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Phenolic glucosides in the course of ligulate flower development in diploid and tetraploid *Matricaria chamomilla*

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ABSTRACT

The main secondary metabolites of *Matricaria chamomilla* ligulate flowers are apigenin-7-*O*-glucoside derivatives and (*Z*)- and (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acids (GMCAs), which are the precursors of herniarin. The quantities of these compounds were determinated in six phases of development of ligulate flowers in diploid and tetraploid cultivar. The content of the apigenin glucoside and its main acylated derivatives in ligulate flowers of diploid plants was found to be significantly higher before the start of flowering in comparison with tetraploid plants. During the flowering and post-flowering phase their content decreased and no difference between diploid and tetraploid plants was observed. The (*E*)-izomer was the dominant form of 2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acid. These secondary stress metabolite precursors were accumulated in higher concentrations in young growing ligulate flowers, but during flowering and post-flowering phases their content decreased. Significantly higher content was found in tetraploid plants in comparison with diploid plants. Aglycones of glycosides were found in low concentrations.

1. Introduction

Chamomile *(Matricaria chamomilla* L.) is a widely used medicinal plant possessing several pharmacological effects due to presence of active compounds (sesquiterpenes, flavonoids, coumarins, coumaroylspermins and polyacetylenes), which are accumulated in the chamomile drug (*flos chamomillae vulgaris*) (Schilcher, Imming, & Goeters, 2005; Yoo, Lee, Lee, Moon, & Lee, 2008). Chamomile tea is drunk traditionally in cases of gastric and intestinal complaints or use as flavouring agents and adjuvant (Burdock, 1996; Schilcher, 2005). Female ligulate flowers are characteristic components of the anthodia. Several vacuolar glycosides of these ligulate flowers were identified: apigenin-7-*O*-glucoside, apigenin 7-*O*-(4″-acetyl)-glucoside, apigenin 7-*O*-(6″-acetyl)-glucoside, apigenin 7-*O*-(6″-malonyl)-glucoside, apigenin 7-*O*-(6″-caffeoyl)-glucoside, apigenin 7-*O*-(4″-acetyl, 6″-malonyl)-glucoside, (Švehlíková et al., 2004), apigenin 7-*O*-(6″-*O*-rhamnosyl)-glucoside (Weber et al., 2008). (*Z*)- and (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acids (GMCAs) (Kanamori, Terauchi, Fuse, & Sakamoto, 1993), which are the precursors of the coumarin herniarin, are present in all parts of the shoot (Kováčik & Repčák, 2008), but apigenin glucosides accumulate in the ligulate flowers of chamomile only (Redaelli, Formentini, & Santaniello, 1981). The role of the flavone apigenin is probably to assist in the attraction of pollinators during anthodia flowering. Coumarins herniarin and umbelliferone were identified as stress metabolites in *M. chamomilla* (Repčák, Imrich, & Franeková, 2001).

Flavone apigenin was described as a low-toxic and non-mutagenic compound with antiphlogistic and spasmolytic activities. Apigenin is a central benzodiazepine receptor-ligand with anxiolytic effects, and is also responsible for the sedative effect of the chamomile drug (Avallone, Zanoli, Cosi, Cannazza, & Baraldi, 1996; Viola et al., 1995). Apigenin has been shown to have chemopreventive activity against UV-radiation and/or anti-cancer properties against a number of tumour types. Apigenin shows strong cytostatic and anti-angiogenic effects *in vitro* and was found to be an inhibitor of protein kinase and a component in the induction of apoptosis through proteasomal degradation of the human breast cancer cell (Mak, Lejny, Tang, Harwood, & Ho, 2006). Recently, it was found that apigenin inhibits hypoxia-activated pathways linked to cancer progression in human prostate cancer (Mirzoeva et al., 2008). Herniarin has antimicrobial, antifungal, weak antispasmodic (Schilcher et al., 2005) and marked anti-inflammatory effects (Silván, Abad, Bermejo, Sollhuber, & Villar, 1996).

The aim of this paper was to quantify the main secondary metabolites accumulation during the development of ligulate flowers in *M. chamomilla*. The quantities of apigenin glucoside derivatives and GMCAs were estimated in both diploid and tetraploid cultivars.

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2. Materials and methods

Plants of diploid cv. 'Novbona' and tetraploid cv. 'Lutea' of *M. chamomilla* L (Asteraceae) were cultivated in the experimental field in the Botanical Garden of P. J. Šafárik University in Košice (Slovakia). Anthodia were collected in six phases of development:

1. The bracts of involucre are differentiated and ligulate flowers are coiled in lengthwise.
2. The ligulate flowers are still coiled, but longer than the vaulted top of the anthodium; the corollae are parallel with the axis of the anthodia.
3. The corollae of ligulate flowers begin developing; tubular flowers are already differentiated, but their corollae are still closed.
4. Most of the ligulate flowers are blooming; 1/3 of the tubular flowers are opened.
5. The ligulate flowers are still blooming; 3/4 of the tubular flowers are opened.
6. The corollae of ligulate flowers standing bend to the stem; achenes are barren as a rule.

The anthodia were dried at laboratory temperature. Dry mass was determinated after drying to constant weight at 105 °C.

For the qualitative and quantitative analysis of apigenin glucosides and GMCAs dry ligulate flowers were homogenised and extracted with methanol for 30 min at room temperature. The extract was directly analysed by HPLC. A gradient HPLC system from Ecom (Prague, Czech Republic) with a UV detector, was used for analyses carried out with a column Separon SGX C18 7 μm (4 × 250 mm, Tessek, Prague), and a flow rate of 0.7 ml min^{-1} at 30 °C. The gradient consisted of mobile phase A: H_2O–acetonitrile–H_3PO_4 (80:19:1) and B: 80% acetonitrile. Gradient program: 0 min – 15% B, 5 min – 20% B, 10 min – 30% B, 15 min – 35% B, 20 min – 60% B, 25 min – 15% B. Detection was performed at 335 nm. The standard compounds used were apigenin (Sigma) and herniarin (Extrasynthese). Calibration was done by external standardisation. Apigenin derivatives were identified in accordance with Švehlíková et al. (2004, and personal comm.). GMCAs were prepared and identified as described in our previous paper (Repčák et al., 2001).

Quantitative data were analysed using ANOVA and *t*-test. Data expressed as percentages were arcsin transformed before calculation.

3. Results and discussion

The dry mass of chamomile anthodia in the course of evaluated phases increased twice. The weight of the anthodia of tetraploid 'Lutea' was significantly higher in comparison with diploid 'Novbona' (Table 1). The average number of ligulate flowers in the anthodium was 18, varying from 13 to 27 in both diploid and tetraploid cultivars. The weight of ligulate flowers in the anthodium during the flowering stage increased, although the data obtained varied considerably. Higher values were found in tetraploid plants, with significant differences in the first and final developmental phase (Table 1). The relative weight of ligulate flowers in the anthodium increased in the fourth phase, but later decreased. A higher relative weight of ligulate flowers was found in the anthodia of diploid plants than in tetraploid plants before the start of flowering (Fig. 2).

A typical chromatogram of tetraploid chamomile ligulate flower (sixth phase) is shown in Fig. 1. Apigenin-7-*O*-glucoside and apigenin 7-*O*-(6″-malonyl)-glucoside content in the ligulate flowers of diploid plants was found to be significantly higher in the first three phases and then decreased. On the other hand, tetraploid plants accumulated lower amounts of both compounds before the start of flowering. Both the above-mentioned compounds and apigenin 7-*O*-(4″-acetyl, 6″-malonyl)-glucoside were found as the main apigenin derivatives of chamomile in ligulate flowers in amounts of 15–30 mg g^{-1} DM (Table 2). Apigenin 7-*O*-(6″-caffeoyl)-glucoside was found to be higher in diploids and significantly decreased during ontogenesis (Table 2). The other minor derivatives varied as follows: apigenin 7-*O*-(4″-acetyl)-glucoside from 1.4 to 2.7 mg g^{-1} DM of ligulate flower, apigenin 7-*O*-(6″-acetyl)-glucoside from 0.9 to 1.2 mg g^{-1} DM of ligulate flower and apigenin 7-*O*-(X″-acetyl, Y″-malonyl)-glucoside from 1.0 to 3.2 mg g^{-1} DM of ligulate flower. No significant differences due to ploidy and development were found (data not shown). Apigenin (free aglycone) was found in low amounts (0.05–0.4 mg g^{-1} DM).

Table 1
Anthodium and ligulate flowers dry weight [mg] during ontogenesis of diploid 'Novbona' and tetraploid 'Lutea' cultivars of *M. chamomilla* L. $n = 20$ x, average; s_x, standard deviation; n.s., not significant.

Phase of flowering	Diploid		Tetraploid		*t*-Value *P*
	x	Min	x	Min	
	s_x	Max	s_x	Max	
Anthodium					
1	**13.18**	10.3	**20.32**	14.8	−6.872
	2.458	20.3	3.947	31.2	<0.001
2	**17.66**	13.6	**23.19**	16.8	−5.881
	2.265	21.3	3.81	33.1	<0.001
3	**18**	12.7	**22.34**	17.7	−4.567
	3.412	24.9	2.837	27.5	<0.001
4	**26.72**	19.4	**27.8**	18.9	−0.708
	4.744	38.4	5.37	40.9	n.s.
5	**31.28**	17.1	**43.04**	32.3	−7.536
	5.733	46.6	5.983	55.5	<0.001
6	**35.1**	22.6	**50.28**	28.7	−7.784
	5.838	46.3	11.849	79.6	<0.001
Ligulate flowers of anthodium					
1	**1.585**	1	**2.145**	1.4	−3.554
	0.463	2.7	0.532	3.5	<0.001
2	**2.7**	1.7	**2.991**	1.9	−1.608
	0.463	3.5	0.723	4.6	n.s.
3	**2.584**	1.6	**2.79**	1.9	−1.127
	0.544	3.6	0.684	4.8	n.s.
4	**4.138**	2.6	**4.446**	2.9	−1.202
	0.762	5.7	0.932	6.7	n.s.
5	**4.365**	2.9	**6.53**	4.4	−8.39
	0.873	6.7	1.212	9.5	<0.001
6	**3.246**	1.6	**4.46**	2.8	−6.306
	0.705	5	1.149	7.5	<0.001

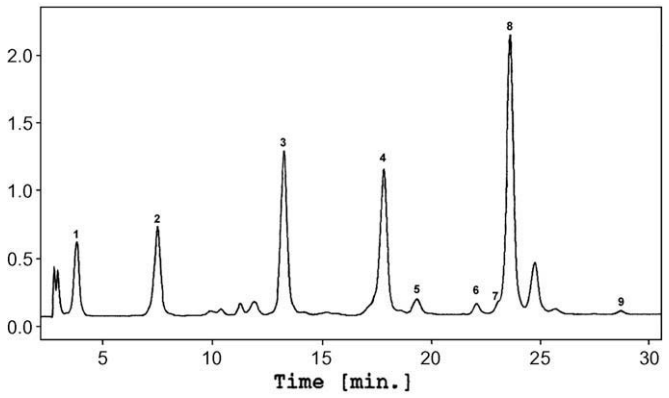

Fig. 1. HPLC chromatogram of the methanol extract of the ligulate flowers of *M. chamomilla*. **1** (*Z*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acid; **2** (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acid; **3** apigenin 7-*O*-glucoside; **4** apigenin 7-*O*-(6″-malonyl)-glucoside; **5** apigenin 7-*O*-(4″-acetyl)-glucoside; **6** apigenin 7-*O*-(6″-caffeoyl)-glucoside; **7** apigenin 7-*O*-(6″-acetyl)-glucoside; **8** apigenin 7-*O*-(4″-acetyl, 6″-malonyl)-glucoside; **9** apigenin.

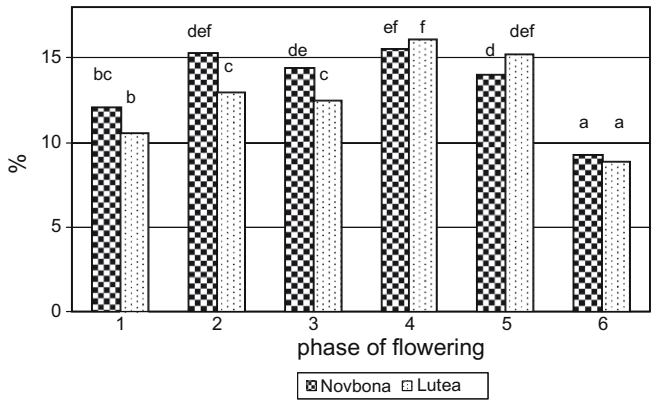

Fig. 2. Percentage weight of ligulate flowers in anthodium during ontogenesis of diploid 'Novbona' and tetraploid 'Lutea' cultivars. Columns sharing the same letters are not significantly different (LSD test, $P < 0.05$) $n = 20$.

The content of GMCAs significantly decreased from juvenile to the post-flowering stage of ligulate flower development. A low concentration of the (*Z*)-isomer has been found in both diploid and tetraploid cultivars. The (*E*)-isomer was the dominant form of 2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acid and its content was significantly higher in tetraploid plants (Table 3). Herniarin content, a product of GMCAs deglucosylation, was found to be lower than 0.1 mg g^{-1} DM.

Polyploidisation is often used to improve productivity of crop plants. Plant height and organ size increase with ploidy level. The higher weight of developing chamomile anthodia and ligulate flowers of tetraploid cultivars in comparison with diploid cultivars were described by Glücknerová, Blažek, and Starý (1965). They also found a greater variation in morphological characters of tetraploid plants, similar to our results.

Various changes in secondary metabolite content evoked by polyploidisation were found. The content of the essential oil in the diploid and tetraploid chamomile drug was similar (Franz, Kirsch, & Isaac, 1985). A higher content of the anthodia essential oil was found in tetraploid plants compared to diploid ones, but only in the phases before the start of flowering (Repčák, Černaj, & Mártonfi, 1993). Also, cuticular flavonoid content increased twice in tetraploid plants in comparison with diploid ones (Repčák, Švehlíková, Imrich, & Pihlaja, 1999). The content of total bound apigenin was found to be higher in ligulate flowers of diploid chamomile plants compared to tetraploid plants (Švehlíková & Repčák, 2000).

Acylation of flavonoid glycosides, which are probably synthesised in the cytoplasm, facilitates transport into the vacuole. Malonylation of apigenin-7-*O*-glucoside is catalysed by isoflavone-7-*O*-glucoside 6″-*O*-malonyltransferase (EC 2.3.1.115). Conformation changes in the sugar parts of the apigenin-7-*O*-(6″-malonyl)-glucoside molecule were observed (Matern, Keller, & Himmelspach, 1983). Decarboxylation of the 6″-malonyl group and formation of apigenin 7-*O*-(6″-acetyl)-glucoside and other acetylated derivatives were described (Švehlíková et al., 2004). In our work a high content of malonylated apigenin-7-*O*-glucosides derivatives and a low content of their degradation products were found in ligulate flowers which were air-dried with adequate air circulation. Specific flavone-7-*O*-*β*-glucosidase was purified and characterised in chamomile with increasing activity during development of flowers (Maier, Carle, Kreis, and Reinhard (1993). Enzymatic cleavage of glucose and releasing of aglycone were observed after harvesting in conditions without adequate air circulation (Schreiber, Carle, & Reinhard, 1990). Stress metabolite herniarin originates from (*Z*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acid, which is hydrolysed by *β*-glucosidase and the released aglycone spontaneously lactonises. Very low amounts of both, aglycones apigenin as well as herniarin presented in our work confirm that the drying method we used did not damage cellular compartmentation.

Table 2
Content of the main apigenin glycosides in ligulate flower dry mass during ontogenesis of anthodia in diploid 'Novbona' and tetraploid 'Luteá cultivars of *M. chamomilla* L. [mg g^{-1} DM]; $n = 8$. x, average; s_x, standard deviation; n.s., not significant; min, minimal value; max, maximal value.

Phase of flowering	Diploid		Tetraploid		*t*-Value *P*
	x s_x	Min max	x s_x	Min Max	
Apigenin-7-O-glucoside					
1	**23.51**	20.49	**15.17**	10.7	−6.804
	2.067	25.87	2.355	17.12	<0.001
2	**27.7**	20.08	**16.9**	11.8	−4.321
	4.52	31.78	4.131	21.73	<0.001
3	**25.16**	20.3	**15.13**	11.25	−5.233
	3.915	31.15	2.585	18.42	<0.001
4	**24.27**	15.63	**22.49**	18.59	−0.743
	5.483	32.4	2.195	24.77	n.s.
5	**19.94**	15.52	**17.53**	15.45	−1.452
	3.408	24.56	2.218	21.5	n.s.
6	**14.21**	11.38	**15.68**	14.27	1.264
	1.97	16.96	2.079	19.6	n.s.
Apigenin 7-O-(6″-malonyl)-glucoside					
1	**29.04**	19.34	**20.81**	14.99	−2.224
	8.365	40.78	3.652	25.25	0.024
2	**29.25**	20.73	**18.25**	10.58	−3.75
	5.027	35.56	5.133	24.43	0.002
3	**32.19**	25.18	**15.29**	10.56	−5.43
	6.566	44.36	3.871	21.17	<0.001
4	**29.03**	17.51	**25.23**	16.54	−0.754
	10.659	46.08	6.666	32.8	n.s.
5	**23.66**	17.05	**15.82**	13	−4.578
	3.683	27.67	2.018	17.4	<0.001
6	**17.18**	14.19	**14.877**	10.9	−1.408
	1.773	18.49	3.59	21.08	n.s.
Apigenin 7-O-(6″-caffeoyl)-glucoside					
1	**4.5**	2.88	**2.76**	2.07	−2.823
	1.366	6.45	0.678	3.69	0.008
2	**4.33**	2.99	**2.7**	1.75	−3.993
	0.819	5.05	0.576	3.54	0.001
3	**4.17**	3.66	**1.24**	0.37	−11.51
	0.363	4.56	0.506	1.75	<0.001
4	**2.22**	1.43	**1.09**	0.78	−4.512
	0.577	3.03	0.211	1.38	<0.001
5	**2.25**	1.69	**0.47**	0.29	−6.992
	0.595	3.15	0.189	0.77	<0.001
6	**0.69**	0.48	**0.58**	0.27	−0.649
	0.274	1.12	0.287	1.11	n.s.
Apigenin 7-O-(4″-acetyl, 6″-malonyl)-glucoside					
1	**25.47**	19.15	**19.11**	16.04	−3.788
	3.364	29.77	2.536	23	0.002
2	**27.29**	23.81	**25.7**	16.95	−0.672
	2.049	29.83	5.431	31.18	n.s.
3	**30.74**	26	**21.8**	16.28	−4.49
	3.638	36.33	3.25	25.9	<0.001
4	**21.57**	15.93	**22.25**	16.84	0.282
	5.143	29.22	3.087	25.36	n.s.
5	**18.77**	11.63	**27.63**	23.29	2.955
	6.514	26.29	3.395	31.62	0.007
6	**15.37**	10.84	**18.44**	13.29	1.223
	4.572	21.55	4.12	23.97	n.s.

Flavonoids were found to be the most active radical-scavengers (Sharififar, Dehghn-Nudeh, & Mirtajaldini, 2009). Therefore the content of apigenin in the drug correlates with tea quality as well as other food preparations. The amount of flavonoids in the chamomile drug was also found to be affected by various ecological factors (Ganzera, Guggenberger, Stuppner, & Zidorn, 2008).

Different quantitative patterns of apigenin-7-*O*-glucosides and GMCAs accumulation in developing ligulate flowers may indicate differences in the functions of these compounds. The high

Table 3
Content of (*Z*)- and (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxy cinnamic acids in ligulate flowers during ontogenesis of anthodia in diploid 'Novboná and tetraploid Ľuteá cultivars of *M. chamomilla* L. [mg g^{-1} DM]; $n = 8$. x, average; s_x, standard deviation; n.s., not significant.

Phase of flowering	Diploid		Tetraploid		
	x	Min	x	Min	t-Value P
	s_x	Max	s_x	Max	
(Z)-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid					
1	**0.183**	0.123	**0.215**	0.146	−1.74
	0.039	0.245	0.034	0.25	n.s.
2	**0.258**	0.154	**0.208**	0.156	1.684
	0.067	0.356	0.044	0.28	n.s.
3	**0.204**	0.156	**0.257**	0.2	−2.32
	0.025	0.236	0.059	0.349	0.019
4	**0.25**	0.154	**0.325**	0.219	−2.372
	0.051	0.301	0.072	0.412	0.017
5	**0.222**	0.13	**0.352**	0.264	−3.766
	0.053	0.318	0.07	0.468	0.001
6	**0.206**	0.085	**0.287**	0.227	−1.93
	0.08	0.346	0.045	0.339	0.04
(E)-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid					
1	**1.175**	0.986	**1.552**	1.296	−3.008
	0.208	1.586	0.149	1.722	0.007
2	**0.766**	0.668	**1.212**	0.831	−3.421
	0.081	0.947	0.278	1.507	0.003
3	**0.501**	0.668	**0.846**	0.685	−5.747
	0.052	0.947	0.122	1.016	<0.001
4	**0.234**	0.141	**0.443**	0.334	−6.471
	0.049	0.307	0.064	0.519	<0.001
5	**0.178**	0.158	**0.273**	0.238	−6.071
	0.016	0.201	0.03	0.333	<0.001
6	**0.156**	0.104	**0.219**	0.207	−3.997
	0.03	0.208	0.014	0.25	0.001

accumulation of the precursor of phytoanticipin herniarin before the start of flowering may be related to the risk of biotic or abiotic stress for growing tissue.

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