 ab	2.33 ab	2.00 bc
	15	2.33abc	2.58 abc	2.83 ab	2.67abc	2.33 abc	2.75 abc	2.50abc
	41	2.50abc	2.50 abc	2.83 ab	2.83 ab	2.42 abc	2.67 abc	2.83 ab
	45	2.58abc	2.58 abc	2.75abc	2.83ab	2.75 abc	2.75 abc	2.75abc
	178	2.17abc	2.17 abc	2.92 ab	2.83ab	2.67 abc	2.75 abc	2.50abc
	228	2.75abc	2.83 ab	3.00 a	2.75abc	2.92 ab	3.00 a	2.67abc
	269	2.42abc	1.83 c	2.8 3 ab	3.00 a	2.67 abc	2.83 ab	2.67abc
	290	2.17abc	2.08 abc	2.75abc	2.83ab	2.58 abc	2.67 abc	2.50abc

Values followed by the same letters are not significantly different

Table 2. Symptoms of infection of carrot plants caused by *A. radicina* in a greenhouse experiment (infestation index Ii, %)

Cultivar	Treatment	2011			2012		
		stage I stage of root expansion	stage II	x	stage I	stage II	x
Bolero	control	1.6 h	5.8 ef	3.7 d	2.0 gh	7.5 de	4.8 d
	inoculation	3.9 fg	9.6 cd	6.8 c	5.6 ef	15.5 b	10.6 b
Koral	control	4.5 f	9.3 cd	6.9 c	4.0 fg	8.5 cd	6.3 c
	inoculation	7.2 de	14.4 b	10.8 b	9.8 c	22.5 a	16.2 a

Explanations as in Table 1

Disease symptoms in the form of brown spots on carrot roots were also most visible in cv. Koral – ca. 17% of roots were infected after storage, and the difference was significant in comparison with cv. Bolero (tab. 3). The results of a greenhouse experiment

show that the analyzed parameters were reliable indicators of pathogenicity of *A. radicina* isolates. The tested isolate was more pathogenic against cv. Koral than cv. Bolero, which validated the results of a laboratory test. Re-isolation of fungi from carrot petioles and roots confirmed the participation of *A. radicina* in the infection process. *A. radicina* is considered the causative agent of serious diseases such as root-rot of carrot seedlings, Alternaria blight and black rot of carrot roots [Pryor 2002]. Agricultural practices, including the choice of variety and fertilization, may also determine plant health and affect the accumulation of phenolics in plants [Gleń 2008, Babik et al. 2011]. In a study by Irzykowska et al. [2007], *A. radicina* was more frequently encountered on carrot seeds cv. Koral, compared with seeds of other cultivars, which confirms that Koral is susceptible to infections caused by the above pathogen. Differences in the susceptibility of carrot varieties to *A. dauci* and *Cercospora carotae* were also reported by Souza et al. [2001] and Gugino et al. [2007]. Foliar and soil application of nitrogen had no significant effect on the total phenolic content of carrot roots, whereas urea application increased the concentrations of phenolic compounds in carrots [Smoleń and Sady 2007].

Table 3. Symptoms of infection of carrot roots caused by *A. radicina* in a greenhouse experiment (Ii, %)

Cultivar	Treatment	2011		2012		× for years	
		after harvest	after storage	sfter harvest	after storage	after harvest	after storage
Bolero	control	0.5 g	3.0 de	0.5 g	4.0 de	0.5 e	3.5 cd
	inoculation	3.0 de	11.5 c	2.5 ef	13.5 b	2.8 d	12.5 b
Koral	control	1.0 fg	4.0 de	0.5 g	4.5 de	0.8 e	4.3 c
	inoculation	5.0 d	16.6 a	4.5 de	17.6 a	4.8 c	17.1 a

After storage; mean values for two sampling dates: 3- and 6-month storage
Values followed by the same letters are not significantly different

Defense mechanisms are induced in plants exposed to biotic and abiotic stress. Crops attacked by pathogens and pests synthesize phenolic compounds characterized by strong cytotoxic properties [Szafrańska et al. 2010]. Barkai-Golan [2001] demonstrated that phenolic compounds with low molecular weight, which are a link in lignin biosynthesis, and free radicals produced during their polymerization, may participate in inducing defense responses in carrot plants by damaging fungal cell membrane, fungal enzymes and toxins.

The chromatogram of phenolic compounds extracted from leaf stalks revealed the presence of six major peaks with retention times of ca. 15.3 min (compound 1), 27.0 min (compound 2), 27.6 min (compound 3), 31.5 min. (compound 4), 39.0 (compound 5) and 47.1 (compound 6 – fig. 1). The spectrum of compound 1 had its maximum intensity at a wavelength of 324 nm, the spectra of compounds 2, 3, 4 and 5 – at

345 nm, and the spectrum of compound 6 – at 315 nm (fig. 3). Based on the retention time of the reference standard and the UV spectrum, compound 1 was identified as chlorogenic acid. Compounds 2, 3, 4 and 5 belonged to one of flavonoid groups, and compound 6 was most probably *p*-cumaric acid ester.

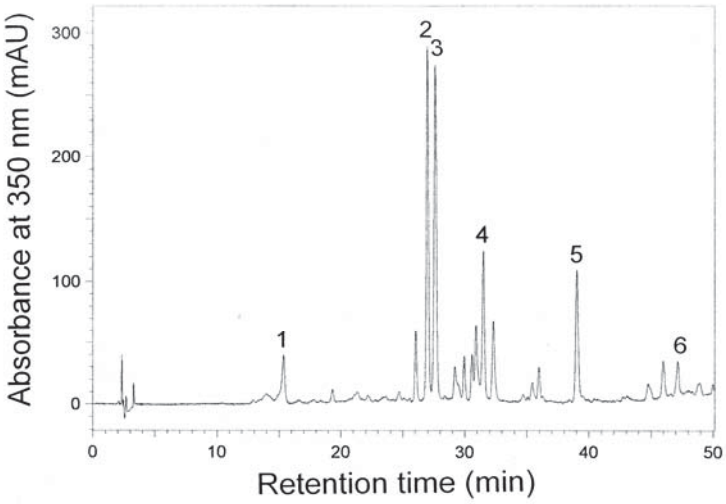

Fig. 1. HPLC chromatogram of phenolic compounds found in carrot petioles (cv. Koral) 4 weeks after inoculation

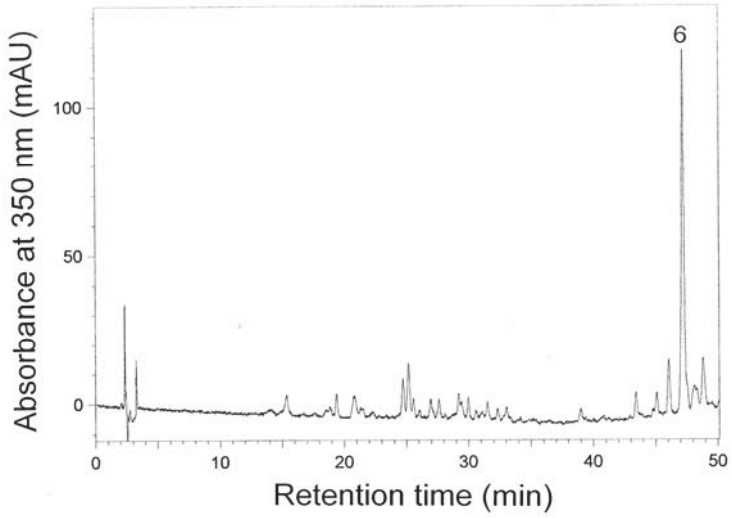

Fig. 2. HPLC chromatogram of phenolic compounds found in carrot petioles (cv. Koral) at harvest

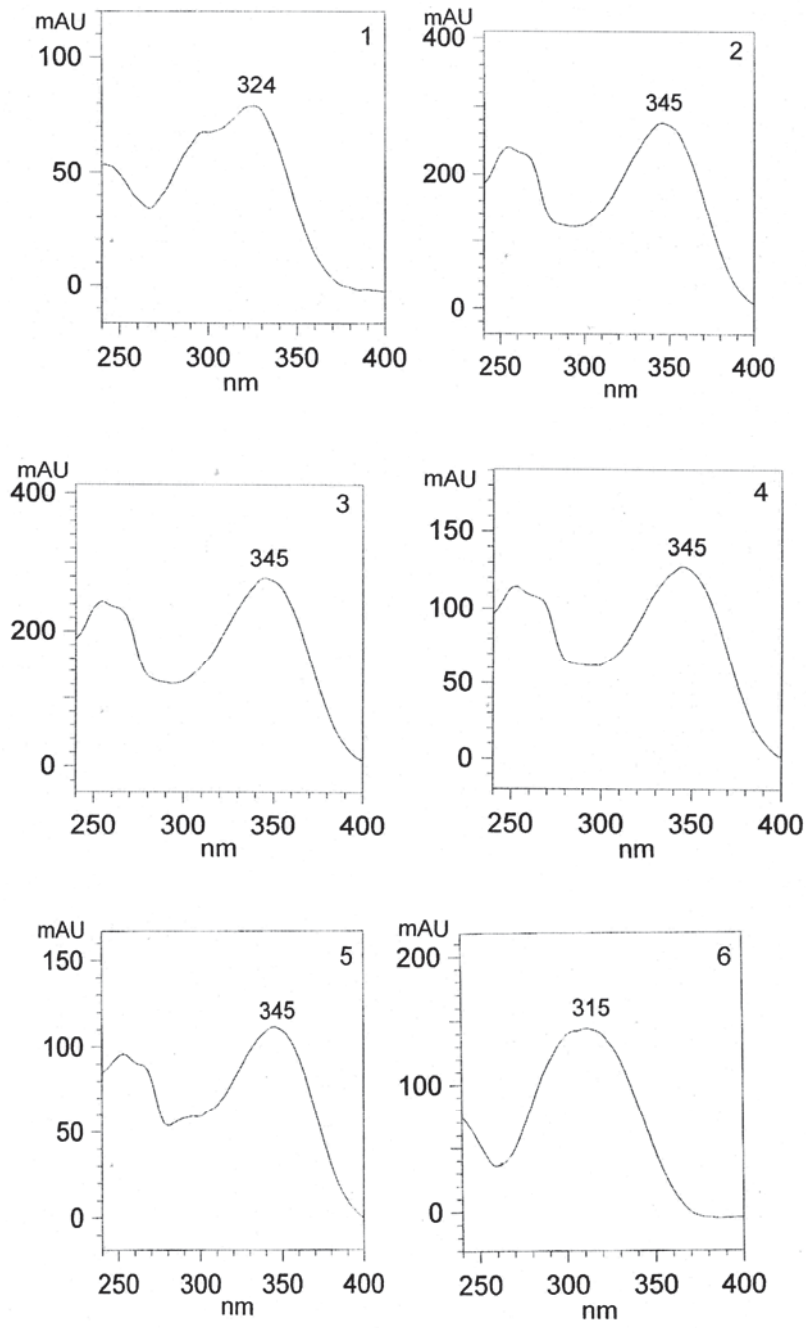

Fig. 3. UV spectra of compounds 1, 2, 3, 4, 5 and 6 found in petioles (cv. Koral) 4 weeks after inoculation

The content of phenolic compounds in carrot petioles of two cultivars is presented in Tables 4 and 5. Compounds 2, 3, 4 and 5 were expressed as rutin equivalents, and compound 6 was expressed as *p*-coumaric acid equivalents. Compounds 2 and 3 dominated in petioles after four weeks of the experiment, while the only phenolic compound present in petioles at harvest was compound 6. Four weeks after inoculation with *A. radicina*, a decrease was noted in the concentrations of the following phenolic compounds in carrot petioles: chlorogenic acid (only in cv. Koral), compound 2 and compound 3 (only in cv. Bolero), compound 4 (only in cv. Bolero) and compound 6 (only in cv. Koral). In cv. Koral, inoculation with the fungal pathogen contributed to an increase in the levels of compound 4 in petioles after four weeks. Inoculation had no effect on the content of compound 6 in petioles at harvest (fig. 2).

Table 4. Content of phenolic compounds in carrot petioles cv. Koral

Phenolic compound	4 weeks after inocultion		At harvest	
	control treatment	experimental treatment	control treatment	experimental treatment
1 (chlorogenic acid)	90.3 ± 11.5 a	36.2 ± 4.5 b	–	–
2	193.4 ± 22.8 a	163.4 ± 12.4 b	–	–
3	138.7 ± 14.5 a	124.4 ± 14.7 a	–	–
4	46.4 ± 4.9 a	58.8 ± 6.1 b	–	–
5	36.8 ± 4.2 a	30.6 ± 3.8 b	–	–
6	12.45 ± 1.6 a	7.75 ± 0.85 b	6.53 ± 0.71 a	8.49 ± 0.94 a

Compounds 2, 3, 4 and 5 were expressed as rutin equivalents, and compound 6 was expressed as *p*-coumaric acid equivalents; Values followed by the same letters are not significantly different.

Table 5. Content of phenolic compounds in carrot petioles cv. Bolero

Phenolic compound	4 weeks after inoculation		At harvest	
	control treatment	experimental treatment	control treatment	experimental treatment
1 (chlorogenic acid)	21.6 ±2.7 a	25.8 ±3.6 a	–	–
2	71.8 ±6.9 a	40.8 +5.2 b	–	–
3	79.1 ±8.5 a	40.2 +4.8 b	–	–
4	34.8 ±3.9 a	21.4 ±2.8 b	–	–
5	32.8 ±4.1 a	20.9 +3.0 b	–	–
6	3.80 ±0.41 a	2.82 ±0.31 a	4.21 ±0.49 a	3.49 ±0.38 a

Explanations as in Table 4

Under stress conditions, major phenolic compounds accumulated in the peel of carrot roots (chlorogenic acid, neochlorogenic acid, caffeic acid and cinnamic acid derivatives) are accompanied by other phenolic acids present in lower concentrations (*p*-coumaric acid, sinapinic acid, benzoic acid and ferulic acid) [Szafrańska et al. 2010, Hallmann et al. 2011, Tarko et al. 2012]. According to Feucht et al. [2004], phenolic compounds can be found in larger amounts in the epidermis, and they are also present in the waxy substance that covers parts of the plant. The concentrations of polyphenols in roots vary during ripening and storage [Stoll et al. 2003, Arscott and Tanumihardjo 2010].

CONCLUSIONS

1. Isolates of *Alternaria radicina* differed in pathogenicity, as shown by laboratory tests.

2. The results of a greenhouse experiment revealed more severe disease symptoms on carrot plants cv. Koral inoculated with *A. radicina* isolates, compared with cv. Bolero.

3. Biotic stress can change metabolism of phenolic compounds in petioles of carrot plant.

4. The content of the predominant phenolic acid (chlorogenic acid) was found to decrease in the leaf stalks of carrots cv. Koral inoculated with *A. radicina*.

5. Statistically non-significant changes in chlorogenic acid levels were noted in the leaf stalks of carrots cv. Bolero in the experimental and control groups.

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ZMIANY ZAWARTOŚCI KWASÓW FENOLOWYCH W ROŚLINACH MARCHWI INOKULOWANYCH *Alternaria radicina* Meier, Drechsler & Eddy

Streszczenie. *Alternaria radicina*, obok innych gatunków grzybów, jest groźnym patogenem części nadziemnej i podziemnej marchwi. Infekcje powodują zmiany, m.in. zawartości związków fenolowych. W teście laboratoryjnym wybrano najbardziej patogeniczne izolaty *Alternaria radicina*, którymi inokulowano siewki marchwi. Przeprowadzono ocenę fitopatologiczną zdrowotności marchwi. Analizowano zawartość fenolokwasów w ogonkach liściowych w terminie 4 tygodni po inokulacji grzybem i podczas zbioru. Wyniki doświadczenia w hali wegetacyjnej wskazują na silniejsze objawy choroby wskutek inokulacji izolatami *Alternaria radicina* na odmianie Koral niż na odmianie Bolero. W ogonkach marchwi odmiany Koral inokulowanych *A. radicina* stwierdzono zmniejszenie zawartości głównego fenolokwasu (kwasu chlorogenowego). Wzrost zawartości kwasu chlorogenowego w ogonkach odmiany Bolero w grupie eksperymentalnej i kontrolnej nie był istotny.

Słowa kluczowes: *Daucus carota* L., zdrowotność roślin, patogeny, związki fenolowe

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