SHORT COMMUNICATION

CHANGES IN PHENOLIC COMPOUNDS IN *NICOTIANA* SPECIES AS A RESPONSE TO WOUNDING AND VIRAL INFECTION

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SUMMARY

The quantitative and qualitative changes in phenolic compounds were compared in *Nicotiana* spp. plants that were either wounded or infected with *Cucumber mosaic virus* (CMV) containing satellite RNA. Significant changes in total phenolic (TP) content were analysed in *N. megalosiphon*, *N. glutinosa* and *N. tabacum* var. Samsun at four different time intervals. The highest decrease of TP was detected in wounded *N. megalosiphon* plants while the highest increase was detected in infected *N. megalosiphon* plants. Depending on treatment and time, a significant increase or decrease of caffeic acid, quercetin or kaempferol was detected in the investigated species. The strongest increase in quercetin concentration was observed in infected *N. glutinosa*. The largest number of significant changes of caffeic acid, quercetin and kaempferol concentration in the analysed time intervals was observed in infected *N. megalosiphon* plants. The defence response to wounding in all investigated *Nicotiana* species resulted in a significant decrease of detected phenolic content. The detected changes in phenolics in virus-infected plants were characteristic for each species, suggesting that each species activates a different defence response to viral infection. The observed changes of phenolics are obvious responses to wounding and viral infection, which could likely be involved in activating a distinct defence response to a specific stress.

Key words: abiotic stress, biotic stress, plant-pathogen interaction, *Cucumber mosaic virus*, satellite RNA, flavonoids, hydroxycinnamic acids, HPLC.

Phenolic compounds are a highly diverse class of secondary metabolites that are widely distributed among plants (Martens *et al*., 2010). Different physiological roles have been attributed to phenolics, including the protection of plants from abiotic and biotic stresses (Dixon, 2001; Agati *et al*., 2012; Treutter, 2005). *Cucumber mosaic virus* (CMV) has the broadest host range of all plant viruses. Some strains of CMV encapsidate satellite RNAs which depend on their helper virus and can modify the symptoms induced by the virus exacerbating them (García-Arenal and Palukaitis, 1999). Host responses in CMV-infected tobacco have extensively been studied at the molecular and physiological levels (Roberts and Wood, 1981; Takahashi and Ehara, 1992). Still, compared to the number of described phenolic compounds (Harborne, 1998), little is known about phenolics in *Nicotiana* plants exposed to abiotic or biotic stress (Ounis *et al*., 2001; Chen *et al*., 2012; Torras-Claveria *et al*., 2012).

When exposed to abiotic or biotic stress, phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid biosynthetic pathway, is strongly induced (Pellegrini *et al*., 1994; Olsen *et al*., 2008). The importance of phenolics for defence response was clearly demonstrated in tobacco plants epigenetically suppressed in PAL expression. Local resistance and systemic acquired resistance to viral infection in these plants were seriously compromised (Pallas *et al*., 1996). Furthermore, the production of flavonoids increases as a response to wounding or pathogen attack (Dixon, 2001; Päsold *et al*., 2010). In addition, abiotic and biotic stress or senescence can lead to increased production of reactive oxygen species (ROS) (Apel and Hirt, 2004; Tamagnone *et al*., 1998). Induced ROS molecules are usually effectively scavenged by phenolics, flavonoids in particular (Agati *et al*., 2012).

Phenolics have distinct roles in plant defence responses and as signalling molecules in the development of plants, which does not exclude possible overlapping roles for phenolics in development and responses to environmental stresses. The aim of this study was to analyse changes in quantitative and qualitative phenolic content in *Nicotiana* plants subjected to abiotic or biotic stress (wounding vs. virus infection) at various stages of plant development. The phenolic pattern was compared between wounded and infected plants to determine whether changes of certain phenolics accompany the intensity of observed symptoms. The long-term aim of this study is to elucidate the significance of phenolics, primarily flavonoids, and their role in plant defence responses.

Nicotiana glutinosa L., *Nicotiana megalosiphon* Van Heurck et Müll. Arg. and *Nicotiana tabacum* L. cv. Samsun were used in this investigation. CMV containing satellite

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Table 1. Quantitative analysis of changes of total phenolics, caffeic acid, quercetin and kaempferol in all, lower and upper leaves of *Cucumber mosaic virus* containing satellite RNA tobacco infected plants compared to wounded (buffer treated) tobacco plants. Leaves were harvested 2, 4, 7, 14 days after inoculation (DAI).

	DAI	Total phenolics[a]			Caffeic acid[b]			Quercetin[c]			Kaempferol[c]		
Nicotiana glutinosa		lower	upper	all leaves	lower	upper	all leaves	lower	upper	all leaves	lower	upper	all leaves
	2	↑ 26.1±1.9	↑ **33.0±2.3***	↑ 29.6±1.1	↓ 2.3±0.2	↑ **3.2±0.4***	↑ 2.8±0.1	↑ 477±17	↑ **1867±186***	↑ **1172±85***	↓ 37±3	↑ 78±5	↑ 58±1
	4	↑ 25.6±2.9	↑ 27.8±4.9	↑ 26.7±3.9	↑ 2.8±0.4	↓ 2.8±0.6	↑ 2.8±0.5	↓ 399±101	↓ 950±133	↓ 674±110	↓ 41±3	↓ 56±8	↓ 49±5
	7	↑ 20.9±4.0	↓ **10.0±0.4***	↓ 15.9±2.2	↓ 2.8±0.1	↓ 1.8±0.1	↓ 2.3±0.1	↓ 388±119	↓ **426±48***	↓ **407±39***	↓ 36±7	↓ 35±1	↓ 35±3
	14	↑ 22.3±1.7	↓ 16.5±0.9	↓ 19.4±1.2	↑ 3.0±0.2	↓ 2.4±0.1	↑ 2.7±0.2	↑ 534±14	↓ 1379±63	↓ 956±33	↑ 53±2	↓ 107±4	↑ 80±3
Nicotiana megalosiphon		lower	upper	all leaves	lower	upper	all leaves	lower	upper#	all leaves	lower	upper#	all leaves
	2	↑ **16.6±0.9***	↑ 18.6±0.9	↑ **17.6±0.4***	↑ 1.1±0.1	↑ 1.5±0.0	↑ **1.3±0.0***	↑ 241±12	↓ 354±25	↑ 297±8	↑ 46±2	↑ **66±3***	↑ **56±2***
	4	↓ 15.3±0.2	↓ 19.1±0.6	↓ 17.2±0.3	↓ **1.0±0.0***	↓ 1.5±0.0	↓ 1.2±0.0	↓ **208±5***	↑ 320±7	↓ 264±6	↓ 50±1	↑ **70±1***	↑ 60±1
	7	↑ **21.2±1.5***	↑ 22.3±0.8	↑ **21.8±1.1***	↑ **1.2±0.0***	↑ 1.9±0.0	↑ **1.5±0.0***	↓ 159±8	↓ **396±19***	↓ **277±9***	↑ 47±1	↑ **79±4***	↑ **63±2***
	14	nd	↓ 20.3±0.4	na	nd	↓ **1.6±0.0***	na	nd	↓ **383±3***	na	nd	↓ **57±1***	na
Nicotiana tabacum		lower	upper	all leaves	lower	upper	all leaves	lower#	upper#	all leaves#	lower#	upper#	all leaves#
	2	↓ 25.3±0.3	↓ 21.8±2.3	↓ 23.5±1.3	↓ 2.7±0.1	↓ 2.0±0.4	↓ 2.3±0.3	↑ 147±5	↓ **97±14***	↓ 122±6	↑ **84±11***	↓ 79±9	↑ 81±9
	4	↓ 23.2±0.6	↓ 21.1±1.0	↓ 22.1±0.6	↓ 2.9±0.1	↑ 2.8±0.2	↓ 2.9±0.1	↓ 36±2	↑ 70±11	↑ 53±5	↑ 53±1	↑ **131±4***	↑ **92±3***
	7	↑ **26.6±1.0***	↓ 20.2±0.5	↑ 23.4±0.5	↑ 3.6±0.1	↑ 2.9±0.1	↑ 3.3±0.1	↓ 235±8	↑ **497±10***	↑ **366±9***	↑ 89±2	↑ **131±1***	↑ **110±2***
	14	↑ 26.2±0.8	↑ 22.5±0.4	↑ 24.3±0.3	↑ 4.5±0.1	↓ 4.1±0.1	↑ 4.3±0.0	↑ **854±44***	↑ **1090±3***	↑ **972±22***	↑ **175±9***	↑ **268±5***	↑ **221±5***

The asterisk (*) denotes significant change of total phenolics, caffeic acid, quercetin or kaempferol content in virus infected plants compared to their respective control (n = 3; $p < 0.05$; factorial ANOVA, post hoc Bonferroni correction). For emphasis these values are presented in boldface. The number sign (#) denotes significant change of quercetin or kaempferol content in lower, upper or all leaves of virus infected plants compared their respective control group (n = 3; $p < 0.05$; factorial ANOVA, post hoc Bonferroni correction).

[a] total phenolics are expressed in mg gallic acid equivalents per gram dry weight.

[b] caffeic acid is expressed in mg/ gram dry weight tissue.

[c] quercetin and kaempferol are expressed in µg/ gram dry weight tissue.

↑ or ↓ arrows indicate higher or lower concentration of total phenolics, caffeic acid, quercetin or kaempferol in *Cucumber mosaic virus* containing satellite RNA infected plants compared to their respective control.

RNA (CMVsat) was propagated in *N. megalosiphon*, a systemic host. A specimen of this virus is deposited at the Faculty of Science, University of Zagreb under number 14A. All plants were grown in an autoclaved 4:1 soil/sand mix, at 22±2°C under a 12-h photoperiod.

Fresh CMVsat inoculum was prepared from systemically infected *N. megalosiphon* leaves in phosphate buffer (0.06 mol dm^{-3}, pH 7.0). Test plants were dusted with silicon carbide prior to wounding, i.e. mock-inoculation with phosphate buffer or inoculation with CMVsat on the fourth and fifth leaf (leaves were numbered from the base towards the apex) and washed thoroughly with distilled water one min after inoculation. Leaves were harvested at 2, 4, 7 and 14 days after inoculation (DAI) from untreated (control for wounded), wounded (control for virus-infected) and virus-infected plants and separated into lower and upper leaves. Lower leaves of untreated, wounded or virus-infected plants harvested at the specified time intervals comprised the inoculated leaves and those below them. Upper leaves from untreated, wounded or virus-infected plants harvested at the specified time intervals comprised all other leaves above those that had been inoculated. For each treatment, three pools of 20 plants were harvested, flash frozen in liquid nitrogen and freeze-dried (Christ Alpha 1-2 LD, Martin Christ, Germany). Tissues were then powdered and stored at −80°C for further experiments. These replicates from untreated, wounded and virus-infected plants were analyzed for each time point.

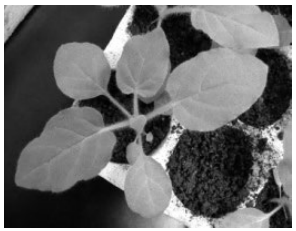
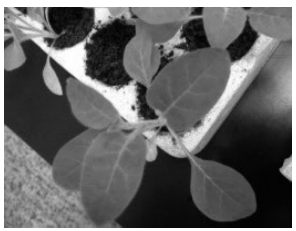
CMV presence was determined using an ELISA kit (Loewe Biochemica, Germany) according to the manufacturer's instructions. Samples were considered positive when the absorbance value was greater than the mean value plus three times the standard deviation of the wounded control samples. The ratio of absorbance values between tested samples and the negative control obtained at 405 nm was used as an estimation of the virus titre in the tested samples.

Powered plant tissues were extracted at a ratio of 1:80 (w/v) with a solvent containing 80% methanol in 1.2 mol dm^{-3} hydrochloride solution. Extraction was carried out in a water bath for 30 min at 80°C under continuous shaking at 150 rpm (SW22, Julabo, Germany). After extraction, the mixture was centrifuged at 15,000*g* for 15 min at 4°C. The supernatant was separated, centrifuged again at 15,000*g* for 15 min at 4°C, collected and used for high-performance liquid chromatography (HPLC) analysis.

For the quantification and confirmation of phenolic compounds, the following standards were used: catechin, chlorogenic acid, caffeic acid, epicatechin, coumaric acid, taxifolin, ferulic acid, myricetin, rosmarinic acid, daidzein, cinnamic acid, quercetin, naringenin, luteolin, genistein, kaempferol, isorhamnetin, pinocembrin, chrysin, galangin (all from Sigma-Aldrich, USA). Extracted components were separated on an Agilent 1100 Series HPLC (Agilent Technologies, USA) system equipped with a quaternary pump, autosampler, multi-wave UV/Vis detector, fraction collector, Zorbax RX-C18 analytical guard column (5 µm, 12.5 × 4.6 mm) and Zorbax RX-C18 column (3.5 µm, 75 × 4.6 mm). The elution profile consisted of solvent A [deionised water containing 0.05 % trifluoroacetic acid (TFA)], solvent B (20% methanol containing 0.05% TFA), solvent C (70% methanol containing 0.05% TFA) and solvent D (100% methanol). The solvent composition (A/B/C/D) changed according to the following gradient: 80/20/0/0 at 0 min, 0/100/0/0 at 4 min, 0/0/100/0 at 23 min, 0/0/0/100 at 27 min, 0/0/0/100 at 32 min, 80/20/0/0 at 33 min and finally 80/20/0/0 at 40 min. The flow rate was 1 cm^3 min^{-1} at 35 °C, the injection volume was 80 mm^3 and the absorbance was measured at 268, 280, 310, 350 and 374 nm. All samples were analysed in triplicate. Obtained chromatograms were analysed using the ChemStation program (Agilent Technologies, USA). For total phenolics (TP) assessment, the response was determined for each sample, compared to the calibration curve of gallic acid, and results were expressed as mg of gallic acid equivalents per gram of dry weight. Qualitative analysis of phenolic compounds in the extracts was performed by a comparison of retention times and absorbance spectra of the investigated phenolics with that of known standards and by co-chromatography of extracts with known standards. Concentrations of the confirmed phenolic compounds were determined based on the calibration curves (peak area vs. concentration) of individual compounds obtained for a wide concentration range. All obtained values are expressed as the mean values ± SD calculated on the basis of three independent evaluations ($n = 3$). Statistical significance of the differences between investigated groups was evaluated by the factorial ANOVA and their means were compared using Bonferroni correction. Differences were considered to be statistically significant at $p < 0.05$.

The functions of phenylpropanoid compounds in plant defence range from preformed or inducible barriers against infection to molecules involved in local and systemic signalling for induction of defence genes (Dixon *et al*., 2002). Several authors enlist various abiotic and biotic stress factors, which can induce the synthesis and accumulation of different phenolics (Dixon, 2001; Hagemeier *et al*., 2001; Stewart *et al*., 2001; Treutter, 2005). Thus, earlier studies have shown a strong induction of phenylalanine ammonia lyase (PAL) following viral infection of tobacco plants (Jaeck *et al*., 1992). In this study, a higher TP content in virus- infected plants compared to wounded plants was observed in *N. glutinosa* plants harvested 2 and 4 DAI, in *N. tabacum* plants harvested 7 and 14 DAI and in *N. megalosiphon* plants harvested 2 and 7 DAI (Table 1). Interestingly, in *N. glutinosa* plants harvested 2 DAI, the observed increase of TP was due to a significant increase of TP in the upper leaves. On the contrary, the significant increase observed in *N. tabacum* and *N. megalosiphon* was due to the significant increase in the lower leaves (Table

Table 2. Quantitative analysis of changes of total phenolics, caffeic acid, quercetin and kaempferol in all, lower and upper leaves of wounded (buffer treated) tobacco plants compared to untreated tobacco plants. Leaves were harvested 2, 4, 7, 14 days after inoculation (DAI).

	DAI	Total phenolics[a]			Caffeic acid[b]			Quercetin[c]			Kaempferol[c]		
Nicotiana glutinosa		lower#	upper#	all leaves#	lower#	upper	all leaves#	lower	upper#	all leaves#	lower	upper#	all leaves#
	2	↓ 24.7±1.2	↓ 23.8±0.2	↓ 24.2±0.7	↓ 2.6±0.2	↓ **2.2±0.1***	↓ **2.4±0.1***	↑ 8±0	↑ 52±9	↑ 30±4	↑ 41±1	↓ 43±1	↓ 42±1
	4	↓ **25.2±1.9***	↓ 28.1±3.7	↓ 26.6±2.7	↓ **2.7±0.3***	↑ 2.8±0.3	↓ 2.8±0.3	↓ 520±79	↓ **1223±169***	↓ **871±122***	↓ 46±3	↓ 63±6	↓ 55±4
	7	↓ **19.3±0.8**	↑ 21.0±0.8	↓ 20.1±0.8	↑ 2.8±0.1	↑ **2.1±0.5***	↑ **2.4±0.3***	↑ 571±508	↓ 1526±566	↓ 1049±427	↑ 50±7	↓ 59±20	↓ 55±14
	14	↓ **17.8±1.2***	↓ 17.8±0.9	↓ 17.8±1.0	↑ **2.6±0.5***	↓ **2.7±0.1***	↓ **2.6±0.3***	↓ 434±80	↓ **2231±145***	↓ **1332±91***	↓ 40±9	↓ **98±9***	↓ **69±8***
Nicotiana megalosiphon		lower#	upper#	all leaves#	lower#	upper#	all leaves#	lower#	upper#	all leaves#	lower#	upper#	all leaves#
	2	↓ **13.5±0.2***	↓ **17.2±0.5***	↓ **15.3±0.3***	↓ **0.9±0.0***	↓ **1.3±0.0***	↓ **1.1±0.0***	↓ **194±4***	↑ **395±7***	↑ 294±5	↓ **37±1***	↑ 55±3	↓ **46±2***
	4	↓ 17.6±0.3	↓ **20.4±0.7***	↓ 19.0±0.3	↓ **1.2±0.0***	↓ **1.5±0.1***	↓ **1.3±0.0***	↓ 461±20	↓ **248±9***	↓ **355±12***	↑ 56±1	↓ **53±2***	↓ **55±1***
	7	↓ 15.1±0.7	↓ 21.6±0.7	↓ 18.3±0.7	↓ **0.9±0.0***	↓ **1.8±0.0***	↓ **1.3±0.0***	↓ 226±9	↓ **562±16***	↓ **394±11***	↓ 42±3	↓ **69±2***	↓ **56±2***
	14	↓ **19.3±0.3***	↓ **20.7±0.4***	↓ **20.0±0.4***	↓ **1.5±0.0***	↓ **1.9±0.0***	↓ **1.7±0.0***	↓ **320±13***	↓ **722±9***	↓ **521±11***	↓ **46±1***	↓ **80±2***	↓ **63±1***
Nicotiana tabacum		lower	upper	all leaves	lower#	upper	all leaves#	lower#	upper#	all leaves#	lower#	upper	all leaves#
	2	↑ 28.0±0.0	↑ 24.8±1.5	↑ 26.4±0.7	↓ **2.9±0.1***	↑ 2.2±0.1	↓ 2.5±0.0	↓ **111±1***	↓ **148±14**	↓ **129±7***	↓ **52±1***	↓ **88±7***	↓ **70±33***
	4	↓ 25.8±0.3	↓ 22.3±2.5	↓ 24.0±1.3	↓ **3.4±0.0***	↑ 2.3±0.2	↓ 2.9±0.1	↓ **51±2***	↓ **38±3***	↓ **44±2***	↓ **52±1***	↓ **65±3***	↓ **59±2***
	7	↓ 20.8±0.1	↑ 22.0±4.4	↑ 21.4±2.1	↓ **3.2±0.0***	↑ 2.8±0.1	↓ **3.0±0.0***	↑ **245±2***	↑ **395±12***	↓ **320±5***	↓ **68±1***	↑ **105±4***	↓ **87±2***
	14	↓ 25.2±1.6	↓ 21.9±0.3	↓ 23.6±0.7	↓ **4.0±0.0***	↑ 4.2±0.0	↓ 4.1±0.0	↑ **268±12***	↑ **829±19***	↑ 548±6	↓ **62±3***	↑ 178±1	↓ **120±2***

The asterisk (*) denotes significant change of total phenolics, caffeic acid, quercetin or kaempferol content in wounded plants compared to their respective control ($n = 3$; $p < 0.05$; factorial ANOVA, post hoc Bonferroni correction). For emphasis this values are presented in boldface. The number sign (#) denotes significant change of total phenolics, caffeic acid, quercetin or kaempferol content in lower, upper or all leaves of wounded plants compared their respective control group ($n = 3$; $p < 0.05$; factorial ANOVA, post hoc Bonferroni correction).

[a] total phenolics are expressed in mg gallic acid equivalents per gram dry weight.

[b] caffeic acid is expressed in mg/ gram dry weight tissue.

[c] quercetin and kaempferol are expressed in µg/ gram dry weight tissue.

↑ or ↓ arrows indicate higher or lower concentration of total phenolics, caffeic acid, quercetin or kaempferol in wounded plants compared to their respective control.

1, values in boldface). These results could indicate a different defence response in the investigated species of the same genus. The observed higher TP content could result from increased expression of at least one gene from two *PAL* gene families (Reichert *et al*., 2009) or the higher activity of the PAL enzyme which is dependent on the intensity of stress (Dixon and Paiva, 1995). On the other hand, the lower TP content in wounded plants compared to the untreated ones was observed in most investigated *Nicotiana* plants. Furthermore, a significant decrease of TP content in wounded *N. glutinosa* and *N. megalosiphon* was observed in both lower and upper leaves (Table 2). In wounded *N. tabacum*, no significant decrease of TP content was detected. In addition, almost all wounded plants had lower contents of caffeic acid, quercetin and kaempferol compared to the untreated ones (Table 2), indicating that in these plants a downregulation of the phenylpropanoid biosynthetic pathway had taken place. It is known that plants produce ROS after wounding (León *et al*., 2001) and that phenolic acids and flavonoids play a role in the detoxification of ROS (Apel and Hirt, 2004; Rice-Evans *et al*., 1996). Thus, the observed decrease in caffeic acid, quercetin and kaempferol concentrations suggests their involvement in the elimination of ROS produced due to mechanical wounding.

Meanwhile, a significantly lower quercetin concentration in the upper leaves of infected *N. megalosiphon* plants compared to the wounded ones suggests an unknown role of quercetin and/or it could indicate downregulation of the expression of chalcone synthase (*CHS*) gene. It was previously shown that viruses can modulate the defence response through downregulation of gene expression for flavonoid synthesis (Dunoyer *et al*., 2004). In *Arabidopsis*, the expression of the *CHS* gene remains downregulated when the CMV 2b protein is present in high concentration (Johansen and Wilson, 2008). Upregulation of the 2b protein is usually needed for the highest titre of CMV in systemic leaves (Soards *et al*., 2002) and for induction and development of strong infection symptoms (Lewsey *et al*., 2010). The highest CMV titre (detemined by ELISA) was detected in systemic leaves of *N. megalosiphon* harvested 7 and 14 DAI (Fig. 1). It was interesting to note that the strongest symptoms observed in infected *N. megalosiphon* were accompanied with strong downregulation of quercetin both in the lower and upper leaves (Table 1). This may indicate that for an efficient systemic spread of CMVsat (Fig. 1B) and symptom development in *N. megalosiphon* plants, strong downregulation of quercetin in upper leaves is needed, since this was not observed in *N. glutinosa* or *N. tabacum* plants (Table 1).

On the other hand, a strong increase in quercetin concentration in infected *N. glutinosa* harvested 2 and 7 DAI and an increase in kaempferol and quercetin concentration with time in infected *N. tabacum* were observed compared with the respective controls (Table 1, figures in boldface). The detection of significant upregulation of

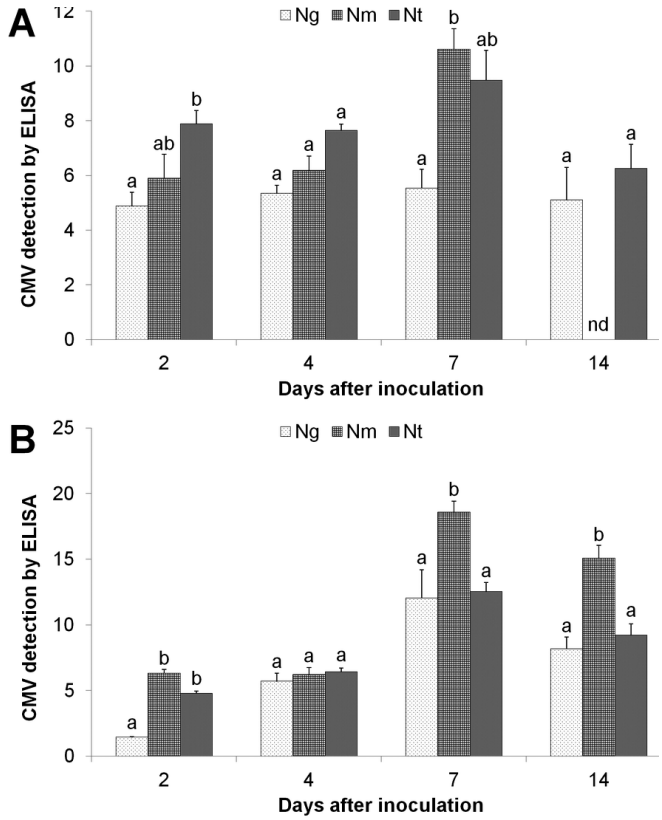


Fig. 1. Time course of *Cucumber mosaic virus* containing satellite RNA (CMVsat) infection and spread in lower (A) and upper (B) leaves of investigated *Nicotiana glutinosa* (Ng), *Nicotiana megalosiphon* (Nm) and *Nicotiana tabacum* (Nt) plants. Leaves were harvested separately at four different time points: 2, 4, 7, 14 days after inoculation. Lower leaves comprised inoculated and other leaves below them and upper leaves comprised all other leaves above inoculated ones. Virus titres were determined using an ELISA-based method measured at 405 nm. The ratio of absorbance values between tested samples and negative control was used to estimate the virus titre. Values are expressed as the mean value + SD calculated based on three independent evaluations. The same letter within a time point denotes no significant difference in virus titre between investigated *Nicotiana* species ($n=3$; $p<0.05$; factorial ANOVA, post hoc Bonferroni correction). nd – not done.

either quercetin or kaempferol in virus- infected plants compared to wounded plants suggests that these flavonoids have an additional defence response role characteristic for plant-virus interaction, in addition to ROS scavenging as a general plant protection response from mechanical wounding (Apel and Hirt, 2004). Diversion of the phenylpropanoid biosynthetic pathway can be induced by differentially activated 4-coumarate:coenzyme A ligase, whose activation depends on the developmental stage of the plant or when external stimuli such as wounding or pathogen infection occur (Lee *et al*., 1995; Lee and Douglas, 1996; Ehlting *et al*., 1999). Rerouting of the phenylpropanoid biosynthetic pathway to increase flavonoid synthesis could be part of the defence response that *N. glutinosa* and *N. tabacum* activate upon infection

with CMVsat, which facilitates the amelioration of infection symptoms. Graham (1998) has shown that the conjugation and later release of kaempferol aglycon could have a direct and regulatory role in plant defence response and it was also reported that flavonoids may exert a modulatory action in cells through actions in the protein kinase signalling pathways (Williams *et al*., 2004) and that they act as inhibitors of protein kinases (Lolli *et al*., 2012).

In conclusion, it was previously shown that CMV can induce different symptoms in ecotypes of the same species and that CMVsat can engage two distinct defence-signalling pathways in *Arabidopsis* (Takahashi *et al*., 1994; Likić *et al*., 2014). Both examples clearly demonstrate the complexity of the plant defence responses. The results presented here demonstrate that significant decreases of caffeic acid, quercetin and kaempferol were induced in all investigated *Nicotiana* species as a part of the defence response to wounding. Though the results obtained from virus-infected plants were not as clear-cut as those from the wounded ones, they do indicate that species of the same genus activate different defence responses to CMVsat infection, and that quercetin and kaempferol could play an important role in the plant-virus interaction. Further elaboration of this subject may contribute to better understanding of the role of flavonoids in plant-pathogen interaction.

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