Estimation of flavonoid and centelloside accumulation in leaves of *Centella asiatica* L. Urban by multiparametric fluorescence measurements

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**A B S T R A C T**

Recently, we have shown the relevance of nitrogen (N), phosphorus (P) and potassium (K) supply levels for resource partitioning between primary and secondary metabolism, and the concentration of centellosides in *Centella asiatica* L. Urban leaves. So far, no efforts have been made to investigate the effects of mineral supply on flavonoid concentrations in this species. Here, we aimed to examine the accumulation of centellosides in *C. asiatica* leaves in vivo by means of non-destructive fluorescence measurements using products of the secondary metabolism, particularly the epidermal flavonols and anthocyanins, as reference. For this purpose we conducted three discrete experiments in a greenhouse having N, P and K levels as experimental factors. Our results reveal that flavonoid and anthocyanin accumulation is affected by N, P and K fertilization in the same way as the centelloside accumulation. More precisely, limitations in plant growth were accompanied by higher flavonoid and anthocyanin concentrations, confirming the proposed trade-off between the plant’s primary and secondary metabolism. The fluorescence-based flavonol (FLAV) and anthocyanin (ANTH,RG) indices correlated fairly with flavonoid and especially with anthocyanin concentrations. Moreover, centellosides were positively correlated with the FLAV and ANTH,RG indices, and with the BFRR,UV index, which is considered as universal ‘stress-indicator’. Thus, here we indicate for the first time, that the fluorescence-based indices FLAV, ANTH,RG as well as BFRR,UV enable the monitoring of flavonoid and centelloside concentrations in leaves of *C. asiatica*. Our results support and highlight the significant potential for further development and application of fluorescence-based sensors in ecophysiological research as well as in the production of medicinal plants.

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1. **Introduction**

*Centella asiatica* L. Urban is a medicinal herb with great economic value. Its bioactive triterpene saponins, the centellosides, are found in a number of commercial drugs and cosmetics. Besides its therapeutic usage, *C. asiatica* is consumed as a vegetable in many cultures because it provides important nutritional values such as fiber, protein, calcium and beta-carotin (Sritongkul et al., 2009). Moreover, the presence of flavonoids, e.g., quercetin and kaempferol derivatives (Zeng and Qin, 2007), which are well known for their potential health benefits in terms of disease prevention (Soto-Vaca et al., 2012), has been reported.

Both, saponins and flavonoids, are carbon-based secondary metabolites. Saponins are biosynthesized via the isoprenoid pathway starting with isopentenyl pyrophosphate and dimethylallyl pyrophosphate. The cyclization of 2,3-oxidosqualene leads to the triterpenoid skeletons, such as α- and β-amyrin. Subsequent modifications, i.e., oxidation, hydroxylation and other substitutions generate the Centella sapogenins, which are finally converted into saponins by glycosylation processes (James and Dubery, 2009; Augustin et al., 2011). Differently, the flavonoids are biosynthesized via the phenylpropanoid pathway. Initially, the amino acid phenylalanine is deaminated and transformed into 4-coumaroyl-CoA, 4-Coumaroyl-CoA and three molecules of malonyl-CoA are condensed to chalcone, which is isomerized to the flavanone...
naringenin. Naringenin is the substrate for further enzymatic reactions leading to a variety of flavonoids, which are classified into several subgroups, including flavonoids and anthocyanins (Dixon and Paiva, 1995; Winkler-Shirley, 1999, 2001).

A number of studies report on the enhanced accumulation of saponins as well as flavonoids in plants in response to biotic and abiotic stresses, such as nutrient deficiency (Dixon and Paiva, 1995; Chalker-Scott, 1999; Treutter, 2005; Szakiel et al., 2011a,b and references therein). Since nutrient deficiency affects growth more than photosynthesis, both the carbon/nutrient balance (CNB) and the growth differentiation balance (GDB) hypotheses assume that the enhanced synthesis of carbon-based secondary metabolites under nutritional stress is attributed to the accumulation of carbohydrates in excess of growth requirements, which can be invested in the formation of secondary defensive compounds (Watson, 1963; Epstein, 1972; Smith, 1973; McKeve, 1979; Bryant et al., 1983; Herms and Mattson, 1992). In contrast, the protein competition model (PCM) suggests that the synthesis of phenolics, including flavonoids, is regulated by the protein–pheno lactum competition for the limiting precursor phenylalanine, rather than by the availability of carbohydrates (Jones and Hartley, 1999). Apart from the specific assumptions, the models propose a trade-off between growth and the synthesis of secondary metabolites (Coley et al., 1985). Accordingly, we have recently demonstrated that the availability of nitrogen (N), phosphorus (P) and potassium (K) strongly influences the resource partitioning between primary and secondary metabolism, and the concentration of centellosides in leaves of C. asiatica (Müller et al., 2013). However, studies on the flavonoid accumulation in C. asiatica plants, particularly in response to the availability of minerals in the growing media, are still lacking.

Traditionally, the relevance of environmental factors for the synthesis of secondary metabolites is investigated by using wet chemical analyses. As a rule, these analyses are costly and very laborious. Thus, in the last years efforts were made to identify non-destructive techniques for the in vivo monitoring of secondary metabolite accumulation in plants. Using the screening technique (Bilger et al., 1997), flavonol and anthocyanin content in fruits and leaves can be estimated by chlorophyll fluorescence excitation (Cerovic et al., 2002; Agati et al., 2005). In contrast, the detection of saponins by fluorescence-based techniques was only mentioned on rare occasions (Crombie et al., 1986; Papadopoulou et al., 1999).

In the present study we aimed to examine the accumulation of centellosides in C. asiatica leaves in vivo by means of fluorescence-based non-destructive measurements using products of the secondary metabolism as reference. For this purpose, three discrete experiments with N, P and K levels as experimental factors were conducted consecutively in a greenhouse. Flavonoid and anthocyanin concentrations in leaves were determined after 8 weeks of treatment application. Non-destructive fluorescence measurements were performed weekly. At the end of the study, the suitability of the fluorescence-based indices for the estimation of flavonoid and centelloside concentrations was evaluated by correlation analyses. We hypothesized that flavonoid accumulation is affected by N, P and K fertilization in the same way as centelloside accumulation, and that centelloside concentrations in leaves of C. asiatica can therefore be estimated in vivo by means of non-destructive recordings of the chlorophyll fluorescence.

2. Materials and methods

2.1. Experimental setup

The experimental setup was organized as described in detail by Müller et al. (2013). Briefly, the experiments on the effects of different levels of either N, P or K supply on the flavonoid and centelloside accumulation in leaves of C. asiatica L. Urban plants were conducted consecutively in a greenhouse. Each experiment consisted of five treatments, which were applied to 40 plants, respectively, for a period of 8 weeks. The control treatments (N100, P100 and K100) corresponded to the standard Hoagland nutrient solution. The N, P or K amounts in the nutrient solutions of the treatments N0, N100, N60 and N150 (and likewise for P and K) were adjusted in terms of 0, 30, 60 and 150% of the amounts applied to the control treatments.

2.2. Non-destructive, fluorescence-based determinations

Fluorescence recordings were conducted weekly under standardized conditions using a multiparametric portable optical sensor (Multiplex Research, FORCE-A, Orsay, France). The petals of two fully expanded leaves of ten plants of each treatment group were labeled at the onset of the experiments enabling the measurements on the same leaves during the whole experimental period. The accumulation of epidermal flavonoids was evaluated by the decadic logarithm of the red to ultraviolet (UV) excitation ratio of the far-red chlorophyll fluorescence (FLAV index), which is proportional to the flavonol content of the leaves (Cerovic et al., 2002). To track the accumulation of anthocyanins we selected the decadic logarithm of the red to green excitation ratio of far-red chlorophyll fluorescence (ANTH_RG index), which is proportional to the anthocyanin content in the tissue (Agati et al., 2005). Furthermore, we determined the ratio between UV-excited blue and the far-red fluorescence (BFRR_UV index), which is a robust indicator of various stress situations, including nutrient deficiency in plants (Chappelle et al., 1984).

To elucidate the relevance of the centellosides for the fluorescence measured in vivo, the fluorescence of asiaticoside and asiatic acid reference compounds as well as of methanolic leaf extracts was determined with a compact fiber-optic fluorescence spectrometer with UV excitation (ION GmbH, Berlin, Germany), as described elsewhere (Büring et al., 2011). The detailed results of the measurements are presented in Fig. A.1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2013.05.001

2.3. Determination of flavonoid and anthocyanin concentration

The flavonoid and anthocyanin concentrations in leaves were determined by wet chemical analysis after 8 weeks of treatment application (WTA). The leaves of eight plants of each treatment group were lyophilized and ground as described elsewhere (Müller et al., 2013). Extraction procedure and analysis were performed according to Solovchenko et al. (2001) and Solovchenko and Schmitz-Eiberger (2003). Briefly, 3 mL of a chloroform/methanol (2/1, v/v) solution were added to 10 mg of the leaf powder. Each sample was mixed thoroughly and passed through a paper filter. The filtrate was combined with 0.6 mL of deionized water and centrifuged at 3000 rpm (4 °C) for 10 min until the chloroform and the water–methanol phases had separated. The filter was allowed to dry and then washed with 4 mL of acidic methanol (1 mL HCl (37%) in 100 mL methanol p.a.). For the calculation of the flavonoid concentrations, the absorbencies of the water–methanol phase and the acidic methanol extract were measured at 750 and 360 nm using a UV/VIS spectrophotometer (Lambda 35, Perkin–Elmer, USA). To quantify the anthocyanin concentrations, one drop of HCl (37%) was added to each, the water–methanol phase and the acidic methanol extract, and the absorbencies were assessed at 750 and 530 nm. Subsequently, the flavonoid and anthocyanin concentrations were calculated and expressed on leaf area basis.
2.4. Extraction and determination of saponin and sapogenin concentrations

Saponin and sapogenin concentrations in the leaves (n = 10 plants each treatment group) were determined after 8 WTA according to Müller et al. (2013). 50 mg of leaf powder were extracted under sonication with methanol/water (9/1, v/v; VWR, West Chester, USA). The leaf concentrations of asiaticoside, madecassoside, asiatic acid and madecassic acid were determined by high performance liquid chromatography (HPLC). The HPLC device (Agilent, Series 1100, Waldbronn, Germany) consisted of a binary pump, a degasser, an autosampler, a thermostated column oven and a multiwavelength-UV-detector. A Nucleodur C18 reversed phase column (250 mm × 4 mm, 5 μm; Macherey & Nagel, Düren, Germany) was used as stationary phase and a gradient program, composed of water and acetonitrile, both acidified with 0.01% formic acid (VWR, West Chester, USA), was used as mobile phase. Separation was performed at a temperature of 30 °C and at a flow rate of 1 mL min⁻¹. Detection of saponins and sapogenins occurred at 206 nm. Quantification of asiaticoside and asiatic acid and, according to Rafamantanana et al. (2009), also of madecassoside and madecassic acid, was done using external calibration curves of asiaticoside and asiatic acid reference compounds (Sigma–Aldrich, St. Louis, MO, USA). The isomers madecassoside and asiaticoside B as well as madecassic acid and terminolic acid were considered as one compound, respectively. Total centelloleosides were calculated summing up the determined leaf concentrations of asiaticoside, madecassoside, asiatic acid and madecassic acid.

2.5. Statistics

Statistical analyses were performed with PASW statistics software (Version 20.0, SPSS Inc., Chicago, USA). Means were compared by analysis of variance (one-way ANOVA). Significant differences among the treatments groups, as well as among the measuring dates, were determined according to Duncan’s multiple range test. Correlations between selected parameters were tested with the Pearson correlation coefficient. Graphs were drawn with Sigma Plot 11.0 (Systat Software Inc., Richmond, CA, USA).

3. Results

3.1. Flavonoid and anthocyanin accumulation

At 8 WTA significantly higher flavonoid and anthocyanin concentrations were determined in the leaves of those plants, which were provided with a fertigation solution lacking either N, P or K (Fig. 1A–C). One exception was the treatment N150, which induced flavonoid concentrations as high as treatment N0 (Fig. 1A). In the experiment on N supply, the lowest flavonoid and anthocyanin concentrations were observed in the treatment N30, followed by N60. In response to the P supply, the lowest flavonoid concentrations were determined in P60, P100 and P150 (Fig. 1B). The anthocyanin concentrations were generally low in those treatments providing any P to the plants (P30, P60, P100 and P150). Concerning the K supply, the lowest flavonoid concentrations by trend were induced by the treatment K30 (Fig. 1C). Except for K0, and analogous to the experiment on N supply, the flavonoid concentrations raised with higher K concentrations in the fertigation solution (Fig. 1A and C). The anthocyanin concentrations did not differ significantly among the treatments K30, K60, K100 and K150.

3.2. Fluorescence-based flavonol (FLAV) and anthocyanin (ANTH_RG) indices

In general, we noticed a strong impact of the N supply on the FLAV and ANTH_RG indices (Fig. 2A and D). Plants without any N supply (N0) revealed an immediate, strong increase in the FLAV values, which remained significant until 3 WTA (p ≤ 0.05). In contrast, the other treatments induced only a slight rise, e.g., observed until 3 WTA in case of N30, until 2 WTA in case of N60, and during the first experimental week in case of N100 and N150 (p ≤ 0.05). Thereafter, the values remained at a similar level (Fig. 2A). Differences among the N supply treatments were detected especially in the time-frame of 3–5 WTA. At the end of the experiment, FLAV was about 3 times higher at N0 in comparison to the other treatment groups. Similarly, the ANTH_RG index was significantly higher in the N0 treatment (1–8 WTA, p ≤ 0.05), followed by the N30 treatment (2–8 WTA, p = 0.05) (Fig. 2D). At specific dates (2–5 WTA and 7 WTA, p = 0.05), the treatment N60 was slightly superior to the treatments N100 and N150; the latter ones exhibited similar values over the whole measuring period.

As shown in Fig. 2B and E, also the P supply strongly influenced the FLAV and ANTH_RG indices. In the absence of P supply we observed a strong increase in both, FLAV (1–6 WTA, p ≤ 0.05) and ANTH_RG (2–3 WTA and 5–7 WTA, p ≤ 0.05), fluorescence-based

![Fig. 1. Effect of N (A), P (B) and K (C) supply levels on flavonoid and anthocyanin concentrations in leaves of C. asiatica. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount, 0% (P0), 30% (P30), 60% (P60), 100% (P100), or 150% (P150) of the P amount and 0% (K0), 30% (K30), 60% (K60), 100% (K100), or 150% (K150) of the K amount in the standard Hoagland solution. Evaluation was done at 8 weeks of treatment application. Mean ± standard error (n = 8). Values followed by the same letter do not differ statistically according to Duncan’s multiple range test (p ≤ 0.05).](image-url)
indices. The FLAV index obtained from the treatment P30 by trend increased from 4 to 8 WTA, while it slightly decreased in the treatments P100 and P150 from 5 to 8 WTA (Fig. 2B). Finally, the P30 treatment group had higher values as compared to the other P supply levels. In case of ANTH_RG, no significant differences were observed among the treatments providing P to the plants. Analogous to the results described for the N and P supply, the absence of K in the fertigation induced a rise in the FLAV index during the whole experimental period (Fig. 2C). At 8 WTA, the highest FLAV index was detected in the treatment K0, followed by the treatment K150. Similarly, values of ANTH_RG were higher in the absence of K supply (3–8 WTA, p ≤ 0.05) (Fig. 2F). The remaining treatments did not differ significantly until 6 WTA. At the end of the experiment, the highest ANTH_RG index was obtained from the treatment K0, followed by K150.

3.3. Correlation analysis

In the scope of each experiment we conducted correlation analyses between the fluorescence-based indices recorded from single leaves, and the concentrations of flavonoids and anthocyanins determined from a composite sample (Table 1). As first relevant information, the strong correlation between the FLAV index and the concentration of anthocyanins became obvious in all three experiments. With one exception (experiment on N supply), the correlations between the FLAV index and the flavonoid concentration were strong (K supply) or very strong (P supply). Moreover, the ANTH_RG index correlated fairly with the anthocyanin concentration, especially in the experiments evaluating the supply of P and K. The BFRR_UV index, a robust indicator of plant stress, showed strong to very strong correlations with the anthocyanin (in the experiments on N, P and K supply, respectively) and flavonoid concentrations (P supply).

In each experiment we conducted correlation analyses between the centellosides and the flavonoids, anthocyanins as well as the fluorescence indices, obtaining extensive and informative correlation matrices (Tables 2–4). As a rule, we demonstrate moderate to very strong positive correlation coefficients between the individual compounds of the centellosides and the flavonoids and anthocyanins. In addition, reliable correlation coefficients were observed between the centellosides and the fluorescence-based indices. In general, we noticed different reaction patterns as related to the supply of minerals. Roughly, in the experiment evaluating the N supply the strongest correlation coefficients were detected for the saponins, asiaticoside and madecassoside (Table 2), while the K supply induced strongest correlation to the sapogenins, asiatic acid.
Table 1
Pearson correlation coefficients between flavonoid and anthocyanin concentrations and the fluorescence-based indices FLAV, ANTH_RG and BFRR_UV. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount, 0% (P0), 30% (P30), 60% (P60), 100% (P100), or 150% (P150) of the P amount and 0% (K0), 30% (K30), 60% (K60), 100% (K100), or 150% (K150) of the K amount in the standard Hoagland solution. Evaluation was done at 8 weeks of treatment application. Number of samples: fluorescence indices, n = 20 plants each treatment group; flavonoid and anthocyanin concentrations, n = 8 plants each treatment group.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Fluorescence index</th>
<th>Flavonoids (nmol cm⁻²)</th>
<th>Anthocyanins (nmol cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N supply</td>
<td>FLAV</td>
<td>0.31</td>
<td>0.73**</td>
</tr>
<tr>
<td></td>
<td>ANTH_RG</td>
<td>0.02</td>
<td>0.44**</td>
</tr>
<tr>
<td></td>
<td>BFRR_UV</td>
<td>0.39</td>
<td>0.68**</td>
</tr>
<tr>
<td>P supply</td>
<td>FLAV</td>
<td>0.89**</td>
<td>0.89**</td>
</tr>
<tr>
<td></td>
<td>ANTH_RG</td>
<td>0.83**</td>
<td>0.93**</td>
</tr>
<tr>
<td></td>
<td>BFRR_UV</td>
<td>0.84</td>
<td>0.95**</td>
</tr>
<tr>
<td>K supply</td>
<td>FLAV</td>
<td>0.61**</td>
<td>0.75**</td>
</tr>
<tr>
<td></td>
<td>ANTH_RG</td>
<td>0.58**</td>
<td>0.74**</td>
</tr>
<tr>
<td></td>
<td>BFRR_UV</td>
<td>0.53</td>
<td>0.84**</td>
</tr>
</tbody>
</table>

* Level of significance: p ≤ 0.05.
** Level of significance: p ≤ 0.01.

Table 2
Pearson correlation coefficients between flavonoid and anthocyanin concentrations, fluorescence-based indices (FLAV, ANTH_RG and BFRR_UV) and centelloside concentrations (asiaticoside, madecassoside, asiatic acid, madecassic acid and total centellosides) in leaves of C. asiatica. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount in the standard Hoagland solution and evaluated at 8 weeks of treatment application. Number of samples: fluorescence indices, n = 20 plants each treatment group; flavonoid and anthocyanin concentrations, n = 8 plants each treatment group; centellosides, n = 10 plants each treatment group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Centellosides (mg g⁻¹ DM)</th>
<th>Flavonoids (nmol cm⁻²)</th>
<th>Anthocyanins (nmol cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asiaticoside</td>
<td>Madecassoside</td>
<td>Asiatic acid</td>
</tr>
<tr>
<td>Flavonoids (nmol cm⁻²)</td>
<td>0.28</td>
<td>0.30</td>
<td>0.57**</td>
</tr>
<tr>
<td>Anthocyanins (nmol cm⁻²)</td>
<td>0.74**</td>
<td>0.77**</td>
<td>0.51</td>
</tr>
<tr>
<td>FLAV</td>
<td>0.95**</td>
<td>0.95**</td>
<td>0.22</td>
</tr>
<tr>
<td>ANTH_RG</td>
<td>0.88**</td>
<td>0.87**</td>
<td>−0.01</td>
</tr>
<tr>
<td>BFRR_UV</td>
<td>0.93**</td>
<td>0.94**</td>
<td>0.17</td>
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* Level of significance: p ≤ 0.05.
** Level of significance: p ≤ 0.01.

Table 3
Pearson correlation coefficients between flavonoid and anthocyanin concentrations, fluorescence-based indices (FLAV, ANTH_RG and BFRR_UV) and centelloside concentrations (asiaticoside, madecassoside, asiatic acid, madecassic acid and total centellosides) in leaves of C. asiatica. Plants were fertigated with treatment solutions containing 0% (P0), 30% (P30), 60% (P60), 100% (P100), or 150% (P150) of the P amount in the standard Hoagland solution and evaluated at 8 weeks of treatment application. Number of samples: fluorescence indices, n = 20 plants each treatment group; flavonoid and anthocyanin concentrations, n = 8 plants each treatment group; centellosides, n = 10 plants each treatment group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Centellosides (mg g⁻¹ DM)</th>
<th>Flavonoids (nmol cm⁻²)</th>
<th>Anthocyanins (nmol cm⁻²)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Asiaticoside</td>
<td>Madecassoside</td>
<td>Asiatic acid</td>
</tr>
<tr>
<td>Flavonoids (nmol cm⁻²)</td>
<td>0.89**</td>
<td>0.89**</td>
<td>0.57**</td>
</tr>
<tr>
<td>Anthocyanins (nmol cm⁻²)</td>
<td>0.81**</td>
<td>0.84**</td>
<td>0.51**</td>
</tr>
<tr>
<td>FLAV</td>
<td>0.90**</td>
<td>0.92**</td>
<td>0.75**</td>
</tr>
<tr>
<td>ANTH_RG</td>
<td>0.85**</td>
<td>0.86**</td>
<td>0.68**</td>
</tr>
<tr>
<td>BFRR_UV</td>
<td>0.84**</td>
<td>0.86</td>
<td>0.72**</td>
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* Level of significance: p ≤ 0.05.
** Level of significance: p ≤ 0.01.

Table 4
Pearson correlation coefficients between flavonoid and anthocyanin concentrations, fluorescence-based indices (FLAV, ANTH_RG and BFRR_UV) and centelloside concentrations (asiaticoside, madecassoside, asiatic acid, madecassic acid and total centellosides) in leaves of C. asiatica. Plants were fertigated with treatment solutions containing 0% (K0), 30% (K30), 60% (K60), 100% (K100), or 150% (K150) of the K amount in the standard Hoagland solution and evaluated at 8 weeks of treatment application. Number of samples: fluorescence indices, n = 20 plants each treatment group; flavonoid and anthocyanin concentrations, n = 8 plants each treatment group; centellosides, n = 10 plants each treatment group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Centellosides (mg g⁻¹ DM)</th>
<th>Flavonoids (nmol cm⁻²)</th>
<th>Anthocyanins (nmol cm⁻²)</th>
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<tr>
<td></td>
<td>Asiaticoside</td>
<td>Madecassoside</td>
<td>Asiatic acid</td>
</tr>
<tr>
<td>Flavonoids (nmol cm⁻²)</td>
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<td>0.65**</td>
<td>0.78**</td>
</tr>
<tr>
<td>Anthocyanins (nmol cm⁻²)</td>
<td>0.46**</td>
<td>0.51**</td>
<td>0.87**</td>
</tr>
<tr>
<td>FLAV</td>
<td>0.51**</td>
<td>0.55**</td>
<td>0.79**</td>
</tr>
<tr>
<td>ANTH_RG</td>
<td>0.45**</td>
<td>0.47**</td>
<td>0.76**</td>
</tr>
<tr>
<td>BFRR_UV</td>
<td>0.51**</td>
<td>0.57**</td>
<td>0.81**</td>
</tr>
</tbody>
</table>

* Level of significance: p ≤ 0.05.
** Level of significance: p ≤ 0.01.
and madecassid acid (Table 4). In the experiment evaluating the P supply, stronger correlations were observed to the saponins, but also the correlations to the sapogenins were significant (Table 3).

As shown, the FLAV, ANTH_RG and the BFRR_UV indices strongly correlated with the total centellolose concentration. The lowest correlation coefficients were determined in the experiment employing different levels of K supply. In the remaining experiments, correlation values were higher than 0.84, reaching r > 0.95 for the FLAV index (Tables 2 and 3).

4. Discussion

The present study aimed to investigate the accumulation of centellolides in *C. asiatica* leaves in vivo by non-destructive fluorescence measurements using products of the secondary metabolism as reference. For this purpose we established three sequential experiments with different N, P, and K fertilization levels. Thereby, we hypothesized that the accumulation of centellolides is affected by N, P and K fertilization in the same extent as the accumulation of flavonoids and anthocyanins, and that centellolose concentrations in leaves of *C. asiatica* can therefore be assessed in vivo by means of non-destructive fluorescence-based measurements of epidermal flavonols and anthocyanins.

4.1. Flavonoid and anthocyanin accumulation in response to N, P or K supply

In general, flavonoid and anthocyanin concentrations were strongly influenced by N, P and K fertilization, although the effects were more pronounced for the flavonoids. In accordance with the centellolose concentrations (Müller et al., 2013), the highest flavonoid and anthocyanin concentrations were observed in those plants which did not receive either N, P or K fertilization (Fig. 1A–C). These findings agree with a number of studies reporting on the increase of flavonoid and anthocyanin concentrations in various plant species in response to nutrient limitations (Lawanson et al., 1972; Krause and Reznik, 1976; Bongue-Bartelsman and Phillips, 1995; Stewart et al., 2001; Misson et al., 2005; Lea et al., 2007; Müller et al., 2007). Recently, it was proposed that low N concentrations in leaves, as observed in the plants of the treatments N0, P0 and K0 (Müller et al., 2013), may act as a signal triggering the synthesis of phenolic compounds (Rubio-Wilhelmi et al., 2012). In our study the high flavonoid and anthocyanin concentrations were accompanied by strong limitations in growth (Müller et al., 2013) and exemplify the proposed trade-off between primary metabolism and the synthesis of carbon-based defensive compounds (Coley et al., 1985).

In response to the N supply, the lowest flavonoid and anthocyanin concentrations were determined in the leaves of the treatment N30, followed by N60 (Fig. 1A). By considering herb and leaf yield (Müller et al., 2013) and its relation to the synthesis of secondary compounds, we expected the lowest flavonoid and anthocyanin concentrations in the treatment groups N100 and N60. One probable reason for this mismatching is the strong increase in the plant’s growth rate in N30 during the last 2 weeks of the experiment (Table A.1). The enhanced resource requirements for growth processes might have resulted in a reduced availability of carbon to secondary metabolism and consequently in a lower flavonoid and anthocyanin accumulation. As influenced by the P supply, the lowest flavonoid and anthocyanin concentrations were determined in the leaves of the treatments P60, P100 and P150 (Fig. 1B), which concomitantly presented the highest herb and leaf yields (Müller et al., 2013). Accordingly, influenced by the K supply, the lowest flavonoid concentrations (Fig. 1C) and the highest herb and leaf yields (Müller et al., 2013), were induced by the treatment K30. Thus, as predicted by the CNB, GDB and PCM (Watson, 1963; Epstein, 1972; Smith, 1973; McKey, 1979; Bryant et al., 1983; Hermns and Mattson, 1992; Jones and Hartley, 1999) and illustrated for the centellolides (Müller et al., 2013), the flavonoid and anthocyanin concentrations were inversely related to plant growth, irrespective of N, P and K supply.

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4.2. Temporal development of the FLAV and ANTH_RG indices

Analogous to the flavonoid and anthocyanin concentrations (Fig. 1), the highest FLAV and ANTH_RG indices were induced by the treatments N0, P0 and K0 (Fig. 2A–F). Considering the experiment on N supply, differences in the FLAV index among the treatments were particularly observed in the time-frame of 3–5 WTA. Complementary, the ANTH_RG index indicated a more clear separation between the treatment groups starting at 2 WTA (Fig. 2D). The high sensitivity of anthocyanin synthesis to N supply has also been reported in other studies (Lea et al., 2007). Finally, the lowest ANTH_RG index was observed in the treatment groups N60, N100 and N150, while the lowest FLAV index was recorded from the treatments N60 and N100. The correlation analysis revealed at best weak positive correlations between the FLAV and ANTH_RG indices and the flavonoid and anthocyanin concentrations (Table 1). This in turn is possibly related to the enhanced growth rate, particularly in the treatment N30, during the last 2 weeks of the experiment (Table A.1). More precisely, the FLAV and ANTH_RG indices were recorded from adult leaves, while the extracts for the destructive determination of flavonoid and anthocyanin concentrations were obtained from the leaves of the entire plants. A number of studies report on an increase in flavonoid accumulation with leaf age (Vogt and Gült, 1994; Ounis et al., 2001; Laitinen et al., 2002). Thus, our results suggest that the flavonoid concentrations of the older leaves were less influenced by the previously mentioned reduced availability of carbon to secondary metabolism, resulting in the higher fluorescence-based indices. Assuming that especially young leaves were affected by the trade-off, we propose that the higher proportion of young leaves as compared to old leaves lowered the flavonoid concentrations in the leaf extracts, resulting in the discrepancies between the findings obtained by non-destructive and destructive determinations.

As related to the P supply, the lowest FLAV, and by trend also the lowest ANTH_RG index, were recorded from the treatments P60, P100 and P150. This was in accordance with the destructively assessed flavonoid and anthocyanin concentrations (Fig. 1B), leading to the strong positive correlations between non-destructive and destructive measurements (Table 1).

In dependence on K supply, the highest FLAV and ANTH_RG values were observed in the treatment K0, followed by K150 (Fig. 2F). The correlation analysis revealed moderate to strong positive associations between the parameters FLAV and flavonoids as well as ANTH_RG and anthocyanins (Table 1). Interestingly, in the experiments on N and K supply the FLAV index was stronger associated with anthocyanin than with flavonoid concentrations (Table 1). The FLAV index is proportional to the epidermal flavonol content of the leaves (Cerovic et al., 2002), while the analytical determination comprises the total flavonoid concentration. Further, one hypothesis is that other compounds than flavonols had a stronger relevance for the total concentration of flavonoids, resulting in this way to the weak correlations between both parameters.

Nevertheless, although the non-destructive and destructive determination of flavonoid and anthocyanin concentrations was not based on the same sample and did not target exactly the same classes of flavonoids, in most cases we found moderate to strong positive correlations between the FLAV and ANTH_RG indices and...
the flavonoid and anthocyanin concentrations (Table 1). Therefore, and in accordance with published results (Agati et al., 2011 and references therein), we propose that the fluorescence-based indices are suitable for the estimation of total flavonoid concentrations in leaves of C. asiatica.

4.3. FLAV and ANTH, RG indices: robust indicators for the monitoring of centelloside concentrations?

Irrespective of the supply of minerals we found strong or very strong correlations between the total centelloside concentrations and the fluorescence-based indices FLAV, ANTH, and BFRR,UV (Tables 2–4). Screenings of the asatioside and asatiaz acid reference compounds in solution and of the methanolic leaf extracts with a compact fiber-optic fluorescence spectrometer revealed that the centellosides themselves had an apparent weak fluorescence in the blue spectral range (Fig. A.1A–C). However, this fluorescence intensity may not play a significant role for the in vivo fluorescence determinations with the multiparametric optical fluorescence sensor. With one exception (experiment on N supply), we also observed a close relationship between the total centellosides and the flavonoids and anthocyanins.

As a rule, positive correlations were also detected between the fluorescence-based indices, the flavonoids and anthocyanins, and the individual compounds of the centellosides; thereby, we noticed significant variations depending on the supply of either N, P or K. In the experiments evaluating the N and P supply, especially the saponins were positively associated with the FLAV, ANTH, RG and BFRR,UV indices and with the flavonoid (P supply) and anthocyanin (N and P supply) concentrations (Tables 2 and 3). In contrast, in response to the K supply positive correlations were particularly established between the sapogenins and the FLAV, ANTH, RG and BFRR,UV indices, the flavonoids and the anthocyanins (Table 4). Accordingly, in the experiments on N and P supply especially the saponins were negatively correlated with herb and leaf yield, while in the K experiment strong negative correlations were observed between the sapogenin concentrations and the yield parameters (Müller et al., 2013). This emphasizes that, although centellosides are synthesized via the isoprenoid pathway (Jaslow & Dubery, 2009; Augustin et al., 2011) and flavonoids via the phenylpropanoid pathway (Dixon & Paiva, 1995; Winkel-Shirley, 1999, 2001), their accumulation was analogously affected by changes in resource partitioning between primary and secondary metabolism. Moreover, the positive relationship between the BFRR,UV index, considered as a robust ‘stress-indicator’ in various situations (Chappelle et al., 1984; Büring et al., 2013), and the leaf flavonoid, anthocyanin as well as centelloside concentrations (Tables 1–4), corroborates the simultaneous accumulation of these secondary compounds in response to nutrient stress.

When summarizing, our hypothesis that flavonoid accumulation is affected by N, P and K fertilization in a similar extend as centelloside accumulation was confirmed. The fluorescence-based FLAV and ANTH, RG indices were positively correlated with anthocyanin concentrations. The positive relationship between the FLAV index and flavonoid concentrations was less pronounced. Nevertheless, centellosides were generally positively correlated with the FLAV, ANTH, RG and BFRR,UV indices. Thus, by our findings we demonstrate for the first time, that the fluorescence-based indices FLAV, ANTH, RG as well as BFRR,UV are useful for the estimation of centelloside concentrations in leaves of C. asiatica.

In recent years, strong efforts have been made to improve the overall quality of medicinal plants and their biosynthetic products. Amongst others, the basic and applied research play a key role in supporting the selection of more appropriate or promising genetic material as well as in the improvement of plant performance and the natural drug concentration. In this context, non-destructive techniques for the in vivo and in situ monitoring of the ‘plant quality’ open up new possibilities for the timely adjustment of cultivation techniques. To our knowledge, the present study is the first attempt to track the accumulation of saponins in leaves non-destructively by fluorescence-based techniques. The potential for further development and application of the fluorescence fingerprinting in research and production of medicinal plants is broad, starting at the selection of genotypes and their response to variable cultivation scenarios up to the contributions for chemical phenotyping. However, in view of this great potential, further research is encouraged to proof the suitability of the technique and the identified parameters for the prediction of other types of secondary compounds, also, in a broader range of plant species.

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