

Quantitative Changes of Secondary Metabolites of *Matricaria chamomilla* by Abiotic Stress

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The responses of young plants of diploid and tetraploid *Matricaria chamomilla* cultivars to abiotic stress were studied. The course of quantitative changes of main leaf secondary metabolites was evaluated within an interval from 6 h before to 54 h after spraying the leaf rosettes with aqueous CuCl₂ solution. The content of herniarin in the treated plants rose approximately 3 times, simultaneously with a decline of its precursor (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acid. The highest amounts of umbelliferone in stressed plants exceeded 9 times and 20 times those observed in control plants of the tetraploid and diploid cultivar, respectively. Due to stress the concentration of ene-yne-dicycloether in leaves decreased by more than 40%. The pattern of quantity changes of the examined compounds in tetraploid and diploid plants was similar.

Key words: *Matricaria chamomilla*, Secondary Metabolites, Abiotic Stress

Introduction

By means of the secondary metabolite biosynthetic pathways, plants produce a wide range of compounds of various chemical classes effective in their defence against infection (Harborne, 1999). Some of these substances are constitutive metabolites termed phytoanticipins: they are present in plants before challenge by pathogens or are formed after infection solely from preexisting constituents, *e.g.* from the less toxic glycoside precursors (VanEtten *et al.*, 1994; Morrissey and Osbourn, 1999). Other ones, called phytoalexins, are produced by *de novo* expression of the enzymes involved in their biosynthesis, only when the plant organism is attacked (Grayer and Kokubun, 2001). Due to their antibiotic properties, phytoalexins and phytoanticipins play an important role in plant resistance against fungi and other microorganisms. Experimental studies revealed that both the accumulation of phytoalexins and the release of toxic aglycones from glycoside precursors can be induced also by non-biological stress factors, such as UV light or treatment with heavy metal ions (Purkayastha, 1995; Higgins *et al.*, 1995).

Matricaria chamomilla L. (Asteraceae) belongs to a major group of cultivated medicinal plants. Chamomile anthodia (*flos chamomillae*) are used therapeutically, mainly due to their anti-inflamma-

tory and spasmolytic effects. Sesquiterpenes, flavonoids, coumarins, and polyacetylenes are considered the most important constituents of the chamomile drug (Schilcher, 1987). The coumarins are represented in *Matricaria chamomilla* by herniarin (7-methoxycoumarin), umbelliferone (7-hydroxycoumarin), and other minor ones (Redaelli *et al.*, 1981; Kotov *et al.*, 1991). (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acid (GMCA), the glucoside precursors of herniarin, were described as native compounds in chamomile (Kanamori *et al.*, 1993; Ohe *et al.*, 1995). (*E*)-GMCA isomerizes to the (*Z*)-form by UV light or enzymatically; the (*Z*)-isomer, after hydrolysis by a specific β -glucosidase, spontaneously lactonizes to herniarin. In cells of healthy tissues, GMCA and β -glucosidase are spatially separated. However, they can come into contact when the cell compartmentation is broken, *e.g.* in the case of mechanical or chemical injury.

In the previous paper (Repčák *et al.*, 2001a), umbelliferone was described as a stress metabolite of *Matricaria chamomilla*. Its content in the leaves of mature tetraploid plants cultivated under field conditions increased considerably after both the abiotic stress elicitation by CuCl₂ and the infection by fungal pathogens. In this study we investigate the response of young tetraploid and diploid chamomile plants to the abiotic stress under labo-

ratory conditions. Quantitative changes of main leaf secondary metabolites (GMCA, herniarin, umbelliferone, and polyacetylene ene-yne-dicycloether) are evaluated in relation to time.

Material and Methods

Plant material

Seeds of tetraploid cv. 'Lutea' and diploid cv. 'Novbona' of *Matricaria chamomilla* (supplied by Agricultural farm "Rozkvet", Nová L'ubovňa, Slovakia) were germinated in sand. Seedlings in the stage of one leaf pair were individually transplanted to plastic pots packed with soil. They were grown under a 12-h photoperiod with the soil moisture kept at a value of 60% water holding capacity.

12-week-old healthy plants were used for experiments. To induce a stress reaction, shoots, *i.e.* leaf rosettes, were sprayed with a 2% aqueous solution of CuCl₂. Seven control and seven stressed plants of each tetraploid and diploid cultivar were collected at 6, 18, 30, 42, and 54 h after spraying. The control plants were also collected 6 h before the CuCl₂ treatment. The whole shoot was cut at the soil level and immediately dried at 105 °C for 1.5 h.

Viability assay

Chlorophyll fluorescence measurements were used to assess vitality of stressed plants. The potential quantum yield of photosystem II was detected on whole plants using a kinetic fluorometric CCD camera FluorCam (Photon Systems Instruments, Brno, Czech Republic). Results were expressed as F_v/F_m calculated as the maximal fluorescence (F_m) less the minimal fluorescence (F_o), divided by F_m of dark adapted (10 min) plants: $(F_m - F_o)/F_m = F_v/F_m$. Three replicates were used.

Chromatographic procedure

The content of secondary metabolites in the 70% methanolic extracts of individual plants was estimated by gradient HPLC. Chromatography conditions: column Tessek SGX C18 7 μm (4 × 250 mm); flow rate 0.7 ml · min⁻¹; mobile phase A: acetonitrile/water/H₃PO₄ (19:80:1), B: 45% acetonitrile, C: 90% acetonitrile; all solvents were gradient grade (Merck, Darmstadt, Germany). The linear gradient elution programme was from 100% A to 100% B in 25 min, then to 100% C in 30 min,

isocratic for 5 min, and returning to 100% A in 45 min. Detection was performed at 320 nm. Herniarin (Extrasynthese, Genay, France) and umbelliferone (Merck, Darmstadt, Germany) standard compounds were used for the quantification. (*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acid and (*Z*)- and (*E*)-ene-yne-dicycloether were prepared and identified as described in previous papers (Repčák *et al.*, 1999a, 2001b). The content of both GMCA and ene-yne-dicycloether was evaluated as a sum of quantity of the (*Z*)- and (*E*)-isomers.

Quantitative data were analysed by ANOVA procedure and t-test.

Results and Discussion

Abiotic elicitation was carried out 12 weeks after the seed germination when both tetraploid and diploid chamomile plants reached an average dry weight of 0.17 g (1.11 g fresh weight). CuCl₂ treatment caused specific reactions of leaves, visually observable even after 6 h (diploid) and 12 h (tetraploid), respectively. They included moderate fading, rolling of leaves and leaf segments, later followed by sparse dot necroses of the tissues that were directly affected by aerosol drops. Diploid plants were slightly more damaged by CuCl₂ application than tetraploid ones, probably due to different leaf morphology: more numerous and finer segments.

Chlorophyll fluorescence measurements, expressed as F_v/F_m ratios, have been frequently used in studies focused on the assessment of heavy metal stress in plants. It has been demonstrated that copper stress is responsible for the decrease of this parameter in a wide range of photosynthetic organisms, from unicellular green algae to higher plants (Cook *et al.*, 1997; Bačkor *et al.*, 2003). However, we did not observe a significant decrease in functioning of photosystem II in our study. F_v/F_m ratios determined 5 d after the CuCl₂ application were as follows: 0.69 (standard deviation SD = 0.05) for copper treated plants, 0.70 (SD = 0.05) for control, unstressed plants. Similar results were obtained 7 d after the stress induction, when F_v/F_m ratios were almost identical, 0.76 (SD = 0.05) for copper treated plants and 0.76 (SD = 0.03) for control plants. From our results it is visible that chamomile plants survived CuCl₂ treatment and remained alive.

Our study of the accumulation of GMCA, herniarin, umbelliferone and ene-yne-dicycloether in young tetraploid chamomile shows considerable quantitative changes of these secondary metabolites as a result of abiotic stress. The content of GMCA decreased by more than 25% compared to the control (Fig. 1A) 6 h after spraying the leaves with CuCl_2 . The minimum values, *i.e.* 4.18 and 4.17 $\text{mg}\cdot\text{g}^{-1}$ dry weight, were found after 18 h and 30 h, respectively. Later the amount of GMCA increased so that the difference between stressed and unstressed plants became non-significant at the end of the experiment. The time course of the herniarin quantity changes in CuCl_2 treated chamomile corresponds to that of GMCA. This is in agreement with the fact that GMCA is a precursor of herniarin. As may be seen from Fig. 1B, the increase of the herniarin content was quite rapid – already 6 h after the stress induction it reached a value more than 3 times higher in comparison to the

control. Concerning the increased value of herniarin in control plants (42 h), it can be attributed to random influences of plant material processing rather than to stress factors. It was demonstrated that the drying conditions in particular considerably affect the content of herniarin in the chamomile drug (Repčák *et al.*, 1998). Although umbelliferone occurred in tetraploid control plants only in a small and relatively stable amount (0.009–0.013 $\text{mg}\cdot\text{g}^{-1}$ dry weight), its production after CuCl_2 application increased even more than that of herniarin (Fig. 1C). The highest observed value of the umbelliferone content (0.120 $\text{mg}\cdot\text{g}^{-1}$ dry weight) exceeded 9 times the corresponding value in the control. By comparison of Fig. 1C and Fig. 1B it can be noticed that there was a shift in the time course of umbelliferone and herniarin accumulation in sprayed leaves. The content of herniarin peaked at a time of 18 h and did not differ significantly from the control at the latest time evaluated.

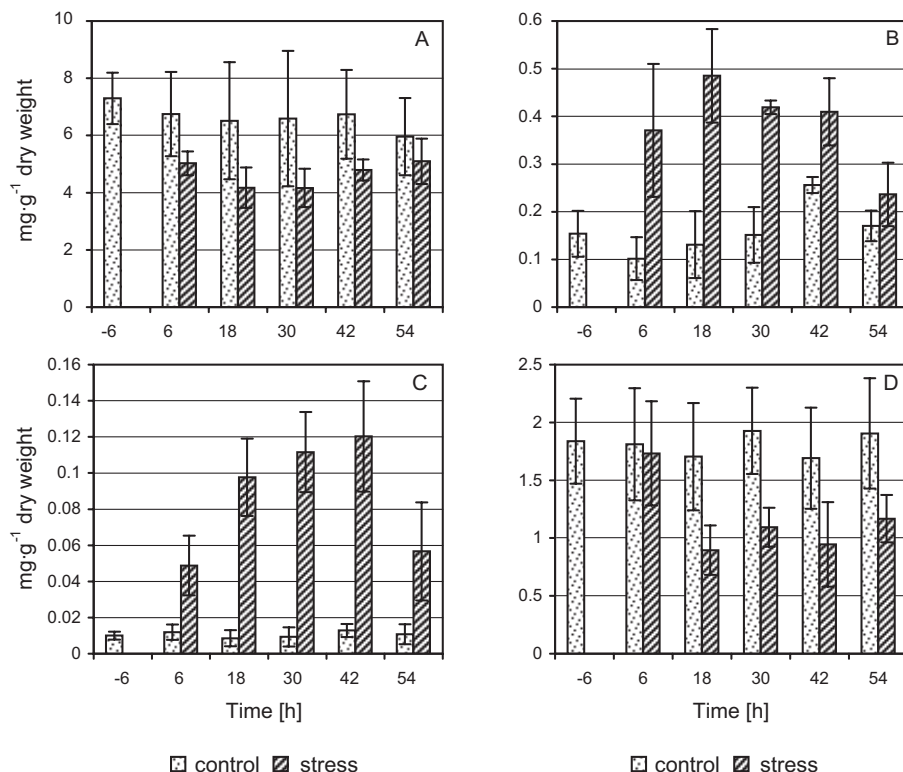


Fig. 1. Time course of secondary metabolites accumulation in the leaf rosettes of *Matricaria chamomilla* tetraploid plants after abiotic stress induced by CuCl_2 spraying. The treatment was carried out at a time of 0 h. A: (Z)- and (E)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acid; B: herniarin; C: umbelliferone; D: (Z)- and (E)-ene-yne-dicycloether. Error bars represent standard deviation.

On the contrary, the umbelliferone content reached its maximum after a time of 42 h and at the end of the experiment it was still on the level of $0.057 \text{ mg} \cdot \text{g}^{-1}$ dry weight, which is more than 5 times higher than that in control plants. As for the ene-yne-dicycloether, the main leaf polyacetylene of chamomile, a significant difference in comparison to the control was found 18 h after stress induction (Fig. 1D). Its content at that time decreased by more than 40% and remained on approximately the same level until the end of the experiment.

The response of diploid cv. 'Novbona' of *Matricaria chamomilla* to abiotic stress, concerning the time course of quantitative changes of secondary metabolites examined, was in principle resembling the tetraploid one. A considerable difference between the cultivars was found in the case of GMCA. The content of (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acid in the diploid plants significantly decreased after the CuCl_2

application, similarly as in the tetraploid plants. However, re-establishment of its level at the end of the experiment was not observed (Fig. 2A). This can be connected with the fact mentioned above, that the diploid plants were more damaged by spraying than the tetraploid ones. As may be seen from Fig. 2B and Fig. 2C, the amount of herniarin as well as of umbelliferone peaked 30 h following the stress-treatment of diploid plants. Thus, the shift in the time course of the accumulation of these two coumarins above-referred to cv. 'Lutea' was not observed in the case of cv. 'Novbona'. The maximum of the umbelliferone content in the diploid cultivar ($0.482 \text{ mg} \cdot \text{g}^{-1}$ dry weight) exceeded 20 times the corresponding value in the control, what is an even more intensive response to stress than that of tetraploid plants. On the contrary, changes of herniarin (Fig. 2B) as well as of ene-yne-dicycloether (Fig. 2D) accumulation in both cultivars were comparable.

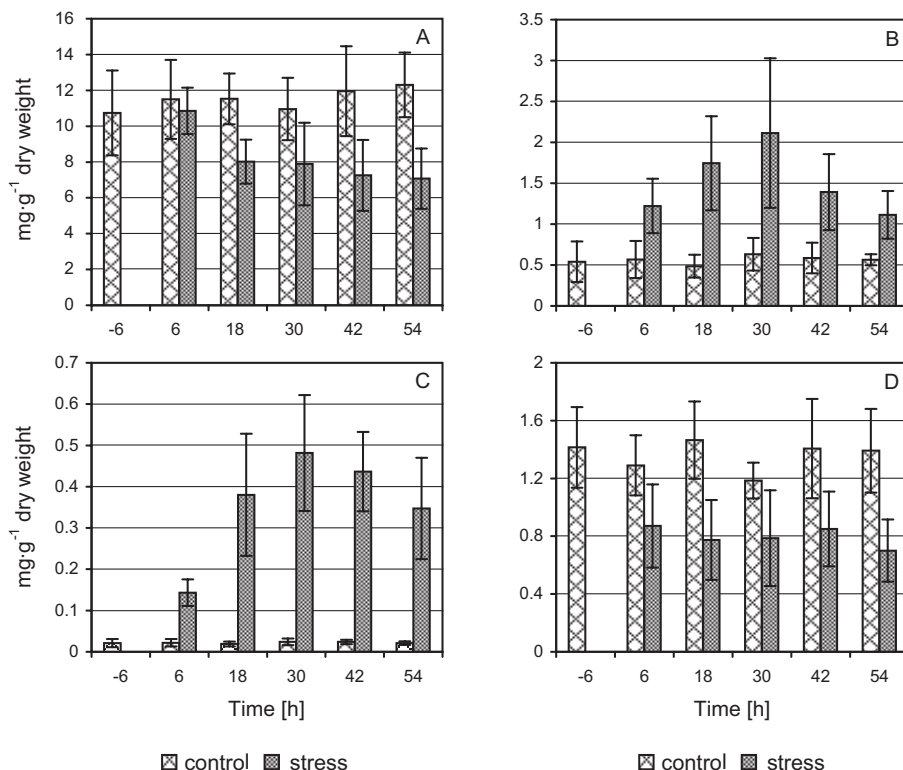


Fig. 2. Time course of secondary metabolites accumulation in the leaf rosettes of *Matricaria chamomilla* diploid plants after abiotic stress induced by CuCl_2 spraying. The treatment was carried out at a time of 0 h. A: (*Z*)- and (*E*)-2- β -D-glucopyranosyloxy-4-methoxycinnamic acid; B: herniarin; C: umbelliferone; D: (*Z*)- and (*E*)-ene-yne-dicycloether. Error bars represent standard deviation.

Several types of phenylpropanoid compounds, including coumarins, are induced in plants by various biotic and abiotic stress factors (Dixon and Paiva, 1995). Umbelliferone and other methoxy-derivatives of coumarin were reported as stress metabolites or phytoalexins from plants of various families and also from *Matricaria chamomilla* (for review see Repčák *et al.*, 2001a). Only little attention was paid to herniarin. According to Patton *et al.* (1997), herniarin is an important factor in host plant resistance as feeding deterrent to the adult beetle *Popilia japonica*.

With respect to the time course of umbelliferone and herniarin quantity changes observed in our experiment, *i.e.* an increase of the metabolite content to a maximum within a few days and its subsequent decline, this pattern of accumulation and degradation is typical for phytoalexins in plants (Bailey, 1982; Harborne, 1999). However, it was different in the case of ene-yne-dicycloether, where only the decline of its amount was detected. The literature data about this polyacetylene accumulation in chamomile leaves are rare, particularly concerning the stress influences. In a previous study (Repčák *et al.*, 2001a), a decrease of the ene-yne-dicycloether content after CuCl₂ application was observed, in contrast to the increased quantity of the substance in plants affected by biotic stress.

The comparison of the secondary metabolite amount in non-sprayed tetraploid and diploid leaf rosettes revealed significant differences between the cultivars. The total average of quantitative data of control plants collected during the whole experiment (in all collection times) is given in Table I. The content of GMCA and umbelliferone was found to be approximately 2 times higher and that of herniarin almost 4 times higher in diploid cultivar as compared to the tetraploid one. On the contrary, plants of diploid cultivar contained 25% less ene-yne-dicycloether.

Polyploidy induction is considered as means of increasing plant production potential, but studies of increases in secondary product content have been ambiguous. While the amount of the coumarin herniarin or methoxylated flavonoids was sig-

Table I. Content of secondary metabolites in the leaf rosettes of tetraploid cv. 'Lutea' and diploid cv. 'Novbona' of *Matricaria chamomilla*.

Compound	Tetraploid	Diploid
	\bar{x}^a SD ^b	\bar{x} SD
(Z)- and (E)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acid	6.622 1.754	11.419 2.089
Herniarin	0.149 0.065	0.563 0.192
Umbelliferone	0.010 0.005	0.022 0.008
(Z)- and (E)-ene-yne-dicycloether	1.816 0.444	1.361 0.273

^a Average content (mg·g⁻¹ dry mass) of the metabolite in control plants collected during whole experiment.

^b Standard deviation.

nificantly higher in tetraploid cv. 'Lutea' of *Matricaria chamomilla* (Repčák *et al.*, 1998, 1999b), the flavonoid apigenin was found to be more abundant in diploid cv. 'Novbona' (Švehlíková and Repčák, 2000). An apparent effect of the ploidy level was not observed in the case of GMCA (Repčák *et al.*, 2001b), similarly to the results regarding chamomile essential oil content and composition (Repčák *et al.*, 1993). All above-mentioned papers refer to anthodia, the pharmacologically relevant parts of the chamomile plant. As far as the secondary metabolite content in leaf rosettes of tetraploid and diploid *Matricaria chamomilla* cultivars is concerned, comparable literature data are not available.

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