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Accumulation of selected phenolics and expression of PAL genes in carrots differing in their susceptibility to carrot fly (*Psila rosae* F.)

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Abstract Three research problems were addressed in this work: an extent of root damage caused by carrot fly (*Psila rosae*) in a collection of carrot breeding accessions as well as correlation between resistance to this pest and accumulation of certain phenolics and mRNAs of the genes coding for phenylalanine ammonia-lyase (PAL). Differences in susceptibility between the studied accessions were statistically significant. The most resistant were lines 7262A and DC 96367. Moreover, no correlation was observed between accumulation of the specific metabolites and the extent of the root damage. However, proportion of certain phenolics exhibited positive correlation with the resistant phenotype. This was shown for the ratios of luteolin 7-*O*-glucoside to kaempferol 3-*O*-glucoside and methyluteolin 7-*O*-glucoside to kaempferol 3-*O*-glucoside. Susceptibility of roots to larval damage also correlated with semi-quantitatively estimated accumulation of *PAL1* and *PAL3* mRNAs in leaves. Both PAL genes were expressed at the highest level in line DC 96367 and less strongly in line 7262A. Distinctly lower accumulation of the PAL transcripts was observed for the susceptible varieties: Dolanka and Karotan.

Keywords Carrot · Gene expression · PAL · *Psila rosae* · Secondary metabolites

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Introduction

Carrot fly (*Psila rosae* F.) is one of the most serious pests of carrot and other *Apiaceae* crops. The actual damage is inflicted on roots which as a result of larval feeding exhibit the presence of brown blemishes. Although breeding experiments have shown significant differences between carrot materials in their susceptibility to carrot fly damages till now there are no cultivars which are resistant to this pest (Ellis and Hardman 1981; Ellis et al. 1984, 1993; Michalik and Wiech 2000). Experiments carried out in England (HRI Wellesbourne), Sweden, Netherlands and Poland pointed at cultivars Sytan and Long Chantenay as the ones which exhibited the high level of resistance. However, none of the tested breeding accessions showed a 100 % resistance to carrot fly damage. In those experiments complete resistance was observed only in case of wild carrots e.g. *Daucus capillifolius* (Gilli.) and *D. glochidiatus* (Labill.) Fisch., Mey. et Ave-Lall. The tolerant cultivars and wild accessions

were utilized as a source of resistance genes in carrot breeding programs resulting in the release of semi-resistant cultivar Flayaway (registered in 1999, Ellis 1999) and some lines which were more resistant than Sytan. The work carried at HRI Wellesbourne proved that resistance to *P. rosae* was a consequence of both genetic and environmental factors indicating that it would be very difficult to improve resistance solely by selection (Finch 1993). There is some evidence that secondary metabolites, especially phenolics, terpenoids and polyacetylenes, may influence the level of resistance. Therefore, biochemical and molecular investigation of these compounds may bring valuable insight into genetic aspects of their accumulation and help to select plants with increased tolerance to the pest. Chlorogenic acid was identified as the main phenolic constituent of carrot roots. Elevated concentration of this compound was correlated with the increased root susceptibility to larval damage (Cole 1985). The concentration of chlorogenic acid was higher in peel than in phloem of the roots (Olsson and Svensson 1997). Similarly Ellis et al. (1991) pointed at chlorogenic acid as a helpful indicator in selection of resistant carrot materials. Moreover, several plant flavonoid glycosides were also shown to act as either attractants or stimulants for herbivores (Delvas et al. 2011, Honda et al. 2011). It was also pointed that it was rather a proportion of some chemicals than their individual content which played an important role in plant–insect interactions. According to Fenny et al. (1988) chlorogenic acid and luteolin-7-*O*-(6′-*O*-malonyl)-β-D-glucopyranoside from carrot foliage served together as oviposition stimulants for black swallowtail butterfly (*Papilio polyxenes* F.), while neither of the pure compounds was active. Similarly, *trans*-methyl-isoeugenol and *trans*-asarone detected in the surface wax of carrot leaves were found to stimulate oviposition of carrot fly (Städler and Buser 1984). In recent years many genes active in the secondary metabolite pathways have been studied (Reymond et al. 2000). Among them, the three PAL genes from *Daucus carota*—*PAL1*, *PAL3* and *PAL4*—were cloned and sequenced (Takeda et al. 1997, Ozeki et al. 2003, Kimura et al. 2008). PAL—phenylalanine ammonia-lyase (EC 4.3.1.5) catalyzes the first reaction in the biosynthesis of many potentially protective secondary compounds such as flavonoides, lignins and phytoalexins.

The purpose of this study was to check if carrot resistance to carrot fly was associated with accumulation of certain phenolics and expression of genes coding for phenylalanine ammonia-lyase (PAL). Both the phenolic content and transcription of PAL genes were studied in leaves of plants from accessions which in the field experiments showed reliable differences in their susceptibility to carrot fly root damage.

Materials and methods

Plant material

Susceptibility of carrot breeding accessions to carrot fly larval damages was studied in the field experiments carried out in 2001, 2002 and 2003. The trials were conducted near Kraków—in Węgrzce and Wieliczka where a high occurrence of the pest had been observed and monitored using yellow sticky traps. Altogether, in the field experiments 29 carrot accessions were tested (Table 1). All of them were included in the Węgrzce trial while in Wieliczka only a subset (ten accessions) of those was evaluated. The biochemical and gene expression analyses were performed for the four accessions grown in both field locations: Dolanka, Karotan, 7262A and DC 96367.

Field experiments

All studied carrot accessions were grown in a randomized block experiment. In Węgrzce seeds were sown on ridges using the Plotmatic 1R seeder (Wintersteiger)—for a given accession 160 seeds were used along a distance of 2 m (the ridges were 65 cm apart). In Wieliczka for each accession 80 seeds were sown manually in a row 1 m long—the rows were 30 cm apart. In Węgrzce and Wieliczka the seeds were sown in the end of April in two and four replications, respectively. The roots were harvested in the end of September or in early October. Upon harvesting each root was inspected in order to determine the number of wounds caused by carrot fly. Based on that each root was assigned into one of the following classes: nine—no wounds, seven—one wound, five—two wounds, three—three to five wounds, 1—more than five wounds. These data were used to calculate the percentage of damaged roots and resistance index for each carrot accession.

The resistance index was calculated using the formula:

Table 1 Plant material used in the study

Accession		Origin	Field experiments		Biochemical analysis	Molecular analysis
			Węgrzce	Wieliczka		
AS 884	BL	PHRO Krzeszowice, Poland	+			
2158	BL	PHRO Krzeszowice, Poland	+			
52274 Fe	BL	PHRO Krzeszowice, Poland	+			
OZ	BL	PHRO Krzeszowice, Poland	+			
1028A	BL, A, ba	PHRO Krzeszowice, Poland	+			
1028B	BL, B	PHRO Krzeszowice, Poland	+			
2163A	BL, A, pet	PHRO Krzeszowice, Poland	+			
2163B	BL, B	PHRO Krzeszowice, Poland	+	+		
10138A	BL, A, ba	PHRO Krzeszowice, Poland	+			
10138B	BL, B	PHRO Krzeszowice, Poland	+			
9370A	BL, A, ba	PHRO Krzeszowice, Poland	+			
9370B	BL, B	PHRO Krzeszowice, Poland	+			
2874A	BL, A, pet	PHRO Krzeszowice, Poland	+			
2874B	BL, B	PHRO Krzeszowice, Poland	+			
1028A × 9370B	F_1	PHRO Krzeszowice, Poland	+			
2163A × NAS	F_1	PHRO Krzeszowice, Poland	+			
10138A × 52257Fe	F_1	PHRO Krzeszowice, Poland	+			
9370A × 2158C	F_1	PHRO Krzeszowice, Poland	+			
2874A × R92	F_1	PHRO Krzeszowice, Poland	+			
2163A × 2158C	F_1	PHRO Krzeszowice, Poland	+			
DC 79002	BL	HRI Wellesbourne, UK, derived from Danvers	+	+		
DC 84022	BL	HRI Wellesbourne, UK, derived from Sytan	+	+		
DC 96280	BL	HRI Wellesbourne, UK, derived from Danvers	+	+		
DC 96367	F_4	HRI Wellesbourne, UK, Sytan × *D. glochidiatus*	+	+	+	+
Satrija	P	ISDI Babtai, Lithuania	+	+		
Dolanka	P	Polan Snowidza, Poland	+	+	+	+
Karotan	P	Rijk Zwaan, The Netherlands	+	+	+	+
7262A	BL, A, pet	USDA ARS	+	+	+	+
7262B	BL, B	USDA ARS	+	+		

BL breeding line, *P* open-pollinated variety, *A* male-sterile maternal line, *B* male-fertile maintainer line, *ba* brown anther type, *pet* petaloid type

$$\text{Resistance index} = \frac{\sum (i \; x \; n_i)}{9 \; x \; N} \times 100,$$

where: *i*—class number (9, 7, 5, 3 and 1)—n_i—number of roots in the *i*-class, *N*—total number of inspected roots.

Differences in the percentage of damaged roots and resistance index were analyzed using two-factorial analysis of variance for each experimental location (with years and genotypes as factors) and three-factorial analysis for both locations (with years, locations and genotypes as factors). In these analyses the least significant difference (LSD) was calculated using the *t* student test.

Chemical analysis

The plant material was collected in July and September of 2003 from the field experiments located in Węgrzce and Wieliczka (see above). Each time, both young and mature (old) leaves were picked—the former from the center and the latter from the periphery of leaf rosettes, respectively. Each 2 g

sample was collected in duplicate and contained the leaf material from 21 plants. The samples were frozen in liquid nitrogen and kept in −80 °C until extraction. Using mortar and pestle the leaves were powdered in liquid nitrogen and then extracted at room temperature with 80 % methanol (20 ml/1 g of fresh weight) for 20 min in an ultrasonic bath (Sonic 5, Polsonic). The resulted suspensions were filtered through a Büchner funnel and concentrated under vacuum to approximately 5 ml. Then the samples were solid phase-extracted (SPE) using cartridges filled with RP C-18 silica gel (Sigma-Aldrich). The cartridges were activated with 5 ml of 96 % methanol and then 5 ml of MilliQ water before application of the samples. At that point 40 µl of 60 µM dimethoxybenzoic acid—DMBA (Sigma-Aldrich)—were added to each sample as the internal standard. The cartridges were washed with 4 ml of MilliQ water and then retained phenolics were eluted out using 3 ml of 96 % methanol. The extracts were evaporated under nitrogen and the resulted solids were dissolved in 1.5 ml of 75 % methanol.

LC/UV and LC/ESI/MS analysis

LC/UV analyses were performed on the Merck–Hitachi HPLC system equipped with the L-7100 gradient pump, L-7450 UV detector and L-7200 sequential autosampler. Five microliters of each sample were injected onto RP C-18 column (250 × 2 mm) (Merck) using 2 ml/min flow rate. Elution was carried out using two solvent mixtures: A (95 % H_2O/4.5 % CH_3CN/0.5 % HCOOH v/v/v) and B (95 % CH_3CN/4.5 % H_2O/0.5 % HCOOH v/v/v). The elution steps were as follows: 0–2 min—20 % solution A and 80 % solution B, 2–30 min—linear gradient from 20 % solution A to 100 % solution B, 30–45 min—100 % solution B. For the LC/ESI/MS analyses the chromatograph was coupled to a mass spectrometer equipped with an electrospray ion source (Waters-Micromass). Nitrogen was used both as the drying and auxiliary gas. The elution protocol was the same as the one used for the LC/UV analysis (see above). Phenolic compounds present in the consecutive samples were identified by their retention times (DMBA was used as the internal standard) or their structures (tentatively elucidated on the basis of the registered mass spectra).

Identification of compound no. 7

For extraction 500 g of fresh young leaves were homogenized and extracted in 5 l of 80 % CH_3OH and the resulted solution was concentrated under vacuum to approximately 5 ml. The obtained sample was further purified and concentrated using solid phase extraction (SPE) (see above). The sequential fractions of metabolites were eluted with the concentration gradient of methanol (from 10 to 80 %). Metabolite content in the consecutive fractions was estimated using the thin layer chromatography (TLC). TLC was carried out on cellulose plates using the following solvent: $HCH_2COOC_2H_5/H_2O/HCH_2OH/NH_3$ in a ratio of 12/3/3/0.5 (v/v/v/v). The plates were examined under UV after fuming with NH_3. The fractions in which compound no. 7 was detected were pooled together and further purified by HPLC on RP C-18 column (the chromatographic conditions are described above). The extracts were evaporated under nitrogen and the resulted solids were diluted in 1.5 ml of 75 % methanol. The LC/UV and LC/ESI/MS analyses were performed as described above.

Quantitation of the identified compounds

The relative amount of a given compound was estimated using LC/UV chromatograms and expressed as the ratio between the pick area of this compound and the pick area of the standard (DMBA). For a given pair of compounds the ratio of their relative amounts was also calculated.

Expression analysis of PAL genes

The plant material was collected in 2003 from both experimental locations—Węgrzce and Wieliczka (see above). The samples were picked up in July—when the second generation of carrot fly was active and in September—just before the root harvest. Each sample contained 2 g of the youngest leaves from 21 plants of a given accession (Table 1). The samples were frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

RNA isolation

The leaf tissue was ground in liquid nitrogen using mortar and pestle, transferred into 50 ml centrifuge

tubes and immediately supplemented with 16 ml of solution D from the Chomczyński and Sacchi (1987) procedure. After vigorous mixing 1.6 ml of 2 M sodium acetate (pH 4), 16 ml of water-saturated phenol and 3.2 ml of chloroform:isoamyl alcohol (49:1 v/v) were added and the samples were again shaken thoroughly. The preparations were kept in ice for 15 min and then centrifuged at 22,000×*g* for 20 min in 4 °C. The upper (aqueous) phase was transferred into new tubes and supplemented with 16 ml of chloroform:isoamyl alcohol (49:1 v/v). The resulting samples were again vigorously mixed and centrifuged as previously. The aqueous phase was again transferred into new tubes and an equal volume of isopropanol was added. The mixture was shaken gently till the white precipitate became visible. After one-hour incubation in ice the samples were centrifuged as previously, the resulting pellets were washed with 80 % ethanol and again centrifuged (22,000×*g*, 10 min, 4 °C). The supernatant was discarded while the pellet was dried under vacuum and dissolved in sterile MilliQ water. The resulted samples were transferred into 1.5 ml Eppendorf tubes, kept in ice for 2 hours and then centrifuged at 24,000×*g* for 10 min in 4 °C. The supernatant was transferred into new Eppendorf tubes and supplemented with 1/3 volume of 8 M LiCl (the final concentration of LiCl was 2 M). The samples were mixed well by inversion and left in 4 °C for at least 18 h to allow RNA precipitation. The RNA precipitate was collected by centrifugation at 22,000×*g* for 15 min in 4 °C. The resulting pellets were washed first with 2 M LiCl and then with 80 % ethanol. Either of the washing steps was followed by centrifugation (24,000×*g* for 15 min in 4 °C). Finally, the RNA pellets were vacuum-dried, dissolved in sterile MilliQ water and stored at −80 °C. RNA concentration was assessed on the basis of the A_{260} measurement.

Probe preparation

The hybridization probes were synthesized using a two step PCR procedure. The primers for DNA amplification were designed on the basis of sequence records for the carrot genes: *PAL1*, *PAL3* and *18S* rRNA (Table 2). In the primary PCR, 25 µl of the reaction mixture contained: 10 mM TrisHCl pH 8.8, 50 mM KCl, 0.08 % NP-40, 2 mM $MgCl_2$, 0.4 mM each dNTP, 0.8 µM each primer, 0.5 U of recombinant *Taq* polymerase (Fermentas) and 15 ng of carrot genomic DNA. The reactions were carried out in the Eppendorf Mastercycler Gradient programmed for 5 min at 94 °C, 35 cycles of: 45 s at 92 °C, 45 s at 57 °C, 2 min at 72 °C followed by 10 min at 72 °C. The amplified fragments were gel-purified using QIAquick PCR Purification Kit (Qiagen), diluted 100-fold and used as a template in the secondary PCR. These reactions were carried out in 50 µl containing: 1× PCR buffer (Qiagen), 2 mM $MgCl_2$, 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dCTP, 0.034 mM dTTP, 0.016 mM DIG-11-dUTP (Roche), 0.25 µM each primer, 1.6 U of HotStarTaq (Qiagen) and 5 µl of the diluted primary PCR product (see above). The temperature profile was as in the primary reaction except initial denaturation, which was extended to 15 min. The PCR products of both reactions were monitored by electrophoresis in a standard agarose gel.

Northern hybridization

Forty micrograms of RNA were brought to 4 µl with sterile MilliQ water and supplemented with 16 µl of denaturation solution (10 µl of formamide, 3.3 µl of 38 % formaldehyde, 1.7 µl of 10× MOPS and 1 µl of sterile MilliQ H_2O). The samples were mixed well and incubated in a thermal cycler at 65 °C for 10 min and then in ice for 5 min. Prior to electrophoresis 2 µl of 6× Loading Dye Solution (Fermentas) supplemented with ethidium bromide (1 mg/ml) was added to each sample. The RNAs were separated for 2 h in 1.5 % agarose containing 2 % formaldehyde. Electrophoresis was carried out in 1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7) at 2 V/cm in 4 °C. With the use of 20× SSC buffer the RNA separations were capillary-blotted onto positively charged nylon membranes (Roche) and then UV-crosslinked according to standard procedures (Sambrook and Russell 2001). The blots were pre-hybridized for 1 h at 65 °C in the Roche standard hybridization buffer and subsequently hybridized overnight at the same temperature with a given DIG-labelled PCR product (see above). The hybridization solution contained 15 ml of the standard hybridization buffer supplemented with 45 µl of the secondary post-PCR mixture. Post-hybridization washes and signal detection were performed according to *DIG Application Manual for Filter Hybridization* (Roche Diagnostics 2000). Finally, the membranes were exposed to an X-ray film (NBX, Zarys) for 10, 20, 60 and 240 min. Optical density of

Table 2 Primers used for probe synthesis

Gene	Literature reference	Sequence record	Sequence (5′ → 3′)	Amplicon length [bp]
PAL1	Takeda et al. 1997	D85850	gga ttg tga gaa caa gaa tg tct gca gag cac cac ctt gt	362
PAL3	Ozeki et al. 2003	AB089813	ctt gaa tgc tgg aat att cg ttc cat gac tgg aga cac tc	1725
18S rRNA	Yokota et al. 1989	X17534	cta gta agc gcg agt cat ca aat gat cct tcc gca ggt tc	221

the hybridization signals was quantified using Gene Profiler v. 403 (Scananalytics).

Results

Variation in plant material susceptibility

In the three-year field experiment carried out in Węgrzce, 29 carrot accessions were tested (Table 3). Only DC 96367 was examined twice—in 2002 and 2003. The mean percentage of damaged roots ranged from 4.7 to 44.9 and the resistance index—from 86.2 to 99.6. Both measures of carrot infestation were correlated, the Pearson coefficient had the value of −0.87 (negative correlation). Differences observed among the studied accessions with respect to the percentage of damaged roots and resistance index were found to be statistically significant. Line 7262A turned out to be the most resistant—its percentage of damaged roots reached 4.7 and resistance index—99.6. The most susceptible were: line OZ with the highest percentage of root damage (44.9) and line AS884 with the lowest resistance index (86.2) (Table 3). Nevertheless, significant genotype-environment interaction was observed (Table 4). Differences in the root damage observed in Węgrzce among the subsequent years were also statistically significant. The respective values reached 24.6, 45.9 and 19.9 in 2001, 2002 and 2003, respectively (Table 3).

In 2002 and 2003 a subset of the accessions analyzed in Węgrzce was also tested in Wieliczka. The subset consisted of ten accessions (Table 1) which according to the first year data from Węgrzce sampled the whole range of phenotypic variation—from strong through moderate susceptibility to resistance. In Wieliczka the mean percentage of damaged roots ranged from 28.0 to 58.3 and the resistance index—from 81.5 to 92.4. The differences observed between the studied accessions with respect to these both parameters were statistically significant. The lowest percentage of damaged roots was noted for line 2163B and the highest resistance index—for line DC 96367. Satrija with 58.3 % of damaged roots and Karotan with the resistance index reaching only 81.5 were the most susceptible accessions. In the Wieliczka trial the percentage of damaged roots and resistance index were highly correlative with the Pearson coefficient of −0.9 (negative correlation). Year-to-year differences in the root damage were also statistically significant. The respective values reached 64.5 and 33 % in 2002 and 2003, respectively (Table 3). No significant interaction between genotypes and environments was observed for the percentage of damaged roots. For the resistance index this interaction was significant only at $P = 0.05$ (Table 4).

Both experimental series (Węgrzce, Wieliczka) were accordant with respect to the resistant behavior of the three accessions: DC 96367, 7262A and 2163B. The relative rank of the seven remaining accessions was to some extent dependent on the experimental location. Both locations were significantly different in the overall extent of infestation—it reached 27 and 48.7 % of damaged roots in Węgrzce and Wieliczka, respectively. The combined calculations performed for both field locations confirmed that in comparison to 2003, year 2002 was over two-fold more provocative in terms of carrot fly activity (Table 3). We did not observe significant interaction between the genotypes and experimental locations as well as between the genotypes and years (Table 4). These interactions had no effect on both the percentage of damaged roots and resistance index.

Phenolic content

This study was performed for four accessions differing in the estimated tolerance to the *Psila rosae* root damage: Dolanka, Karotan, 7262A and DC 96367.

Table 3 Parameters of carrot fly infestation noted for the carrot accessions tested in the field experiments located in Węgrzce and Wieliczka

Accession	Węgrzce (2001–2003)		Wieliczka (2002–2003)		Węgrzce, Wieliczka (2002–2003)	
	% of damaged roots	Resistance index	% of damaged roots	Resistance index	% of damaged roots	Resistance index
OZ	44.9	86.7	–	–	–	–
AS884	43.6	86.2	–	–	–	–
10138B	41.3	90.0	–	–	–	–
9370B	42.8	86.5	–	–	–	–
DC 96280	41.3	89.6	41.2	81.9	41.7	83.5
Satrija	40.8	89.1	58.3	83.3	50.5	85.2
Dolanka	38.1	86.4	45.0	84.4	43.8	84.4
DC 84022	36.7	86.8	51.5	84.4	47.2	84.4
9370 × 2158	34.3	88.7	–	–	–	–
2874 × R92	33.4	89.1	–	–	–	–
10138 × 52257Fe	31.3	89.5	–	–	–	–
1028 × 9370B	30.8	91.0	–	–	–	–
DC 79002	30.4	90.7	46.5	84.6	39.9	87.4
52274Fe	28.9	91.2	–	–	–	–
9370A	28.7	88.9	–	–	–	–
2163A	27.7	92.6	–	–	–	–
7262B	27.2	94.5	49.0	84.3	41.0	88.0
2158	26.8	92.4	–	–	–	–
2874A	26.2	93.2	–	–	–	–
10138A	25.6	92.3	–	–	–	–
Karotan	25.5	91.6	52.4	81.5	42.0	85.4
1028A	25.2	93.9	–	–	–	–
2874B	24.8	90.9	–	–	–	–
1028B	24.5	93.9	–	–	–	–
2163 × 2158	21.1	93.7	–	–	–	–
2163 × NAS	19.5	94.2	–	–	–	–
2163B	17.6	92.3	28.0	89.9	23.9	91.3
DC 96367*	10.8	95.9	29.3	92.4	20.5	94.1
7262A	4.7	99.6	33.8	89.7	21.3	93.6
LSD ($P = 0.05$)	14.28	5.88	14.15	5.31	12.80	5.16
Correlation coefficient (Pearson)	−0.87		−0.9		−0.84	
Means for						
2001	24.6	92.5	–	–	–	–
2002	45.9	85.3	64.5	77.2	55.4	80.3
2003	19.9	94.7	33.0	89.9	25.1	92.4
Means for 10 accessions tested in both locations						
Węgrzce					27.0	91.0
Wieliczka					48.7	83.5
LSD ($P = 0.05$)					5.72	2.36

* In Węgrzce line DC 96367 was tested only in 2002 and 2003 (the remaining accessions were tested in 2001, 2002 and 2003)

Table 4 Results of variance analysis applied to the percentage of damaged roots and resistance index for the carrot accessions tested in Węgrzce and Wieliczka

Field trials	Source	df	% of damaged roots		Resistance index	
			MS	F-value	MS	F-value
Węgrzce 2001–2003	Year (Y)	2	10,346.10	70.20**	1,397.49	55.61**
	Genotype (G)	28	576.83	3.91**	58.55	2.33**
	G × Y	56	387.4	2.63**	43.70	1.74*
	Error	87	147.38		25.13	
Wieliczka 2002–2003	Year (Y)	1	13,164.77	56.03**	2,125.62	64.24**
	Genotype (G)	9	629.70	2.68*	81.81	2.47*
	G × Y	9	375.33	1.60 ^{ns}	72.91	2.20*
	Error	40	234.97		33.09	
Węgrzce, Wieliczka 2002–2003	Year (Y)	1	22,028.57	114.56**	3,524.91	112.67**
	Locations (L)	1	6,939.80	36.09**	822.05	26.28**
	Genotype (G)	9	1214.09	6.31**	116.44	3.72**
	Y × L	1	5116.13	26.60**	714.93	22.85**
	Y × G	9	200.93	1.04 ^{ns}	62.47	1.99 ^{ns}
	L × G	9	318.61	1.66 ^{ns}	27.16	0.87 ^{ns}
	Y × L × G	9	820.51	4.27**	102.00	3.26**
	Error	60	192.29		31.28	

ns not significant

** Significant at the 0.01 probability level

* Significant at the 0.05 probability level

The phenolic content was investigated in young and mature (old) leaves—both were collected in July and September from the plants grown in Węgrzce and Wieliczka. The use of liquid chromatography coupled with mass spectrometry enabled identification of six phenolic compounds. These were: chlorogenic acid as well as glycosides of flavonoids—luteolin (two types), methyluteolin, apigenin and kaempferol (Table 5). The identity of one compound (compound no. 7) was not elucidated. However, the coupled use of liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization (ESI) (Bednarek et al. 2001, Stobiecki and Kachlicki 2006) identified three constituents of that compound: chinolic acid, glucose and caffeic acid methyl ester (Fig. 1).

Relative quantity of the identified compounds was calculated from the LC/UV spectra and expressed as the ratio between the peak area of each compound and that of the standard (dimethoxybenzoic acid—DMBA). The calculated differences in accumulation of the identified compounds were not significant among the studied accessions. Moreover, no correlation was observed between the content of these phenolics and the resistance/susceptibility character of the examined accessions. However, upon closer examination of the accumulation data some specific relationships were noted. We found that in young leaves compound no. 7 was the highest accumulated among the identified phenolics (Fig. 2). In mature leaves accumulation of this compound was reduced and therefore, the phenolic content was dominated by luteolin-7-*O*-glucoside, although contribution of the latter in relation to the other studied compounds did not change with the age of the leaves. The difference between young and mature leaves in accumulation of the compound no. 7 was evident regardless of the date of leaf harvest (July, September) and experimental location (Węgrzce, Wieliczka). We have also looked at accumulation ratios of the studied phenolics and found an interesting correlation for the following two proportions: luteolin-7-*O*-glucoside/kaempferol-3-*O*-glucoside and methyluteoline-7-*O*-glucoside/kaempferol-3-*O*-glucoside. In case of the mature (old) leaves collected in July both ratios were evidently higher for the resistant lines (7262A, DC 96367). This observation was accordant for both experimental localities—Węgrzce and Wieliczka

Table 5 Phenolic compounds detected in carrot leaves with the use of LC/UV, LC/MS and LC/ESI/MS

Compound	Elemental formula	Retention time (t_r) [min]	Molecular mass (M_r)
Chlorogenic acid[a]	$C_{16}H_{18}O_9$	7.0	354
Luteolin-7-*O*-glucoside[a]	$C_{21}H_{20}O_{11}$	26.5	448
Methyluteolin-7-*O*-glucoside[b]	$C_{22}H_{22}O_{11}$	27.5	462
Luteolin-7-*O*-(6′′-malonyl)-glucoside[b]	$C_{24}H_{22}O_{14}$	35.1	534
Apigenin-7-*O*-glucoside[a]	$C_{21}H_{20}O_{10}$	32.9	432
Kaempferol-3-*O*-glucoside[b]	$C_{21}H_{20}O_{11}$	34.1	448
Compound no. 7	–	47.0	568

[a] Identified by comparison with the standard

[b] Identified tentatively

(Fig. 3). For the samples collected in September this correlation was not evident (data not shown).

Accumulation of PAL mRNAs

Expression of PAL genes was studied for the four accessions examined also with respect to the phenolic content—Dolanka, Karotan, 7262A and DC 96367. Leaf samples were collected form both experimental locations (Węgrzce, Wieliczka) in July and September. In all examined samples a single signal was observed for both *PAL1* and *PAL3* probe. The transcript sizes were estimated at 3.0 and 3.5 kb for *PAL1* and *PAL3*, respectively. Accumulation of both mRNAs varied with the tested accessions, time of leaf sampling and field location (Fig. 4).

Among the samples collected in July at Węgrzce the *PAL1* mRNA was observed at low level for Dolanka, for 7262A and DC 96367 the respective signal was barely visible and in case of Karotan it was not detected. The *PAL3* transcripts were detected in all tested accessions but at the very low level requiring 4-hour exposure of the X-ray film. The hybridization signals of the *PAL3* mRNA were slightly higher in Dolanka and Karotan than in 7262A and DC 96367. A different situation was observed for the samples collected in September. The highest accumulation of both transcripts was detected in DC 96367. The signals recorded for 7262A were approximately five-fold weaker than those of DC 96367 and distinctly stronger than those observed for Dolanka and Karotan. Among the samples collected in July at Wieliczka the highest expression of both transcripts was observed for DC 96367. Line 7262A exhibited approximately six-fold lower accumulation of either mRNA. In the samples of Dolanka and Karotan the studied mRNA species were not detected. In all Wieliczka samples collected in September accumulation of both *PAL1* and *PAL3* mRNA was observed. The strongest hybridization signals were obtained for lines 7262A and DC 96367 while the weakest ones for Dolanka (Fig. 4).

Discussion

Our results demonstrate that the studied carrot accessions exhibit significant differences with respect to the

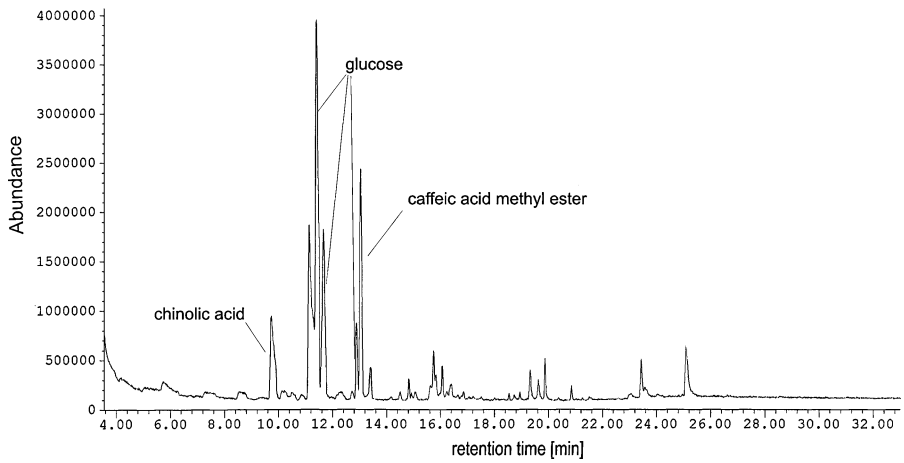

Fig. 1 Total ion chromatogram (LC/MS) of compound no. 7 isolated from carrot leaves

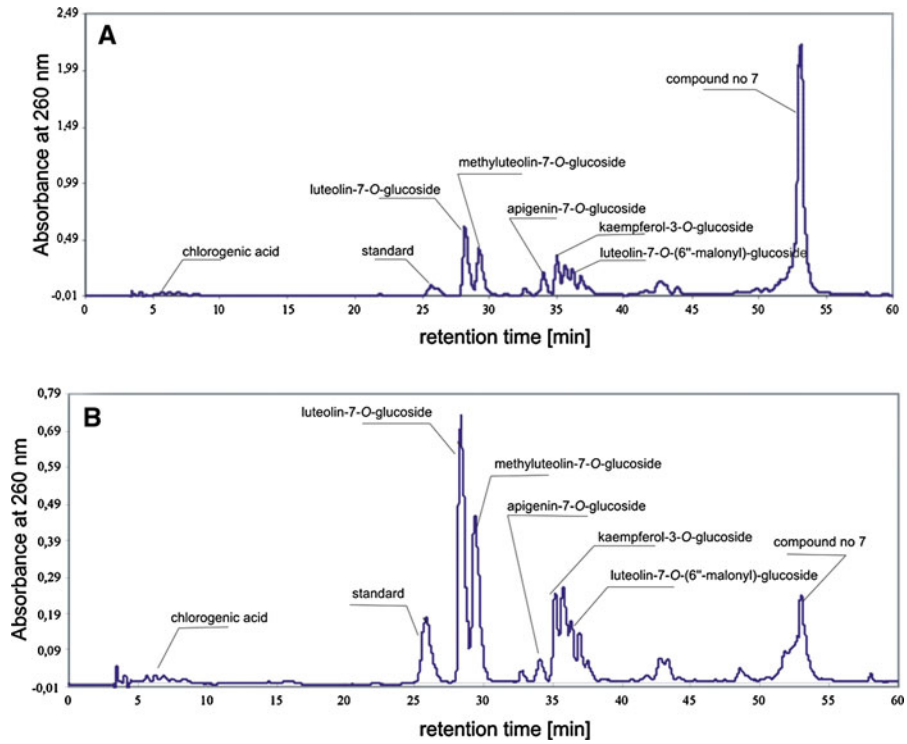

Fig. 2 LC/UV chromatograms of phenolics extracted from young (**a**) and old (**b**) carrot leaves

carrot fly damage. The field experiments were carried out for three years in two different locations. Both time and location of experiments strongly influenced the overall level of root damage. Nevertheless, it was possible to point out some accessions that in these diverse environmental conditions consistently exhibited either high or low level of resistance. The most resistant were DC 96367, 7262A and 2163B. Line 2163 has an orange root color. DC 96367 exhibits a range of root colors—from white through yellow to orange. It was derived from a cross between *Daucus glochidiatus* and cultivated carrot in the program carried out by the Wellesbourne group. Former experiments had shown that *D. glochidiatus* was highly resistant to carrot fly larval damage (Ellis 1999). Line 7262 was derived from a cross between the Turkish accession PI 173687, which had highly variable root colors, and the dark orange inbred derived from Danvers 126. Line 7262 has typical dark purple roots with orange colored phloem, cambium and xylem (Simon et al. 1997). Our data indicate that male-sterile line 7262A is more resistant than its fertile counterpart 7262B. At present it is difficult to judge if it is related to the different cytoplasmic composition of 7262A and 7262B or if it reflects their yet not fully isogenic character. In the Węgrzce trial also five other A/B pairs were examined. Interestingly, the resistance index of the petaloid CMS lines (2163A, 2874A, 7262A, 9370A) was higher than that of their maintainers (2163B, 2874B, 7262B, 9370B). These data indicate that indeed the plasmotype has some effect on carrot resistance to *P. rosae*. Consistently good performance of DC 96367 and 7262A prompted us to use them as the resistance representatives in our chemical and molecular analysis. In terms of practical importance these data indicate that in addition to the well-known Wellesbourne DC 96367 source also line 7262 can be used for introgression of resistance genes in carrot breeding programs. The heaviest root damage was observed for some Polish breeding lines as well as for cultivars Dolanka and Satrija. All this material exhibits a typical orange root color. The high level of root damage was also observed for line DC 96280 which was selected in HRI Wellesbourne from the susceptible cultivar Danvers. DC 79002—another line developed from Danvers and used in Wellesbourne as a susceptible standard (Ellis et al. 1993)—in our experiments showed a low level of root damage which was accordant with the earlier trials carried out in Poland (Legutowska 1991). Such results indicate that

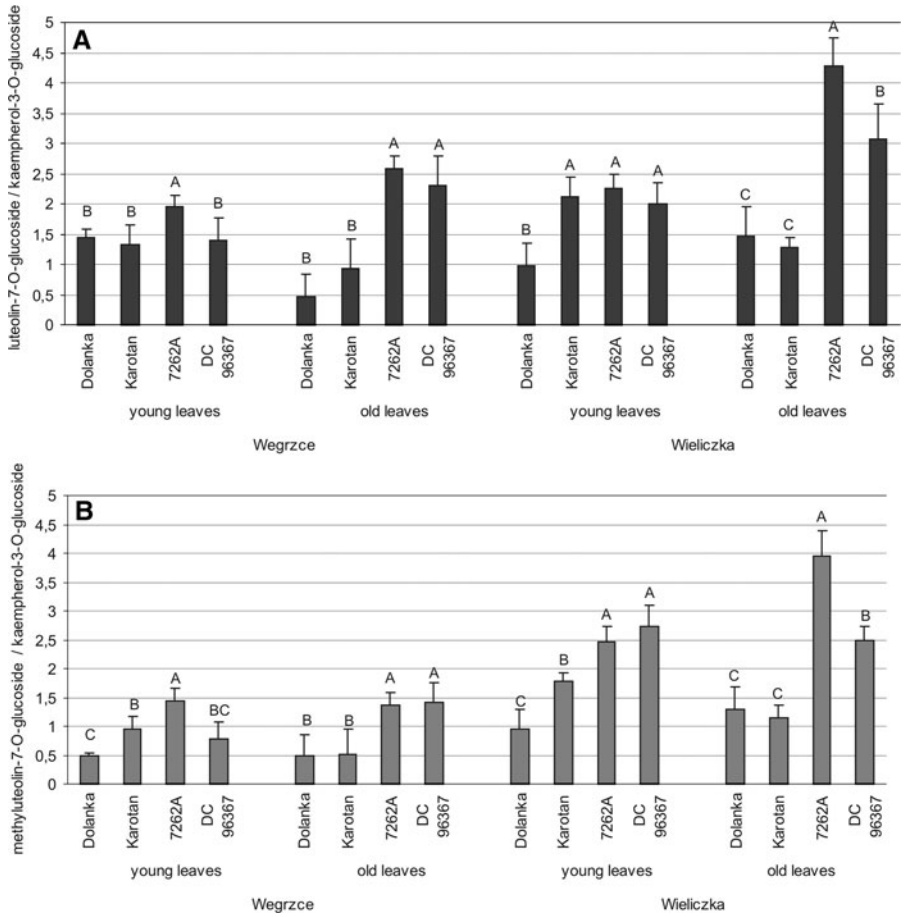

Fig. 3 Luteolin-7-*O*-glucoside/kaempferol-3-*O*-glucoside (**a**) and methyluteolin-7-*O*-glucoside/kaempferol-3-*O*-glucoside (**b**) accumulation ratios calculated for young and mature (old) leaves collected in July from the selected carrot accessions grown in Węgrzce and Wieliczka. Values denoted with different letters are significantly different (Duncan's multiple range test, $P = 0.05$). Standard errors were calculated using the delta method

expression of resistance/susceptibility is strongly modulated by environmental conditions which makes breeding efforts very cumbersome—the more so because very little is known about biochemical and molecular aspects of plant pest resistance.

Numerous reports point to secondary metabolites as the main mediators of plant–pest interactions (Harborne 1997). Carrot plants contain a variety of compounds which may act as stimulants or repellents for pests. Our research demonstrated a spectacular change in the content of secondary compounds during carrot growth. The major phenolic compound detected in young leaves was referred to as compound no. 7 which upon ionization in the mass spectrometer decomposed into chinolic acid, glucose and caffeic acid methyl ester. The phenolic content of mature leaves was dominated by luteolin-7-*O*-glucoside. These observations supplement the data of Kainulainen et al. (1998, 2002) who reported changes in accumulation of volatile terpenoids during vegetation of carrot plants. These authors revealed that younger leaves contain more propenylbenzenes, methylisoeugenol and alfa-asarone than older ones. It is also known that composition of volatile compounds on the surface of leaves influences oviposition of carrot fly (Städler and Buser 1984). The content of volatile constituents was also studied for carrot roots (Buttery et al. 1968). In these studies chlorogenic acid was identified as the main phenolic component accumulated in the peel of carrot roots and moreover, a clear relationship was found between its concentration and carrot fly larval damage. Accordingly to this observation cultivars with higher concentration of root chlorogenic acid were more susceptible to larval damage than low content ones (Cole 1985) indicating that resistance was based on antibiosis. In the present study we focused our attention on foliage metabolites which may condition the antixenosis-based plant resistance like e.g. flavonoid glucosides known to act as oviposition stimulants for pests (Harborne 1997;

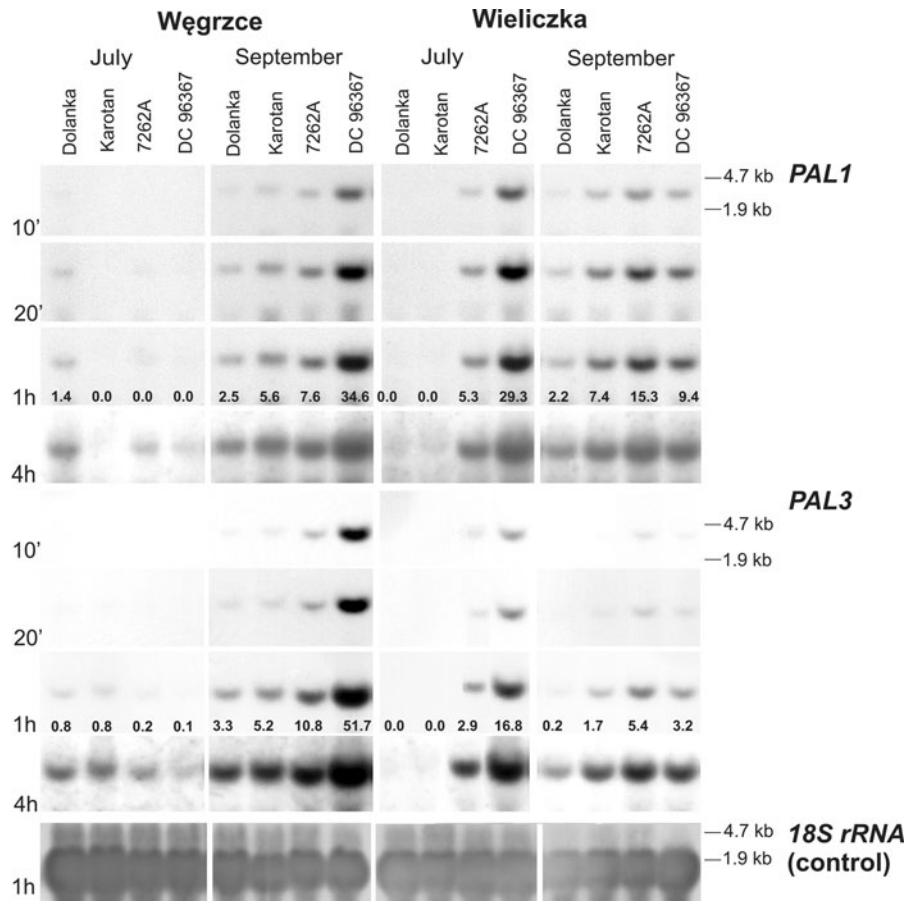

Fig. 4 Northern blots of total cellular carrot RNA hybridized with *PAL1*, *PAL3* and *18S* rRNA (control) gene probes. The RNAs were isolated from harvested in July and September leaves of the selected accessions grown in Węgrzce and Wieliczka. Exposure times of the X-ray film are shown on the left. For one-hour exposure IntOD of the *PAL1* and *PAL3* hybridization signals is indicated

Fulcher et al. 1998; Lattanzio et al. 2000). In our experiments we have identified three flavonoid glucosides (luteolin-7-*O*-glucoside, kaempferol 3-*O*-glucoside and methyluteoline-7-*O*-glucoside) which mutual proportions correlated with the incidence of carrot fly damage. This result is accordant with some earlier reports showing that mixtures of certain compounds emanated from carrot foliage turned out to be more attractive for pests than single compounds (Guerin et al. 1983; Matsuda and Matsuo 1985; Fenny et al. 1988).

A number of genes were shown to be involved in accumulation of secondary metabolites in plant tissues (Hauffe et al. 1991; Logemann et al. 1995; Koopmann et al. 1999). Phenylalanine ammonia-lyase (PAL) catalyzes the first reaction in the biosynthesis of many plant phenylpropanoid compounds. This enzyme is encoded by the family of genes, which can be expressed differentially during plant development and in response to different environmental cues (Liang et al. 1989, Olsen et al. 2008, Huang et al. 2010). It is also possible that the individual PAL genes are expressed in different sets of cells (Logemann et al. 1995; Kao et al. 2002). In our experiments carrot leaves exhibited similar expression patterns of *PAL1* and *PAL3* suggesting common regulation of both genes. The respective transcript sizes were different confirming that the detected mRNAs originated from distinct genes. Our data showed that the studied carrot accessions differed in accumulation of the *PAL1* and *PAL3* mRNAs and that in most cases these differences correlated with the overall tolerance to carrot fly. Generally, the highest expression of both PAL genes was observed in the resistant lines DC 96367 and 7262A. Weaker or barely detectable accumulation of these transcripts was shown for much more susceptible varieties—Dolanka and Karotan. Similar results were reported by Chaman et al. (2003) who found that barley cultivars which were resistant to aphids *Schizaphis graminum* exhibited elevated expression

of PAL genes. In addition to the mentioned genotypic differences we also observed temporal dependence of *PAL1* and *PAL3* expression—in most cases accumulation of both studied mRNAs was higher in September than in July. This observation supports some earlier reports indicating that transcription of PAL genes can be activated by stress factors—especially insect feeding (Leszczyński 1985). According to this interpretation accumulation of the PAL mRNA increases over time in response to stressing environmental factors and among those—wounding caused by the larvae of carrot fly. Both transcription level of the PAL genes and activity of the PAL enzyme were shown to correlate with accumulation of specific secondary metabolites in plants. Chlorogenic acid provides an example of a compound which concentration can be associated with the PAL activity (Leszczyński 1985; Bate et al. 1994; Howles et al. 1996; Shadle et al. 2003). However, despite the observed increased expression of the PAL genes in the resistant accessions, we were not able to indicate any single specific chemical compound which accumulation correlated with the resistant phenotype. It may be related to the above-mentioned chemical complexity of the signals sensed by the pest. This may be also due to the fact that the PAL genes control synthesis of many phenolic compounds and only a few were analyzed in the present work. Therefore, it seems that future studies of plant–insect interactions will require metabolomic approaches which provide information about the entire chemical complement of plant tissues.

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