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Relevance of mineral nutrition and light quality for the accumulation of secondary metabolites in *Centella asiatica* and *Hydrocotyle leucocephala*

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Relevance of mineral nutrition and light quality for the accumulation of secondary metabolites in *Centella asiatica* and *Hydrocotyle leucocephala*

The key objective of the present work was to acquire fundamental knowledge on the impact of nutrient supply and light quality on the accumulation of pharmaceutically relevant secondary metabolites, particularly saponins and lignans, using *Centella asiatica* and *Hydrocotyle leucocephala* as examples. Experiments on the impact of N, P, and K supply on saponin and sapogenin (centelloside) accumulation in leaves of *C. asiatica* were conducted in the greenhouse using soilless culture. Thereby, the relationship between plant growth and centelloside accumulation as influenced by nutrient supply was investigated. Furthermore, the suitability of fluorescence-based indices for non-destructive tracking of centelloside accumulation *in vivo* was examined. For this purpose, different levels of N, P, and K supply were selected as experimental factors. In order to investigate the effects of light quality on saponin and lignan accumulation, experiments were conducted in technically complex sun simulators providing almost natural irradiance. Here, we postulated that high intensity of photosynthetic active radiation (PAR) and ambient level of ultraviolet (UV)-B radiation additively promote the accumulation of centellosides in leaves of *C. asiatica*. The specific UV-B response in terms of flavonoid accumulation was monitored *in vivo* by fluorescence recordings. Finally, the impact of different PAR/UV-B combinations on the concentration and distribution pattern of selected phenylpropanoids, and in particular the lignan hinokinin, was examined in leaves and stems of *H. leucocephala*. The results ascertained in the single chapters can be summarized as follows:

1. The higher levels of N, P, or K supply (in the range from 0 to 150% of the amount in a standard Hoagland solution) enhanced net photosynthesis (Pn) and herb and leaf yield of *C. asiatica*. However, exceeding nutrient-specific thresholds, the high availability of one single nutrient caused lower leaf N concentrations and a decline in Pn and plant growth. Irrespective of N, P, and K supply, the leaf centelloside concentrations were negatively associated with herb and leaf yield. Moreover, negative correlations were found between saponins and leaf N concentrations, and between sapogenins and leaf K concentrations.

2. The accumulation of both flavonoids and anthocyanins was affected by N, P, and K fertigation in the same way as the centelloside accumulation, indicating that limitations in plant growth were generally accompanied by higher secondary metabolite concentrations. The fluorescence-based flavonol (FLAV) and anthocyanin (ANTH_RG) indices correlated fairly with flavonoid and particularly with anthocyanin concentrations. Moreover, the 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, the indices FLAV, ANTH_RG, as well as BFRR_UV enabled the *in situ* monitoring of flavonoid and centelloside concentrations in leaves of *C. asiatica*.

3. UV-B radiation favored herb and leaf production of *C. asiatica*, and induced higher values of the fluorescence-based FLAV index. Similarly, the ANTH_RG index and the saponin concentrations were raised under high PAR. In contrast, UV-B radiation had no distinct effects on saponin and sapogenin concentrations. In general, younger leaves contained higher amounts of saponins, while in older leaves the sapogenins were the most abundant constituents.

4. The concentration of the selected phenylpropanoids in *H. leucocephala* depended on the plant organ, the leaf age, the light regimes, and the duration of exposure. The distribution pattern of the compounds within the plant organs was not influenced by the treatments. Based on the chemical composition of the extracts a principal component analysis enabled a clear separation of the plant organs and harvesting dates. In general, younger leaves mostly contained higher phenylpropanoid concentrations than older leaves. Nevertheless, more pronounced effects of the light regimes were detected in older leaves. As assessed, the individual compounds responded very differently to the PAR/UV-B combinations. Hinokinin was most abundant in the stems, where its accumulation was slightly enhanced under UV-B exposure.

Relevanz der Mineralstoffversorgung und der Lichtqualität für die Akkumulation von Sekundärmetaboliten in *Centella asiatica* und *Hydrocotyle leucocephala*

Ziel dieser Arbeit war es, grundlegendes Wissen in Bezug auf den Einfluss des Nährstoffangebots und der Lichtqualität auf die Akkumulation von pharmazeutisch relevanten Sekundärmetaboliten, insbesondere Saponinen und Lignanen, zu erlangen, wobei *Centella asiatica* und *Hydrocotyle leucocephala* als Modellpflanzen dienten. Versuche zum Einfluss des N-, P- und K-Angebots auf die Saponin und Sapogenin (Centellosid)-Akkumulation in *C. asiatica* Blättern wurden im Gewächshaus in hydroponischer Kultur durchgeführt. Dabei wurde die Beziehung zwischen Pflanzenwachstum und Centellosid-Akkumulation in Abhängigkeit vom Nährstoffangebot untersucht. Weiterhin wurde die Eignung von Fluoreszenz-basierten Indizes für die nicht-destruktive Erfassung der Centellosid-Akkumulation *in vivo* geprüft. Dazu wurde ein unterschiedliches N-, P- und K-Angebot als experimenteller Faktor gewählt. Um die Effekte der Lichtqualität auf die Saponin- und Lignan-Akkumulation zu untersuchen, wurden Experimente in technisch komplexen Sonnensimulatoren durchgeführt, die eine nahezu natürliche Strahlung generierten. Die Studien basierten auf der Hypothese, dass eine hohe photosynthetisch aktive Strahlung (PAR) und eine ambiente Ultraviolett (UV)-B Intensität die Centellosid-Akkumulation in *C. asiatica* Blättern additiv fördern. Die spezifische UV-B Antwort, d.h. die Akkumulation von Flavonoiden, wurde mit Hilfe von Fluoreszenz-Messungen *in vivo* verfolgt. Schließlich wurde der Einfluss von verschiedenen PAR/UV-B Kombinationen auf die Konzentration und das Verteilungsmuster von ausgewählten Phenylpropanoiden, insbesondere dem Lignan Hinokinin, in den Blättern und Stängeln von *H. leucocephala* untersucht. Die in den einzelnen Kapiteln ermittelten Ergebnisse können wie folgt zusammengefasst werden:

1. Ein höheres N-, P- bzw. K-Angebot (im Bereich von 0 bis 150% der Nährstoffmenge in einer Standard Hoagland-Nährlösung) erhöhte die Nettophotosyntheserate (Pn) und den Kraut- und Blattertrag von *C. asiatica*. Bei Überschreitung nährstoffspezifischer Schwellenwerte hatte die hohe Verfügbarkeit der einzelnen Nährstoffe niedrigere Blatt N-Konzentrationen und eine Abnahme der Pn und des Pflanzenwachstums zur Folge. Unabhängig vom N-, P- und K-Angebot war die Centellosid-Konzentration negativ mit dem Kraut- und Blattertrag assoziiert. Des Weiteren wurden negative Korrelationen zwischen den Saponinen und der Blatt N-Konzentration und zwischen den Sapogeninen und der Blatt K-Konzentration gefunden.

2. Die Flavonoid- und Anthozyan-Akkumulation wurde durch die N-, P- und K-Fertigation auf die gleiche Weise beeinflusst wie die Centellosid-Akkumulation, was darauf hinweist, dass ein limitiertes Pflanzenwachstum generell mit einer höheren Konzentration an Sekundärmetaboliten einherging. Die Fluoreszenz-basierten Flavonol- (FLAV) und Anthozyan- (ANTH_RG) Indizes korrelierten gut mit den Flavonoid- und insbesondere mit den Anthozyan-Konzentrationen. Zudem korrelierten die Centelloside positiv mit den FLAV und ANTH_RG Indizes sowie dem BFRR_UV Index, der als universeller ‚Stressindikator' betrachtet wird. Somit ermöglichten die Indizes FLAV, ANTH_RG und BFRR_UV die *in situ* Beobachtung der Flavonoid- und Centellosid-Konzentration in den Blättern von *C. asiatica*.

3. UV-B Strahlung förderte die Kraut- und Blattproduktion von *C. asiatica*, und induzierte höhere Werte des Fluoreszenz-basierten FLAV Index. Ebenso waren der ANTH_RG Index und die Saponin-Konzentration unter hoher PAR Intensität erhöht. Im Gegensatz dazu hatte UV-B Strahlung keine eindeutigen Effekte auf die Saponin- und Sapogenin-Konzentrationen. Grundsätzlich enthielten jüngere Blätter höhere Saponin-Konzentrationen, während in älteren Blättern die Sapogenine die am häufigsten vorkommenden Substanzen waren.

4. Die Konzentration der ausgewählten Phenylpropanoide in *H. leucocephala* war abhängig von Pflanzenorgan, Blattalter, Lichtzusammensetzung und Behandlungsdauer. Das Verteilungsmuster der Substanzen zwischen den Pflanzenorganen wurde nicht durch die Behandlungen beeinflusst. Basierend auf der chemischen Komposition der Extrakte ermöglichte eine Hauptkomponenten-analyse eine klare Trennung der Pflanzenorgane und Erntetermine. Grundsätzlich enthielten jüngere Blätter meist höhere Phenylpropanoid-Konzentrationen als ältere Blätter. Stärkere Effekte der Lichtzusammensetzung wurden jedoch in älteren Blättern detektiert. Wie festgestellt, reagierten die einzelnen Substanzen sehr unterschiedlich auf die PAR/UV-B Kombinationen. Hinokinin kam am häufigsten im Stängel vor, wo die Akkumulation unter UV-B Strahlung leicht erhöht war.

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List of abbreviations

ANOVA	analysis of variance
ANTH_RG	decadic logarithm of the red to green excitation ratio of far-red chlorophyll fluorescence
BFRR_UV	ultraviolet excitation ratio of blue-green and far-red chlorophyll fluorescence
C	carbon
C. asiatica	*Centella asiatica* L. Urban
$Ca(NO_3)_2$	calcium nitrate
cm	centimeter
CNB	carbon-nutrient balance
CO_2	carbon dioxide
CoA	coenzyme A
$CuSO_4$	copper(II) sulfate
cv.	cultivar
°C	degree Celsius
DM	dry mass
DMAPP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
e.g.	exempli gratia, for example
EC	electrical conductivity
ESI-MS	electrospray ionization - mass spectrometry
et al.	et alii (m.), et aliae (f.), and others
etc.	et cetera
fam.	family
$FeSO_4$	iron(II) sulfate
Fig. (sg.), Figs. (pl.)	figure (sg.), figures (pl.)
FLAV	decadic logarithm of the red to ultraviolet excitation ratio of far-red chlorophyll fluorescence
FPP	farnesyl diphosphate
FRF	far-red fluorescence
g	gram
GDB	growth-differentiation balance
Glu	glucose
GPP	geranyl diphosphate
H	hydrogen
h	hours
H. leucocephala	*Hydrocotyle leucocephala* Cham. & Schlecht.
H_2MoO_4	molybdic acid
H_2O	water
H_3BO_3	boric acid
H_3COO^-	acetate anion
HCA	hydroxycinnamic acid
HCl	hydrogen chloride
HNO_3	nitric acid
HPLC	high-performance liquid chromatography
HY	herb yield
i.e.	id est, that is
IPP	isopentyl diphosphate

IR	infrared radiation
K	potassium
K_2O	potassium oxide
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
kV	kilovolt
L	liter
LT	leaf type
LY	leaf yield
m	meter
M	molar (mole per liter)
[M]	molar mass
m/z	mass-to-charge ratio
MeOH	methanol
MEP	methylerythritol phosphate
mg	milligram
MgO	magnesium oxide
$MgSO_4$	magnesium sulphate
min	minutes
mL	milliliter
mm	millimeter
$MnSO_4$	manganese(II) sulfate
MoO3	molybdenum(VI) oxide
mS	millisiemens
MVA	mevalonate
mW	milliwatt
µg	microgram
µm	micrometer
µmol	micromole
N	nitrogen
n	number of replications
n.s.	not significant
NaCl	natrium chloride
$(NH_4)_2SO_4$	ammonium sulphate
$(NH_4)H_2PO_4$	ammonium dihydrogen phosphate
nm	nanometer
nmol	nanomole
OH	hydroxide
OPPP	oxidative pentose phosphate pathway
%	percent
% m m^{-1}	percent mass per mass
P	phosphorous
p	probability of error
P_2O_5	phosphorus pentoxide
p.a.	pro analysi
PAM	pulse-amplitude-modulated
PAR	photosynthetic active radiation
PC	principal component
PCA	principal component analysis
PCM	protein competition model
PFA	perfluoroalkoxy

Pn	net photosynthesis
ppm	parts per million
r	Pearson's correlation coefficient
rel.	relative
Rha	rhamnose
ROS	reactive oxygen species
rpm	revolutions per minute
s	second
S	sulfur
spp.	species
syn.	synonym
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet
V	volt
v/v	volume per volume
VIS	visible
W	watt
WTA	weeks of treatment application
$ZnSO_4$	zinc sulfate

A Introduction

1 Plant secondary metabolites and their importance for medicinal purposes

Plant secondary metabolites are chemicals produced by plants in a vast diversity of more than 200,000 structures (Hartmann, 2007). Contrary to primary metabolites, secondary metabolites are not essential for growth processes but enable the plant to adapt to the environment, *e.g.*, by serving as feeding deterrents against herbivores, protective agents against pathogens or abiotic factors, pollinator attractants, antioxidants, or chemical signals (Croteau et al., 2000; Wink, 2003).

Owing to the bioactivity of these chemicals, plants have been utilized as medicines for thousands of years. Initially, these medicines were administered as crude drugs, such as teas, tinctures, poultices, powders, and other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004). In more recent history, progress in analytical chemistry enabled the isolation and the pharmaceutical usage of single compounds, starting with the isolation of morphine from opium poppy in the early 19th century (Hamburger and Hostettmann, 1991; Hamilton and Baskett, 2000; Li and Vederas, 2009). Despite the success of drugs derived from natural sources, the tremendous development of synthetic pharmaceutical chemistry and microbial fermentation in the 20th century led to a declining interest of the pharmaceutical companies in natural product research (Hamburger and Hostettmann, 1991). However, in recent years herbal drugs have gained renewed attention, mainly because of ecological awareness and an increased demand in alternative therapies (Hamburger and Hostettmann, 1991; Calixto, 2000; Roggo, 2007). It has been estimated that to date only a small percentage of the ca. 250,000 species of higher plants has been investigated for pharmacological active constituents. Thus, there is still an enormous potential for the discovery and development of new drugs from plant resources (McChesney et al., 2007; Li and Vederas, 2009).

2 The need for a well-directed cultivation of medicinal plants

To meet the increasing market demand for herbal medicines, a large proportion of the raw material is collected from wild plant populations (Schippmann et al., 2006; Cordell, 2009). As a consequence, uncontrolled harvesting, limited cultivation, and insufficient attempts of replacement of the plants result in depletion of wild stock, extinction of endangered species, and shrinking of biodiversity (Rates, 2001; Schippmann et al., 2006; Cordell, 2009). Beyond, the collected material often does not match high quality standards because of contaminants like heavy metals, toxic or hazardous substances, microbes, or undesirable plant species.

Further problems are the insecurity of long-range availability of plant material as well as variable or unsatisfactory contents of the target biochemicals (Calixto, 2000; McCaleb et al., 2000; Gurib-Fakim, 2006; McChesney et al., 2007; Cordell, 2009; Prasad et al., 2012). Therefore, a well-directed cultivation of the medicinal plants would contribute to the continuous availability and to an improved quality of safe raw material (Calixto, 2000; Rates, 2001). However, in dependence on the compound class, the content of bioactive constituents may be affected, *e.g.*, by light, temperature, and nutrient supply, as well as time of harvest and the physiological stage of the plant (Li et al., 2008; Selmar and Kleinwächter, 2013). Thus, to achieve high yields of the desired secondary compounds, a precise knowledge on optimum conditions for its biosynthesis and for plant development is necessary.

3   Selected plant species, active constituents, and medicinal usage

3.1 *Centella asiatica*

Centella asiatica L. Urban (syn.: *Hydrocotyle asiatica* L., fam.: Apiaceae) is a perennial creeping herb (Fig. 1), which flourishes in marshy areas of tropical to subtropical regions (Cepae, 1999; James and Dubery, 2009).

Fig. 1. *Centella asiatica* L. Urban. Insert: Inconspicuous pale purple flowers arranged in shortly petiolate umbels.

The aerial parts of *C. asiatica*, or even the entire plant, have been used for therapeutic applications since ancient times. In some cultures, the herb is also consumed as a vegetable (Sritongkul et al., 2009). In folk medicine *C. asiatica* is used for many purposes, including the treatment of skin disorders, respiratory problems, nervous disorders, infectious diseases, and gastro-intestinal diseases. Furthermore, different pharmacopoeias and traditional systems of medicine report on the usage of the plant, *e.g.*, in the therapy of leprous ulcers, venous

disorders, and hepatic cirrhosis. Beyond, its efficacy in the treatment of wounds, burns, ulcerous skin ailments, and stomach or duodenal ulcers, as well as in the prevention of keloid and hypertrophic scars, has already been confirmed in clinical studies (Hausen, 1993; WHO, 1999 and references therein).

The pharmacological activity of *C. asiatica* is attributed mainly to pentacyclic triterpene saponins, the centellosides, which are preferentially accumulated in the leaves of the plant. The most important saponins are asiaticoside and madecassoside, and their respective genins asiatic acid and madecassic acid (Fig. 2) (Inamdar et al., 1996). In addition, *C. asiatica* contains mono- and sesquiterpenoids (Oyedeji and Afolayan, 2005), polysaccharides (Wang et al., 2004), polyacetylenes (Siddiqui et al., 2007; Govindan et al., 2007), sterols (Srivastava and Shukla, 1996; Rumalla et al., 2010; Sondhi et al., 2010), phenolic acids, and flavonoid derivatives (Kuroda et al., 2001; Matsuda et al., 2001; Yoshida et al., 2005; Subban et al., 2008). The latter are generally considered to promote human health and to prevent cardiovascular diseases and cancer (Ross and Kasum, 2002; Fraga et al., 2010). Besides, the nutritional value of the plant is related to its notable contents of fiber, protein, calcium, and beta-carotin (Sritongkul et al., 2009).
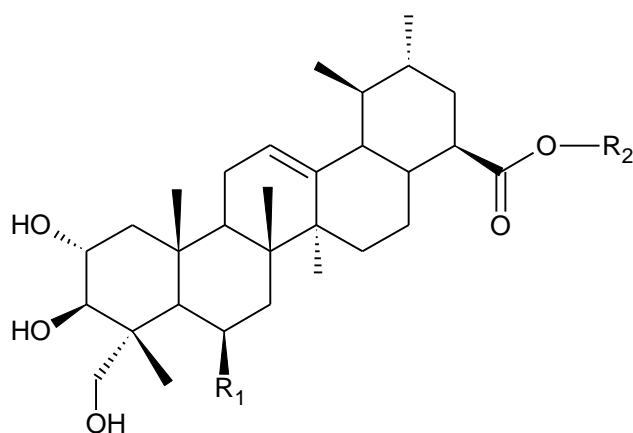
asiaticoside	R1 = H	R2 = Glu-Glu-Rha
madecassoside	R1 = OH	R2 = Glu-Glu-Rha
asiatic acid	R1 = H	R2 = H
madecassic acid	R1 = OH	R2 = H

Fig. 2. Chemical structure of asiaticoside, madecassoside, asiatic acid, and madecassic acid. Glu, glucose; Rha, rhamnose.

During the last years, *C. asiatica* based drugs and cosmetics have gained significant economic interest worldwide (James and Dubery, 2009; Devkota et al., 2010a; Singh et al., 2010). Despite of this, the commercial cultivation of the plant is largely underexplored and the market's demand is predominantly satisfied by wild harvesting from nature. Thus,

unrestricted exploitation of the drug has markedly depleted spontaneous populations of *C. asiatica* and may lead to the extinction of valuable genotypes (Singh et al., 2010; Thomas et al., 2010). On the other hand, centelloside concentrations in the raw material are known to vary in dependence on the collected genotypes, geographic regions, and growth conditions (Randriamampionona et al., 2007; Devkota et al., 2010a, b; Thomas et al., 2010). Consequently, the raw material is often of poor quality owing to low contents of bioactive compounds. Therefore, research-based developments of cultivation techniques are needed in order to encourage the commercial production of *C. asiatica* raw material containing high amounts of the bioactive compounds.

3.2 Hydrocotyle leucocephala

Hydrocotyle leucocephala Cham. & Schlecht. (fam.: Araliaceae) is a perennial stoloniferous creeper (Fig. 3), indigenous to South America. The aquatic plant is able to grow even submerse and occurs abundantly in wet and marshy habitats (Alvarez, 2001; Ramos et al., 2006).

Fig. 3. *Hydrocotyle leucocephala* Cham. & Schlecht.. Insert: Small white flowers arranged in simple long petiolate umbels.

The leaves of *H. leucocephala* are edible and, owing to their peppery taste, they are used as a spice or for the preparation of a soda in some tropical countries. In Colombia the plant is used as medicinal herb because of its diuretic, antihelminthic, and antidiarrheal properties (Ramos et al., 2006).

Up to now, a number of secondary compounds have been isolated from the aerial parts of *H. leucocephala*, including three diacetylenic compounds, two monoterpenoids, seven leucoceramides, six leucocerebrosides, one sterol, one nor-isoprenoid, one megastigmane derivative, four flavonoids, and the dibenzylbutyrolactone lignan (–)-hinokinin (Fig. 4). Some of them, *e.g.*, hinokinin were shown to possess immunosuppressive activity (Ramos et al.,

2006). Beyond, hinokinin is considered to be a potent agent, *e.g.*, against human hepatitis-B virus (Huang et al., 2003) and *Trypanosoma cruzi*, the pathogen of Chagas disease (e Silva et al., 2004; Saraiva et al., 2007). Moreover, hinokinin was shown to have anti-inflammatory and analgesic properties (da Silva et al., 2005). Thus, *H. leucocephala* is a promising source for several secondary metabolites, which potentially might be considered for the development of new drugs. So far, neither there is information on the propagation and cultivation of the species, nor on the significance of growth conditions for the accumulation of biochemicals in the tissue.
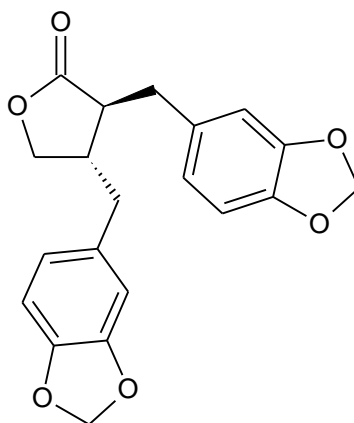
Fig. 4. Chemical structure of (–)-hinokinin.

4   Biosynthesis of the active constituents

4.1 Saponins

Pentacyclic triterpene saponins, including the centellosides, are synthesized *via* the isoprenoid pathway starting with isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two biosynthetic routes for the generation of IPP and DMAPP have been characterized, *i.e.*, the cytosolic mevalonate (MVA) pathway, which uses acetyl-CoA as biosynthetic precursor, and the plastidal methylerythritol phosphate (MEP) pathway, by which IPP and DMAPP are formed from pyruvate and glyceraldehyde phosphate. While in the MEP pathway both IPP and DMAPP are produced simultaneously, the MVA pathway only yields IPP, which is finally converted into DMAPP. In higher plants both pathways are operative, and even a metabolic cross-talk between them may exist (Hemmerlin et al., 2012; Vranová et al., 2013). However, evidences show that under standard growth conditions triterpenes, such as saponins, are synthesized mainly in the cytosol utilizing IPP from the MVA pathway (Rohmer, 1999; Trojanowska, 2000; Chappell, 2002; Kirby and Keasling, 2009; Hemmerlin et al., 2012).

The condensation of one IPP molecule and one DMAPP molecule, respectively, leads to geranyl diphosphate (GPP), and the subsequent addition of another IPP unit originates farnesyl diphosphate (FPP). Then, the linkage of two FPP units generates squalene, which is epoxygenated to 2,3-oxidosqualene. The cyclization of 2,3-oxidosqualene leads to the tetracyclic dammarenyl cation, which is transformed *via* several intermediates into the pentacyclic α- and β-amyrins. The latter undergo various modifications, *i.e.*, oxidation, hydroxylation, and other substitutions to form the *C. asiatica* sapogenins. Finally, the sapogenins are converted into saponins by glycosylation processes (James and Dubery, 2009; Augustin et al., 2011).

4.2 Lignans

Dibenzylbutyrolactone lignans, such as hinokinin, belong to the group of phenylpropanoids. The biosynthetic precursor, coniferyl alcohol, is formed in the general phenylpropanoid and the cinnamate/monolignol pathway. At first, the deamination of the aromatic amino acid phenylalanine leads to cinnamic acid, which is hydroxylated *via p*-coumaric acid into caffeic acid. Caffeic acid is transformed into ferulic acid, which is converted *via* feruloyl-CoA and coniferyl aldehyde into coniferyl alcohol (Sakakibara et al., 2007; Suzuki and Umezawa, 2007).

The formation of (−)-hinokinin starts with the enantioselective dimerization of two coniferyl alcohol units mediated by a dirigent protein, resulting in (+)-pinoresinol. Subsequently, (+)-pinoresinol is reduced *via* (+)-lariciresinol to (−)-secoisolariciresinol. The dehydrogenation of (−)-secoisolariciresinol leads to (−)-matairesinol, and finally (−)-hinokinin originates from the generation of two methylenedioxy bridges, either *via* (−)-pluviatolide or *via* (−)-haplomyrfolin, depending on the benzene ring on which the first methylenedioxy bridge is formed (Suzuki and Umezawa, 2007; Bayindir et al., 2008).

5 Effects of abiotic factors on the accumulation of plant secondary metabolites

Plants are sessile organisms and inevitably exposed to a diversity of environmental factors. In order to cope with rapid changes of their surroundings, plants have evolved a wide spectrum of acclimation responses, including the accumulation of secondary metabolites (Wink, 2003; Hartmann, 2007).

Saponins are generally considered to be accumulated to protect the plant from pathogens and herbivores (Augustin et al., 2011). Besides, evidences indicate that the synthesis of saponins, including centellosides, might be affected by abiotic factors, such as soil fertility

and light conditions (Mathur et al., 2000; Devkota et al., 2010a, b; Siddiqui et al., 2011; Szakiel et al., 2011; Maulidiani et al., 2012; Prasad et al., 2012). In contrast to the saponins, the function and the inducibility of lignans in herbaceous plants is rather unknown. Some authors assume that lignans play a role in plant defense against herbivory and pathogen attack (Gang et al., 1999; Harmatha and Nawrot, 2002). However, precise information is scarce; beyond, knowledge on the impact of abiotic factors on lignan accumulation is completely lacking. Since lignans belong to the group of phenylpropanoids and UV-B radiation is known to induce the expression of key-enzymes of the phenylpropanoid pathway, *e.g.*, phenylalanine ammoniumlyase (Chappell and Hahlbrock, 1984; Strid et al., 1994; Jenkins et al., 2001), it is conceivable that the synthesis of lignans might be affected by the spectral composition of light.

5.1 Nutrient supply

Nitrogen (N), along with phosphorus (P) and potassium (K), is one mineral required by the plants in large amounts. N is an essential constituent of proteins, nucleic acids, nucleotids, chlorophyll, co-enzymes, and phytohormones. P is incorporated into various organic compounds, including nucleic acids, sugar phosphates, adenosine phosphates, and phospholipids. In addition, it controls several key enzyme reactions and is necessary for the transfer of carbohydrates in leaf cells. K plays a major role in osmoregulation and is important for cell extension and stomata movement. It stimulates phloem loading of sucrose, affects the rate of mass flow-driven solute movement within the plant, and activates a number of enzymes (Epstein and Bloom, 2005; Marschner, 2012 and references therein).

Variations in N, P, and K availability may influence resource allocation between primary and secondary metabolism, and consequently affect the concentration of secondary metabolites in the plant tissues (Coley et al., 1985; Lattanzio et al., 2009). Efforts to explain the patterns of resource allocation led to the emergence of several hypotheses, such as the carbon-nutrient balance hypothesis (CNB) (Bryant et al., 1983), the growth-differentiation balance hypothesis (GDB) (Herms and Mattson, 1992), and the protein competition model (PCM) (Jones and Hartley, 1999). Both the CNB and the GDB assume that in conditions of low nutrient availability growth is more restricted than photosynthesis. Consequently, fixed carbon is accumulated in excess of growth requirements and is invested in the synthesis of carbon-based secondary metabolites, such as terpenoids or phenols (Watson, 1963; Epstein, 1972; Smith, 1973; McKey, 1979; Bryant et al., 1983). In contrast, the PCM suggests that the synthesis of phenolic compounds is rather inversely related to the formation of proteins, since

both compete for the same limited precursor phenylalanine (Jones and Hartley, 1999). Nevertheless, all the three hypotheses assume a trade-off between growth and the biosynthesis of secondary metabolites (Coley et al., 1985).

In the literature, a number of studies report on the enhanced biosynthesis of phenols, *e.g.*, flavonoids, in response to nutrient limitations, paralleled by constraints in plant growth (Muzika, 1993; Haukioja, 1998; Hale et al., 2005). On the contrary, results on terpenoid formation as influenced by nutrient supply are less consistent (Mihaliak and Lincoln, 1985; Muzika, 1993; Haukioja, 1998). Analogous to that, there are contradictory findings on the effects of nutrient availability on the accumulation of saponins in plants. In this context, the application of cattle manure enhanced plant growth and berry yield of *Phytolacca dodecandra* L'Hérit, but it generally decreased the content of triterpene saponins in the berries (Ndamba et al., 1996). On the contrary, the content of steroidal furostanol and spirostanol saponins in roots of *Asparagus racemosus* Willd. increased in response to N, P, and K fertilization (Vijay et al., 2009). Accordingly, N and P fertilization led to an enhancement in plant growth and steroidal saponin content in shoots of *Tribulus terrestris* L. (Georgiev et al., 2010). Moreover, the application of moderate doses of N and P, particularly when applied in combinations, promoted saikosaponin contents in roots of *Bupleurum chinense*; on the other hand, the additional increase in nutrient supply in turn decreased saponin production (Zhu et al., 2009).

The inconsistent findings summarized above might be explained by the divergent experimental designs, genotypes, and growing conditions, as well as by the target organ which was investigated. At all, this diversity makes comparisons with *C. asiatica*, which accumulates saponins preferentially in the leaves, very difficult. Moreover, even fertilization studies of *C. asiatica* revealed divergent findings. On the one hand, some studies report on a negative impact of fertilization and nutrient rich soil on saponin and sapogenin concentrations in *C. asiatica* plants (Devkota et al., 2010a, b). Similar results were observed for multiple shoot cultures having higher asiaticoside concentrations at lower N levels in the culture media (Prasad et al., 2012). On the other hand, there are also studies reporting on a positive impact of N fertilization on plant growth as well as the accumulation of saponins and sapogenins in *C. asiatica* plants (Siddiqui et al., 2011). Hence, precise information on the relationship among plant growth, physiology, and centelloside biosynthesis in response to mineral nutrition is urgently needed.

5.2 Light quality

Plants are photoautotrophic organisms and depend on the absorption and utilization of sunlight as a source of energy driving photosynthesis. Moreover, light is an informational signal directing growth, differentiation, and metabolism of the plants (Kendrick and Kronenberg, 1994; Fankhauser and Chor, 1997).

Sunlight reaching the Earth's surface encompasses ultraviolet-B (UV-B, 280–315 nm), ultraviolet-A (UV-A, 315–400 nm), photosynthetic active (PAR, 400–700 nm), and infrared radiation (IR, >700 nm) (Fig. 5). Since wavelengths below 290 nm are efficiently absorbed by the stratospheric ozone layer, only a small proportion of UV-B radiation is transmitted to the Earth's surface (Pyle, 1997; McKenzie et al., 2003). Nevertheless, UV-B radiation is the most energetic component of the daylight spectrum and has the potential to affect growth, development, reproduction, and survival of many organisms, including plants (Caldwell et al., 2007). The effects of UV-B radiation on plants depend on various factors, *e.g.*, the fluence rate, duration of exposure, the wavelengths, and the interaction with other environmental signals, such as other spectral wavelengths (Caldwell et al., 2003).

As a consequence of ozone depletion, UV-B radiation reaching the Earth's surface has increased during the last decades. Therefore, numerous studies published during the 1970-2000s dealt with the impact of enhanced UV-B levels on plants. These studies revealed that high fluence rates of UV-B generate high levels of reactive oxygen species and may damage macromolecules, such as DNA, proteins, and membrane lipids, which consequently leads to alterations in photosynthesis and reductions in growth (Teramura and Sullivan, 1994; Jansen et al., 1998 and references therein). However, during recent years, ozone depletion has been reduced significantly, and the dramatic forecasts were not confirmed. These facts, along with major advances in experimental manipulation of UV-B radiation, led to a shift of the scientific focus towards the influence of lower but ecologically relevant UV-B levels on plants (Jansen and Bornman, 2012). Correspondingly, it was elucidated that harmful effects are predominantly induced by above-ambient UV-B doses, which trigger the expression of stress-related genes mediated by unspecific pathways, similar to those of wound-signaling and pathogen-defense. Differently, environmentally relevant fluence rates of UV-B radiation activate specific, photomorphogenic signaling pathways, which induce a range of genes involved in UV protection and/or the amelioration of UV damage (Jenkins and Brown, 2007; Jenkins, 2009).
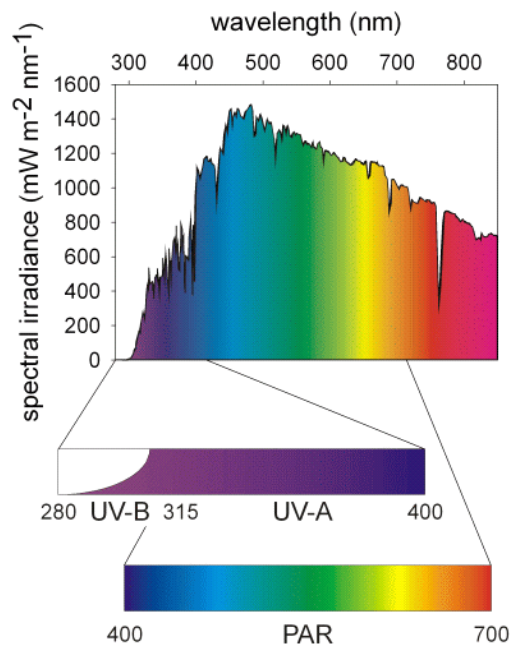
Fig. 5. Typical spectrum of global irradiance and ranges of UV-B, UV-A, and PAR measured at the Helmholtz Zentrum München, Germany (11.6 East, 48.2 North, 489 m above sea level) on a sunny spring day (Albert et al., 2006).

A well-known protective mechanism in plants against the UV-B wavelengths is the increased accumulation of phenolic compounds, including flavonoids (Li et al., 1993; Frohnmeyer and Staiger, 2003). Beyond, flavonoid synthesis was also shown to be induced by high PAR intensity even in the absence of the UV-B range (Nitz et al., 2004; Götz et al., 2010; Agati et al., 2011). Nevertheless, the presence of UV-B radiation additively promoted flavonoid accumulation (Nitz et al., 2004; Götz et al., 2010; Agati et al., 2011), which substantiates the necessity of the additional consideration of other wavelengths when evaluating the UV-B impact on plants.

Analogous to the flavonoids, even the accumulation of saponins was proposed to be influenced either by UV-B radiation or by PAR intensity. Accordingly, the glycyrrhizin concentrations in *Glycyrrhiza uralensis* roots were enhanced after UV-B exposure (Afreen et al., 2005). Moreover, cultivation of *Phytolacca dodecandra* L'Hérit in shade led to a lower berry yield and to lower triterpene saponin concentrations than cultivation in full sunlight (Ndamba et al., 1996). Similarly, the roots of *Panax quinquefolius* plants grown in the understory of a broadleaf forest contained higher ginsenoside concentrations when the plants were exposed to longer sun flecks as compared to those exposed to shorter periods of direct sunlight; although the overexposure to light intensity led to a decrease in the concentrations (Fournier et al., 2003). In contrast, fruits of *Diospyros abyssinica* (Hiern) F. White in the

upper crown of the tree, and therefore exposed to higher light intensity, were found to contain less triterpenoid saponins (derivatives of betulin and betulinic acid) than lower crown fruits, which were exposed to lower light intensity (Houle et al., 2007).

Finally, similar to the reports on nutrient supply, investigations on light revealed divergent findings concerning the impact of its intensity and quality on saponin concentrations in plants. Accordingly, some studies on *C. asiatica* indicate a promoting effect of higher light intensities on centelloside accumulation (Sritongkul et al., 2009; Devkota et al., 2010b; Maulidiani et al., 2012), while others report on the opposite, *i.e.*, higher yields of herbage paralleled by higher concentrations of asiaticoside under 50% shading as compared to full sunlight (Mathur et al., 2000). Beyond, the controlled combination of UV-B and PAR, and their influence on the accumulation of saponins has not been investigated, yet.

With regard to the lignans, experiments on the effects of light supply on biosynthesis are lacking. However, since lignans belong to the group of phenylpropanoids, it has to be elucidated whether the accumulation of lignans is influenced by different light regimes in the same extent as the accumulation of other phenylpropanoids, such as flavonoids.

6 Potential use of non-destructive fluorescence recordings for research and cultivation of medicinal plants

Chlorophyll fluorescence is an optical signal that provides information on the physiological status of the plant. The general principles of fluorescence are reviewed elsewhere (*e.g.*, Krause and Weis, 1991; Maxwell and Johnson, 2000; Murchie and Lawson, 2013).

The pulse-amplitude-modulated (PAM) fluorometry is one of the most common techniques used to measure the light-induced chlorophyll fluorescence reflecting the photosynthetic performance of the plant tissue (Krause and Weis, 1991; Baker and Rosenqvist, 2004). However, since the PAM method requires dark-adaptation of the leaf prior to measurement for optimum results, it often imposes practical limitations. During the last decade, advances in fluorescence measurement techniques have led to the development of new portable optical sensors, enabling stable measurements under daylight conditions without the necessity of dark-adaptation of the leaf (Buschmann et al., 2000). One of these sensors is the Multiplex® device (Force-A, Orsay, France). The Multiplex®, a multiparametric fluorescence sensor, measures the fluorescence intensity in the three spectral bands, *i.e.*, red, far-red, and blue or green, after excitation with different light sources (ultraviolet, blue or green, and red). The fluorescence signals recorded in these bands are specific to the species

which is being evaluated. While the red and far-red fluorescence emanates from chlorophyll molecules, the blue and green fluorescence are related to phenolic compounds, particularly the cell wall bound cinnamic acids (Morales et al., 1996; Lichtenthaler and Schweiger, 1998).

However, the absolute fluorescence signals are strongly affected by the distance between sensor and leaf, the allocation site of the molecules in the tissue, and the leaf structure. Thus, the calculated fluorescence ratios establish more robust and reliable indices, and are therefore more suitable, *e.g.*, for the estimation of the chlorophyll, flavonol, and anthocyanin content in plant tissues (Cerovic et al., 1999; Lichtenthaler et al., 2012). With this rationale, the chlorophyll content is reflected by the simple fluorescence ratio of far-red and red fluorescence excited either with green or red light (Lichtenthaler et al, 1986; Buschmann, 2007). Further, the content of epidermal flavonols can be evaluated by the decadic logarithm of the red to UV excitation ratio of the far-red chlorophyll fluorescence (Cerovic et al., 2002), while the content of anthocyanins is related to the decadic logarithm of the red to green excitation ratio of far-red chlorophyll fluorescence (Agati et al., 2005).
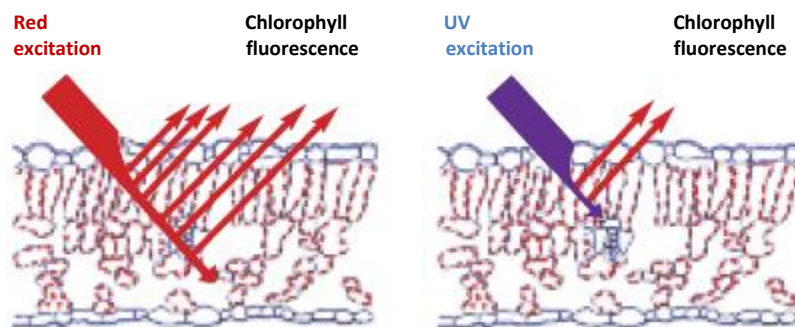
Fig. 6. Cross section of a leaf. The comparison of the red and UV excitation quantifies the screening effect due to polyphenols and therefore the content of the latter in the epidermis (modified after Force-A, 2010).

The estimation of the content of epidermal flavonols and anthocyanins is based on the screening properties of the compounds on chlorophyll, which is localized below the epidermis (Burchard et al., 2000; Bilger et al., 2001). With this method the intensity of chlorophyll fluorescence excited with red light (not absorbed by flavonols and anthocyanins) is compared with the intensity of chlorophyll fluorescence excited either with UV (absorbed by flavonols) or green light (absorbed anthocyanins) (Fig. 6). In this way, the excitation light reaching the chloroplasts is attenuated by the constituents located in the epidermis. Consequently, the higher the concentration of absorbing compounds per leaf area, the lower is the intensity of the chlorophyll fluorescence.

As highlighted above, plant tissues accumulate secondary metabolites, including flavonols and anthocyanins, in order to adapt to their surroundings. Changing environmental conditions induce alterations in secondary metabolite concentrations, which consequently lead to changes in the fluorescence intensities. In this context, the potential of specific fluorescence-based indices has been tested in several studies, *e.g.*, for the early detection of N deficiency, drought stress, and pathogen infection in agricultural crops. Furthermore, the usefulness of the multiparametric fluorescence system (Multiplex®) was even proven for the monitoring of the maturity of apples (Betemps et al., 2012), olives (Agati et al., 2005), and grapes (Ben Ghozlen et al., 2010; Bramley et al., 2011; Baluja et al., 2012).

Although there is a great potential for the application of the fluorescence techniques in physiological studies, selection of genotypes, and cultivation of medicinal plants, respective experiments are lacking. Traditionally, the accumulation of secondary metabolites in the plant tissues is investigated by using wet chemical analyses. As a rule, these analyses are costly and very laborious. Thus, the easy tracking of desired compounds *in situ* by means of non-destructive techniques like the multiparametric fluorescence would promote the applied research on medicinal plants. Moreover, it would support and facilitate crop management in terms of determination of appropriate timing of fertilization, light application, and harvest time, in the sum resulting in an improvement in plant and product quality.

7 Objectives of the study

Since the content of bioactive constituents in medicinal plants may be affected by environmental factors, time of harvest, and developmental stage of the plant, the precise knowledge on optimum conditions for plant growth and biosynthesis of the desired secondary metabolites is necessary. Both *Centella asiatica* and *Hydrocotyle leucocephala* accumulate biochemicals, *e.g.*, the centellosides and the lignan hinokinin, in the aerial organs, with considerable pharmaceutical potential. The available literature provides evidences that the concentration of centellosides might be influenced either by nutrient supply and/or by light conditions. Moreover, analogous to flavonoids, different UV-B/PAR combinations may possibly have regulatory properties on lignan synthesis. However, experiments on the impact of abiotic factors on lignan accumulation are completely lacking. Further, the findings on saponin concentrations, including centellosides, as affected by nutrient and light supply are scarce and/or contradictory; the combination of UV-B and PAR, and its impact on constituent accumulation has not been investigated, yet. Hence, fundamental research on the inducibility of saponin and lignan synthesis is required, serving as basis for more practical investigations

targeting the increase in compound concentrations in the plant tissue. Moreover, light and nutrient supply might be controlled more precisely during cultivation to steer both primary and secondary metabolism of medicinal plants.

The objective of this work was to examine the relevance of nutrient supply and light quality for the biosynthesis of pentacyclic triterpene saponins and sapogenins using *C. asiatica* as example. We further aimed to elucidate the causal relationship between the plant's primary metabolism and the accumulation of secondary compounds as influenced by the growth conditions. Moreover, we targeted the applicability of the multiparametric fluorescence technique for the non-destructive estimation of centelloside accumulation *in vivo* using products of the secondary metabolism as reference. Finally, we aimed to explore the effects of light quality on the accumulation of selected phenylpropanoids, including the dibenzylbutyrolactone lignan hinokinin, in *H. leucocephala* plants cultivated under controlled conditions.

The study was divided into four experimental chapters, each one having its own hypothesis, as follows:

1. Higher doses of either N, P, or K in the range of 0 to 150% of the amount in a standard Hoagland solution favor herb and leaf yield of *Centella asiatica* but decrease saponin and sapogenin concentrations in the leaves. Thereby, we focused on the causal relationship among photosynthesis, leaf N, P, and K concentrations, herb and leaf production, and centelloside accumulation in the leaves of *C. asiatica*.

2. Flavonoid accumulation is affected by N, P, and K fertigation in the same way as centelloside accumulation, and centelloside concentrations in leaves of *C. asiatica* can therefore be estimated *in vivo* by means of non-destructive recordings of the chlorophyll fluorescence.

3. Ambient level of UV-B radiation and high PAR intensity additively promote the accumulation of saponins and their respective genins in leaves of *C. asiatica*. Furthermore, we elucidated the causal relationship among the accumulation of centellosides in leaves, photosynthesis, as well as herb and leaf yield of *C. asiatica*. Aiming a monitoring of the specific UV-B response of the plants, we additionally recorded the accumulation of epidermal flavonols and anthocyanins *in vivo* by multiparametric fluorescence measurements.

4. The accumulation of hinokinin in *Hydrocotyle leucocephala* plants is enhanced under ambient level of UV-B and high PAR intensity. Here, we proof the impact of different UV-B/PAR combinations on the concentration of selected phenylpropanoids, namely phenolic acids, flavonols, and hinokinin in leaves which had emerged either before or during the experiment, and in stems.

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B Centelloside accumulation in leaves of *Centella asiatica* is determined by resource partitioning between primary and secondary metabolism while influenced by supply levels of either nitrogen, phosphorus, or potassium[1]

1 Introduction

Centella asiatica (Apiaceae) is a stoloniferous medicinal herb, which grows in tropical and subtropical regions. It has been used in traditional medicine in the therapy of various physical and mental ailments presumably since prehistoric times. Its medicinal properties are attributed to pentacyclic triterpene saponins, also referred to as centellosides, among which asiaticoside and madecassoside and their respective genins asiatic acid and madecassic acid are of particular interest (Inamdar et al., 1996). Several studies reported on the antioxidant (Flora and Gupta, 2007; Pittella et al., 2009), anti-inflammatory (Li et al., 2009), neuroprotective (Shinomol and Muralidhara, 2008; Dhanasekaran et al., 2009; Haleagrahara and Ponnusamy, 2010), and cardioprotective (Bian et al., 2008; Cao et al., 2010) activity of these compounds. Since centelloside accumulation occurs preferentially in the leaves of *C. asiatica* (Gupta et al., 1999; Aziz et al., 2007; Mangas et al., 2008), the aerial parts of the plant are harvested and used for diverse purposes.

During the last years, *C. asiatica* based pharmaceutical and cosmetic products have gained popularity worldwide (James and Dubery, 2009; Devkota et al., 2010a; Singh et al., 2010). Nevertheless, the cultivation of *C. asiatica* for commercial purposes is widely underexplored. As a consequence, the market's demand is largely satisfied by collection from natural populations, which implicates a large variation in concentrations and compositions of centellosides due to genetic variation and growth conditions (Randriamampionona et al., 2007; Devkota et al., 2010a, b; Thomas et al., 2010). Hence, to assure a continuous availability of plant material with high concentrations and desirable compositions of centellosides a well-directed cultivation of *C. asiatica* is needed. So far, a few studies published in the last years aimed at the identification of suitable agronomic practices focussing on the biomass production of *C. asiatica*. However, findings on the effects of nutrient supply on saponin production in plants obtained in *in vitro* or field studies are still scarce and the results even contradictory (Devkota et al., 2010a, b; Szakiel et al., 2011 and references therein; Siddiqui et al., 2011; Prasad et al., 2012). The inconsistency of these findings is probably related to physical and/or chemical interactions between nutrients and

soil, since most of the experiments were conducted using soil as substrate. As a result, the precise knowledge on the relevance of defined nutrients for plant physiology, growth, and centelloside biosynthesis of *C. asiatica* is still lacking.

In general, nitrogen (N), phosphorus (P), and potassium (K) are those mineral elements which are required by plants in largest amounts and play an important role in assuring appropriate growth and development of the plants. The general importance of these minerals for plant primary metabolism is reviewed elsewhere (Epstein and Bloom, 2005; Marschner, 2012 and references therein). To predict the effects of environmental factors, including nutrient availability, on plant secondary metabolism, several hypotheses, such as the carbon-nutrient balance (CNB) (Bryant et al., 1983) and the growth-differentiation balance hypothesis (GDB) (Herms and Mattson, 1992), have evolved. These hypotheses assume a trade-off between primary and secondary metabolism, *i.e.*, in conditions of high nutrient availability growth predominates, but if nutrients are limiting, growth is more restricted than photosynthesis, leading to a build-up of fixed carbon, which can be invested in the synthesis of carbon-based defensive compounds (Watson, 1963; Epstein, 1972; Smith, 1973; McKey, 1979; Bryant et al., 1983). The assumptions were tested by a number of studies revealing that especially phenolic compounds are increasingly synthesized under adverse conditions, paralleled by constraints in growth (Muzika, 1993; Haukioja, 1998; Hale et al., 2005).

Accordingly, saponins are known to accumulate in response to biotic as well as abiotic stresses (Francis et al., 2002; Sparg, et al. 2004). They are synthesized *via* the isoprenoid pathway starting with isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Enzymatic conversions of IPP and DMAPP lead to 2,3-oxidosqualene, which is then cyclized to generate the triterpenoid skeletons such as α- and β-amyrin. The latter undergo several modifications, *i.e.*, oxidation, hydroxylation, and other substitutions originating the *Centella* sapogenins. Finally, the sapogenins are converted into saponins by glycosylation processes (James and Dubery, 2009; Augustin et al., 2011).

The viability of the CNB and GDB hypotheses to predict terpenoid accumulation in plants is considered more critically (Muzika, 1993; Gershenzon, 1994; Haukioja, 1998). Nevertheless, since the formation of centellosides requires substantial amounts of substrate, energy, and specific enzymes (Gershenzon, 1994; James and Dubery, 2009), it is conceivable, that the more plant growth is promoted by high N, P, or K availability, the more resources are required for growth processes at the expense of centelloside synthesis. Therefore, the objectives of the present study were to examine the significance of N, P, or K supply for herb and leaf production and for saponin and sapogenin concentrations in leaves of *C. asiatica*

under more controlled conditions in the greenhouse and under employment of soilless culture. Furthermore, we aimed to exploit the causal relationship among photosynthesis, leaf N, P, and K concentrations, herb and leaf production, and centelloside accumulation in leaves in response to N, P, or K supply. For this purpose, our experiments were conducted in hydroponic culture to eliminate interactions between the nutrients and the substrate. Moreover, we chose well-defined nutrient solutions in order to be able to investigate the relevance of an individual nutrient for photosynthesis, growth, and saponin and sapogenin biosynthesis of *C. asiatica*. In the framework of three discrete greenhouse experiments, the application rates of either N, P, or K were varied while maintaining the other nutrients at a constant level. Net photosynthesis, leaf and herb production, and centelloside concentrations were monitored in periodic intervals. Our work was based on the hypothesis that the increasing application of N, P, or K from 0 to 150% of the amount in a standard Hoagland solution favors herb and leaf yield of *C. asiatica* but decreases saponin and sapogenin concentrations in leaves.

2   Materials and methods

2.1 Plant material

Stock plants of *Centella asiatica* L. Urban were purchased from a commercial nursery (Rühlemann's Kräuter & Duftpflanzen, Horstedt, Germany); the genome-based identification of the species was done by A.N. Nicolas (Institute of Systematic Botany, The New York Botanical Garden, NY, USA). Four weeks before starting the experiments, plantlets were propagated vegetatively to assure genetically identical plants. As soon as the cuttings had rooted, they were fertilized with half strength Hoagland nutrient solution. At the onset of the experiments, 200 homogeneous plants having four to six fully expanded leaves were transplanted into rock wool cubes (600 cm^3, Grodan, Hedehusene, Denmark) each placed on a trivet. Forty plants per treatment group were completely randomized on a greenhouse table.

2.2 Experimental and growth conditions

Experiments on the effect of N, P, and K supply were performed sequentially from June 2011 until May 2012 for a period of eight weeks, respectively. Each experiment consisted of five treatments represented by five nutrient solutions differing in the level of either N, P, or K. A standard Hoagland nutrient solution was chosen for the control treatments (= 100% N, P, and K). It contained 0.50 M KH_2PO_4, 1.00 M KCl, 1.33 M $Ca(NO_3)_2\cdot4H_2O$, and 0.25 M $(NH_4)_2SO_4$. The nutrient solutions of the other treatments were modified, resulting in 0, 30,

60, and 150% of the N, P, or K amount applied to the control treatments. Thus, the treatments are referred to as N0, P0, K0, *etc.* and N150, P150, K150, respectively. In the N and P experiments, adjustments were made by changing the amounts of the above-specified compounds. In the K experiment, the lack of P in the nutrient solutions of the treatments K30 and K60 caused by the reduction of KH_2PO_4 was compensated by the addition of 0.35 M and 0.20 M $(NH_4)H_2PO_4$, respectively. Accordingly, the amount of $(NH_4)_2SO_4$ was reduced to 0.08 and 0.15 M, respectively. The supply of micronutrients was similar for all treatments. Hence, the nutrient solutions contained 1 M $MgSO_4 \cdot 7H_2O$, 0.05 M KCl, which was substituted by 0.05 M NaCl in the K0 treatment, 0.025 M H_3BO_3, 0.002 M $MnSO_4 \cdot 4H_2O$, 0.002 M $ZnSO_4 \cdot 7H_2O$, 0.0005 M $CuSO_4 \cdot 5H_2O$, 0.0005 M H_2MoO_4 (85% MoO_3), and 0.4 M $FeSO_4 \cdot 7H_2O$.

Starting the experiments, the five nutrient solutions were applied to forty plants in each treatment group until substrate saturation. In order to assure an adequate nutrient and water supply, the plant's consumption was documented on a weight basis, and application rates were calculated with regard to plant biomass production. The application of the nutrient solutions was carried out twice or three times a week according to the plant's demand. Irradiation was supplied by natural light supplemented by 400 W high-pressure sodium lamps (Son-T-Agro 400, Philips International B.V., Amsterdam, Netherlands) to reach a photoperiod of 16 h and to maintain a photosynthetic active radiation (PAR) of 300 μmol m^{-2} s^{-1} at 40 cm above table surface. Temperature and relative humidity were recorded by Data Loggers (TGU-4550 Tinytag, Ultra 2, Gemini DATA LOGGERS, Chichester, West Sussex, UK) at plant height. The average day/night temperature was 23.8 °C/18.1 °C during the N experiment, 25.3 °C/17.7 °C during the P experiment, and 24.2 °C/17.5 °C during the K experiment. The day/night relative humidity was on average 48.8%/61.2% in the N experiment, 60.4%/82.9% in the P experiment, and 56.7%/71.2% in the K experiment.

2.3 Sampling and sample preparation

The leaves and stems of ten experimental plants of each treatment group were harvested separately after 2, 4, 6, and 8 weeks of treatment application (WTA). Immediately after harvest, the samples were frozen at –25 °C and lyophilized (GAMMA 1-16 LSC, Christ, Osterode, Germany). After lyophilization the dry weight of the leaves and stems was determined gravimetrically. Herb yield was calculated by summing up leaf and stem dry weight. Subsequently, N, P, and K concentrations, as well as saponin and sapogenin concentrations in leaves were analyzed from the same sample.

2.4 Determination of N, P, and K concentrations in leaves

Concentrations of the key mineral elements in leaves were determined after 8 WTA. The leaf samples were lyophilized and ground separately with a mixer mill (MM 2000, Retsch, Haan, Germany). Prior to N analysis, leaf powder was dried overnight at 105 °C, and 3 mg was placed in a tin capsule. The N concentrations were determined using a Carlo Erba CN 1180 Elemental Analyzer (Carlo Erba, Milan, Italy). To analyze the concentrations of P and K, plant samples were acid-digested using a microwave digestion system (MarsXpress, CEM, Matthews, USA). 0.05 g of leaf powder was weighed into PFA Teflon® vessels and provided with 10 mL of 65% m m^{-1} HNO$_3$ and 4 mL of 35% m m^{-1} H$_2$O$_2$. Digestion was done at 180 °C. After cooling, the contents of the vessels were transferred into 25 mL flasks and filled up with deionized water. The P concentrations were determined with a continuous flow auto-analyzer (TrAAcs 800, Bran-Luebbe, Hamburg, Germany) and the K concentrations were analyzed by atomic absorption spectroscopy (AAnalyst 300, Perkin Elmer Inc., Massachusetts, USA). The detailed results of the analyses are presented in Table S1.

2.5 Determination of saponin, sapogenin, and total centelloside concentrations in leaves

After lyophilization, the leaf material of ten plants was ground, as described above. Subsequently, 50 mg of the leaf powder was placed in a 1.5-mL micro centrifuge tube (VWR, West Chester, USA) and extracted under sonication for 15 min with 0.5 mL of MeOH–H$_2$O (9/1, v/v). Extraction was repeated three times. After centrifugation at 6000 rpm (Rotilabo® mini centrifuge, Carl Roth GmbH, Karlsruhe, Germany) for 10 min, the supernatants were combined and filled up to a volume of 2 mL. Before injection in the high-performance liquid chromatograph (HPLC) the extracts were filtered through a 0.2 μm regenerated cellulose syringe filter (Phenex, Phenomenex Inc., Aschaffenburg, Germany). Every sample was assayed in duplicate.

Asiaticoside, madecassoside, asiatic acid, and madecassic acid are the quality determining saponins and sapogenins of *C. asiatica* (Stahl-Biskup, 2006) and were chosen as representatives for all centellosides. The chemical structure of the four compounds is shown in Fig. 1 (ACD/ChemSketch 12.0, Advanced Chemistry Development Inc., Toronto, Ontario, Canada).
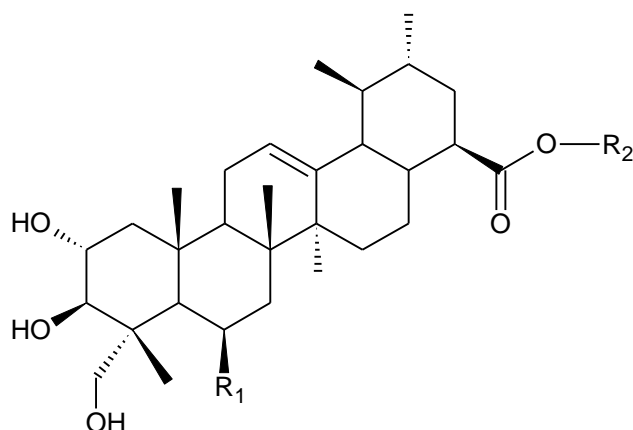
asiaticoside   R1 = H   R2 = Glu-Glu-Rha
madecassoside   R1 = OH   R2 = Glu-Glu-Rha
asiatic acid   R1 = H   R2 = H
madecassic acid R1 = OH   R2 = H

Fig 1. Chemical structure of asiaticoside, madecassoside, asiatic acid, and madecassic acid. Glu, glucose; Rha, rhamnose.

Prior to quantification, the compounds were identified by mass spectrometry. The main ions of asiaticoside in negative ESI-MS were $[M–H]^-$ with m/z 957, $[M+H_3COO]^-$ with m/z 1003, $[M–Glu–Glu–Rha–H]^-$ with m/z 487, and $[M–Glu–Glu–Rha–H_2O–H]^-$ with m/z 469. The main ions of asiatic acid were $[M–H]^-$ with m/z 487 and $[M–H_2O–H]^-$ with m/z 469. Analogous ions augmented by the mass of 16, were found for madecassoside and madecassic acid. These ions were formed in the ion source during ionization (in-source fragmentation). Additionally, the fragmentations 1003 > 487, 957 > 487, 957 > 469 and 1019 > 503, 973 > 503 for asiaticoside and madecassoside, respectively, were found in selected reaction mode. The detected ions as well as the retention time were the same in the sample extracts and in the reference compounds of asiaticoside and asiatic acid (Sigma Aldrich, St. Louis, Missouri, USA) and are in agreement with published data (Mauri and Pietta, 2000; Rafamantanana et al., 2009).

The concentrations of the saponins and sapogenins in leaves were determined using an HPLC device (Agilent, Series 1100, Waldbronn, Germany) equipped with a binary pump, a degasser, an autosampler, a thermostated column oven, and a multiwavelength-UV-detector. Separation of the four compounds was accomplished with a Nucleodur C18 reversed phase column (250 × 4 mm, 5 μm; Macherey & Nagel, Düren, Germany) at a temperature of 30 °C. Eluent A consisted of water, and eluent B was acetonitrile, both acidified with 0.01% formic acid (VWR, West Chester, USA). The following gradient programme was used for eluent B: 20–35% (0–15 min), 35–65% (15–30 min), 65–80% (30–35 min), 80% (35–40 min), 80–65%

(40–45 min), 65–35% (45–55 min), and 35–20% (55–70 min) (modified after Rafamantanana et al., 2009). The flow rate of the mobile phase was 1 mL min^{-1}, and detection wavelength was set at 206 nm. Reference compounds were used to establish an external calibration curve ranging from 12.3 to 788 µg mL^{-1} for asiaticoside and from 4.9 to 148 µg mL^{-1} for asiatic acid. As described by Rafamantanana et al. (2009), madecassoside and madecassic acid were quantified using the reference substances of asiaticoside and asiatic acid. The isomers madecassoside and asiaticoside B as well as madecassic acid and terminolic acid were considered as one compound, respectively. Total centellosides were calculated by summing up the concentrations of asiaticoside, madecassoside, asiatic acid, and madecassic acid.

2.6 Net photosynthesis

The net photosynthesis (Pn) of ten adult leaves of each treatment group was recorded weekly using a portable photosynthesis system (CIRAS-2, PP Systems International, Amesbury, MA, USA). The petioles of the leaves were labelled to ensure measurement of the same ontogenetic stage of leaves in each treatment and during the whole period of the experiments. The PAR intensity at the measuring site ranged between 800 and 1000 µmol m^{-2} s^{-1}. CO_2 concentration was set at 400 ± 5 ppm, and the air flow rate was 200 mL min^{-1}. To compensate for the natural daytime variations of temperature and air humidity in the greenhouse, plants of the different treatments were measured alternately.

2.7 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 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 Effect of nitrogen supply

In general the increase in N supply up to the treatment N60 stimulated plant growth and led to higher herb and leaf yields (Table 1). At 8 WTA the highest herb (HY) and leaf yields (LY) were obtained from the treatment N60 (HY 13.95 g DM, LY 6.11 g DM), which did not differ statistically from the treatment N100 (HY 13.74 g DM, LY 5.96 g DM). The treatment

N150 showed a lower herb yield of 10.07 g DM and a lower leaf yield of 4.33 g DM. The lowest herb and leaf yields were harvested from the treatment N0 (HY 1.13 g DM, LY 0.44 g DM). These results roughly reflect the net photosynthesis as determined over the whole experimental period (Fig. S1A).

Table 1. Effect of N supply levels on herb and leaf yield 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 after 2, 4, 6, and 8 weeks of treatment application.

	Treatment	Weeks of treatment application							
		2		**4**		**6**		**8**	
Herb yield	N0	0.54 ± 0.03	a	0.82 ± 0.04	a	0.96 ± 0.08	a	1.13 ± 0.06	a
[HY g DM]	N30	0.79 ± 0.04	b	1.25 ± 0.09	b	2.78 ± 0.31	b	5.21 ± 0.46	b
	N60	1.10 ± 0.08	c	2.60 ± 0.12	c	6.98 ± 0.33	c	13.95 ± 0.60	d
	N100	1.28 ± 0.09	c	3.17 ± 0.14	d	8.56 ± 0.34	d	13.74 ± 0.51	d
	N150	1.14 ± 0.07	c	3.10 ± 0.12	d	7.22 ± 0.56	c	10.07 ± 0.93	c
Leaf yield	N0	0.27 ± 0.02	a	0.37 ± 0.02	a	0.39 ± 0.03	a	0.44 ± 0.03	a
[LY g DM]	N30	0.41 ± 0.02	b	0.62 ± 0.05	b	1.26 ± 0.13	b	2.19 ± 0.16	b
	N60	0.57 ± 0.04	c	1.31 ± 0.06	c	3.16 ± 0.14	c	6.11 ± 0.21	d
	N100	0.68 ± 0.05	d	1.58 ± 0.08	d	3.81 ± 0.12	d	5.96 ± 0.21	d
	N150	0.62 ± 0.03	cd	1.56 ± 0.07	d	3.31 ± 0.23	c	4.33 ± 0.38	c

Mean $\pm$ standard error ($n = 10$). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

The concentrations of the two saponins (asiaticoside and madecassoside) augmented during the whole experimental time, especially in the treatment N0 (Fig. 2B and D). The treatments N60, N100, and N150 showed a maximum of saponin concentrations at 6 WTA with a subsequent slight decrease until 8 WTA. The concentrations of the sapogenins (asiatic acid and madecassic acid) developed inversely to the concentrations of the saponins (Fig. 2C and E), with a notable increase in sapogenin concentrations in the leaves of the treatment N150 during the last two weeks. Over the whole experimental period the highest saponin concentrations were observed in the leaves of the treatment N0 (Fig. 2B and D). At 8 WTA this treatment had reached an asiaticoside concentration of 30.24 mg g^{-1} leaf DM and a madecassoside concentration of 40.81 mg g^{-1} leaf DM. The highest sapogenin concentrations were determined in the leaves of the treatment N150 (9.31 mg asiatic acid g^{-1} DM, 10.35 mg

madecassic acid g^{-1} DM), followed by the treatment N0 (7.23 mg asiatic acid g^{-1} DM, 9.13 mg madecassic acid g^{-1} DM) (Fig. 2C and E). Thus, the increase in N supply by trend led to a decrease in saponin concentrations, although there were no significant differences among the treatments N30, N60, N100, and N150. In contrast, disregarding the treatment N0, the sapogenin concentrations increased with the increase in N supply. Hence, at 8 WTA the highest total centelloside concentrations were achieved with the treatment N0 (87.41 mg g^{-1} DM), followed by the treatment N150 (35.50 mg g^{-1} DM) (Fig. 2A).

At the end of the study a positive correlation was found between net photosynthesis (Pn) and HY ($r = 0.77**$), LY ($r = 0.77**$), and leaf N concentrations ($r = 0.77**$), between HY and leaf N concentrations ($r = 0.80**$), as well as between LY and leaf N concentrations ($r = 0.81**$). Regarding the leaf concentrations of saponins and sapogenins a strong positive correlation was observed between asiaticoside and madecassoside ($r = 0.99**$) as well as between asiatic acid and madecassic acid ($r = 0.97**$) (*data not shown*). A negative correlation was assessed between the concentrations of the two saponins and HY, LY, Pn, and especially leaf N concentrations (Table 2). Concerning asiatic acid and madecassic acid a moderate negative correlation was found between the two sapogenins and P and K concentrations in leaves (Table 2).

Table 2. Correlation analysis between centelloside (asiaticoside, madecassoside, asiatic acid, madecassic acid) concentrations, herb and leaf yield (HY, LY), net photosynthesis (Pn), and N, P, and K 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 in the standard Hoagland solution, and evaluated after 8 weeks of treatment application.

	Asiaticoside [mg g^{-1} DM]	Madecassoside [mg g^{-1} DM]	Asiatic acid [mg g^{-1} DM]	Madecassic acid [mg g^{-1} DM]
HY [g DM]	–0.76**	–0.75**	–0.09	–0.20
LY [g DM]	–0.75**	–0.74**	–0.12	–0.23
Pn [µmol m^{-2} s^{-1}]	–0.74**	–0.74**	–0.10	–0.23
N [mg g^{-1}]	–0.96**	–0.96**	–0.33*	–0.50**
P [mg g^{-1}]	–0.24	–0.28	–0.65**	–0.68**
K [mg g^{-1}]	–0.40**	–0.43**	–0.63**	–0.71**

Pearson's correlation coefficients. *Level of significance: $p \leq 0.05$. ** Level of significance: $p \leq 0.01$.
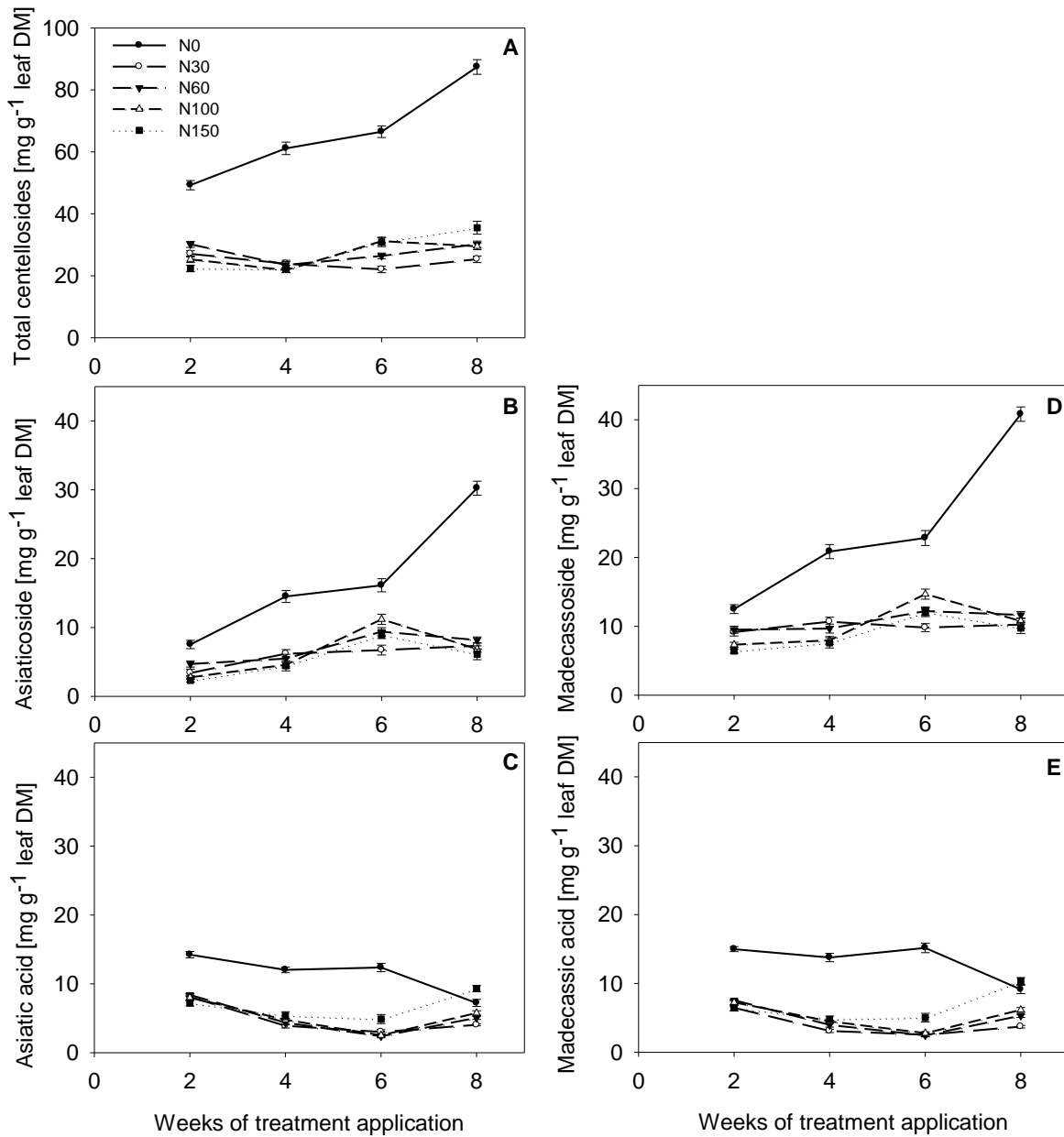
Fig. 2. Effect of N supply levels on total centelloside (A), asiaticoside (B), asiatic acid (C), madecassoside (D), and madecassic acid (E) concentrations in leaves of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount in the standard Hoagland solution, and evaluated after 2, 4, 6, and 8 weeks of treatment application. Mean ± standard error ($n = 10$).

3.2 Effect of phosphorus supply

Herb yield as well as leaf yield augmented with the increase in P supply (Table 3). The highest HY of 11.46 g DM and the highest LY of 4.56 g DM were achieved with the treatment P150 at 8 WTA. Comparatively, the treatments P60 (HY 10.34 g DM, LY 4.06 g DM) and P100 (HY 10.64 g DM, LY 4.14 g DM) induced significantly lower herb and leaf yields, whereas the lowest values were observed in the treatment P0 (HY 1.52 g DM, LY 0.64 g DM).

Table 3. Effect of P supply levels on herb and leaf yield 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 after 2, 4, 6, and 8 weeks of treatment application.

	Treatment	Weeks of treatment application							
		2		**4**		**6**		**8**	
Herb yield	P0	0.63 ± 0.05	a	0.99 ± 0.09	a	1.27 ± 0.10	a	1.52 ± 0.10	a
[HY g DM]	P30	0.83 ± 0.06	b	2.22 ± 0.18	b	5.68 ± 0.22	b	8.31 ± 0.16	b
	P60	0.89 ± 0.07	b	2.54 ± 0.12	bc	6.76 ± 0.16	c	10.34 ± 0.14	c
	P100	0.98 ± 0.04	b	2.77 ± 0.17	cd	7.24 ± 0.18	d	10.64 ± 0.23	c
	P150	0.96 ± 0.05	b	3.07 ± 0.09	d	7.30 ± 0.14	d	11.46 ± 0.20	d
Leaf yield	P0	0.30 ± 0.03	a	0.46 ± 0.04	a	0.54 ± 0.05	a	0.64 ± 0.04	a
[LY g DM]	P30	0.44 ± 0.03	b	1.04 ± 0.08	b	2.40 ± 0.11	b	3.16 ± 0.05	b
	P60	0.45 ± 0.03	b	1.15 ± 0.06	bc	2.87 ± 0.08	c	4.06 ± 0.05	c
	P100	0.51 ± 0.02	b	1.29 ± 0.08	cd	3.04 ± 0.08	cd	4.14 ± 0.10	c
	P150	0.51 ± 0.03	b	1.46 ± 0.05	d	3.11 ± 0.07	d	4.56 ± 0.10	d

Mean ± standard error (*n* = 10). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

Irrespective of P supply, the asiaticoside and madecassoside concentrations increased, while the asiatic acid and madecassic acid concentrations decreased during the eight weeks of the experiment (Fig. 3B–E). This trend was more pronounced for the treatments P0 and P30; an exception was presented by the treatment P0 at 6 WTA, when saponin concentrations were extraordinary low and sapogenin concentrations were remarkably high (Fig. 3B–E). The highest asiaticoside and madecassoside concentrations were observed at 8 WTA in the leaves of the treatment P0 (28.25 mg g^{-1} DM and 33.26 mg g^{-1} DM, respectively), followed by the treatment P30 (19.45 mg g^{-1} DM and 22.59 mg g^{-1} DM, respectively). Concerning the asiatic

acid and madecassic acid concentrations, a very constant ranking of the treatments was observed during the whole experimental period (Fig. 3C and E). The highest asiatic acid and madecassic acid concentrations were found in the leaves of the treatment P0 (20.42 mg g^{-1} DM and 22.80 mg g^{-1} DM, respectively) at 6 WTA. Finally, the increase in P supply up to the treatment P60 resulted in a decline in saponin as well as sapogenin concentrations (Fig. 3B–E). The total centelloside concentrations reached the maximum at 8 WTA in the leaves of the treatment P0 (78.03 mg g^{-1} DM), followed by P30 (53.42 mg g^{-1} DM) (Fig. 3A).

At 8 WTA we observed a positive correlation between Pn and HY ($r = 0.94$**), LY ($r = 0.94$**), and N, P, and K concentrations in leaves ($r = 0.91$**, $r = 0.81$**, $r = 0.84$**, respectively). HY was also positively associated with N, P and K concentrations ($r = 0.93$**, $r = 0.81$**, $r = 0.87$**). Corresponding positive correlations were observed between LY and N, P, and K concentrations ($r = 0.93$**, $r = 0.82$**, $r = 0.87$**). Furthermore, strong positive correlations were observed between asiaticoside and madecassoside ($r = 0.99$**) and between asiatic acid and madecassic acid concentrations ($r = 0.99$**) (*data not shown*). Strong negative correlations were assessed between the two saponins and HY, LY, Pn, and N, P, and K concentrations in leaves (Table 4); in case of the two sapogenins, the correlations were high ($0.6 < r < 0.8$).

Table 4. Correlation analysis between centelloside (asiaticoside, madecassoside, asiatic acid, madecassic acid) concentrations, herb and leaf yield (HY, LY), net photosynthesis (Pn), and N, P, and K concentrations 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 after 8 weeks of treatment application.

	Asiaticoside [mg g^{-1} DM]	Madecassoside [mg g^{-1} DM]	Asiatic acid [mg g^{-1} DM]	Madecassic acid [mg g^{-1} DM]
HY [g DM]	–0.92**	–0.93**	–0.74**	–0.75**
LY [g DM]	–0.92**	–0.93**	–0.74**	–0.75**
Pn [μmol m^{-2} s^{-1}]	–0.90**	–0.91**	–0.73**	–0.75**
N [mg g$^-$1]	–0.94**	–0.94**	–0.72**	–0.75**
P [mg g$^-$1]	–0.85**	–0.84**	–0.67**	–0.72**
K [mg g$^-$1]	–0.82**	–0.83**	–0.78**	–0.81**

Pearson's correlation coefficients. *Level of significance: $p \leq 0.05$. **Level of significance: $p \leq 0.01$.
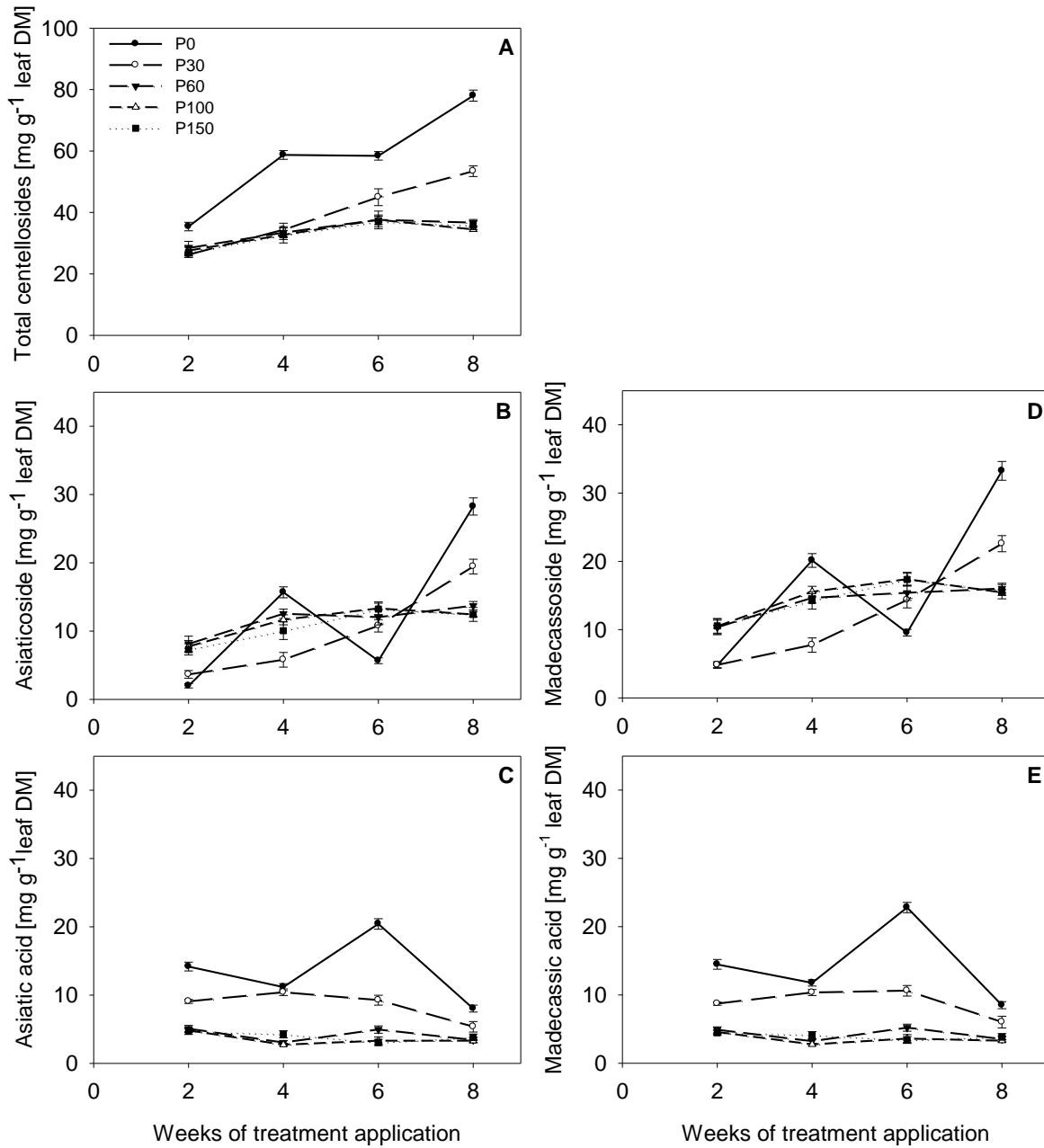
Fig. 3. Effect of P supply levels on total centelloside (A), asiaticoside (B), asiatic acid (C), madecassoside (D), and madecassic acid (E) concentrations in leaves of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (P0), 30% (P30), 60% (P60), 100% (P100), or 150% (P150) of the P amount in the standard Hoagland solution, and evaluated after 2, 4, 6, and 8 weeks of treatment application. Mean ± standard error (*n* = 10).

3.3 Effect of potassium supply

Potassium supply induced no significant differences among the treatments concerning herb and leaf yield until 2 WTA (Table 5). At 8 WTA the maximum herb and leaf yields were obtained from the treatment K30 (HY 19.51 g DM, LY 8.14 g DM), followed by the treatments K60 and K100. The significantly lowest herb and leaf yields were harvested from the treatment K0 (HY 2.33 g DM, LY 1.20 g DM).

Table 5. Effect of K supply levels on herb and leaf yield 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 after 2, 4, 6, and 8 weeks of treatment application.

	Treatment	Weeks of treatment application							
		2		**4**		**6**		**8**	
Herb yield	K0	0.70 ± 0.05	n.s.	1.61 ± 0.17	a	1.99 ± 0.26	a	2.33 ± 0.47	a
HY [g DM]	K30	0.69 ± 0.07		3.60 ± 0.23	c	10.28 ± 0.33	c	19.51 ± 0.46	d
	K60	0.73 ± 0.07		3.16 ± 0.16	bc	9.15 ± 0.36	c	16.47 ± 0.79	c
	K100	0.62 ± 0.04		2.96 ± 0.18	b	9.55 ± 0.18	c	15.96 ± 0.45	c
	K150	0.65 ± 0.05		2.94 ± 0.24	b	7.61 ± 0.62	b	12.59 ± 0.87	b
Leaf yield	K0	0.42 ± 0.03	n.s.	0.88 ± 0.08	a	1.03 ± 0.11	a	1.20 ± 0.22	a
[LY g DM]	K30	0.38 ± 0.04		1.84 ± 0.10	c	4.54 ± 0.12	d	8.14 ± 0.26	d
	K60	0.38 ± 0.03		1.65 ± 0.07	bc	4.06 ± 0.14	c	6.64 ± 0.38	c
	K100	0.35 ± 0.03		1.55 ± 0.08	b	4.35 ± 0.08	cd	6.92 ± 0.25	c
	K150	0.37 ± 0.04		1.52 ± 0.11	b	3.58 ± 0.26	b	5.58 ± 0.30	b

Mean $\pm$ standard error ($n = 10$). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$); n.s.: not significant.

In the time course, the concentrations of the two saponins (asiaticoside and madecassoside) decreased until 4 WTA, irrespective of K supply (Fig. 4B and D). Subsequently, the concentrations strongly rose until 6 WTA, followed either by a steady state or a slight increase in case of the treatments K100 and K150. The concentrations of asiatic acid and madecassic acid developed inversely to the saponin concentrations until 6 WTA (Fig. 4C and E), with a following increase in sapogenin concentrations in the leaves of the treatment K0 and a steady state in case of the treatments K30, K60, K100, and K150 until 8 WTA. The highest saponin concentrations (31.74 mg asiaticoside g^{-1} DM and 31.69 mg madecassoside g^{-1} DM) were reached with the treatment K0 at 6 WTA. At 8 WTA, the

treatment K0 still presented the highest saponin concentrations, while the lowest concentrations were detected in the leaves of the treatment K30 (Fig. 4B and D). The maximum asiatic acid and madecassic acid concentrations were also observed in the leaves of the treatment K0, which were especially high at 4 WTA (19.10 mg asiatic acid g^{-1} DM and 17.15 mg madecassic acid g^{-1} DM) (Fig. 4C and E). At the end of the study the highest concentrations were still detected in the leaves of this treatment, while the other treatments did not differ significantly in sapogenin concentrations. Disregarding the treatment K0, the increase in K supply by trend led to an increase in saponin concentrations. At 8 WTA, the highest total centelloside concentrations (78.11 mg g^{-1} DM) were detected in the leaves of the treatment K0, followed by the treatments K150 (53.50 mg g^{-1} DM), K60 (48.08 mg g^{-1} DM), K100 (43.46 mg g^{-1} DM), and K30 (35.31 mg g^{-1} DM) (Fig. 4A).

Table 6. Correlation analysis between centelloside (asiaticoside, madecassoside, asiatic acid, madecassic acid) concentrations, herb and leaf yield (HY, LY), net photosynthesis (Pn), and N, P, and K concentrations 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 after 8 weeks of treatment application.

	Asiaticoside [mg g^{-1} DM]	Madecassoside [mg g^{-1} DM]	Asiatic acid [mg g^{-1} DM]	Madecassic acid [mg g^{-1} DM]
HY [g DM]	–0.50**	–0.51**	–0.82**	–0.81**
LY [g DM]	–0.49**	–0.50**	–0.83**	–0.82**
Pn [μmol m^{-2} s^{-1}]	–0.16	–0.19	–0.40*	–0.39*
N [mg g^{-1}]	–0.75**	–0.72**	–0.20	–0.21
P [mg g^{-1}]	–0.31	–0.24	0.35*	0.34
K [mg g^{-1}]	0.22	0.16	–0.54**	–0.52**

Pearson's correlation coefficients. *Level of significance: $p \leq 0.05$. **Level of significance: $p \leq 0.01$.

The correlation analysis at 8 WTA revealed weak positive correlations between Pn and HY ($r = 0.47^{**}$), LY ($r = 0.48^{**}$), and K concentration in leaves ($r = 0.38^{*}$). Furthermore, HY and leaf N concentrations ($r = 0.52^{**}$), and LY and leaf N concentrations ($r = 0.49^{**}$) were associated positively. Strong positive correlations were found between asiaticoside and madecassoside ($r = 0.98^{**}$) and between asiatic acid and madecassic acid concentrations in leaves ($r = 0.99^{**}$) (*data not shown*). Negative associations were observed between saponin concentrations and HY, LY, and especially leaf N concentrations (Table 6). Asiatic acid and

madecassic acid concentrations strongly correlated negatively with HY and LY; slight negative correlations were also found between sapogenin concentrations and Pn. Furthermore, sapogenin concentrations were associated negatively with leaf K concentrations (Table 6).
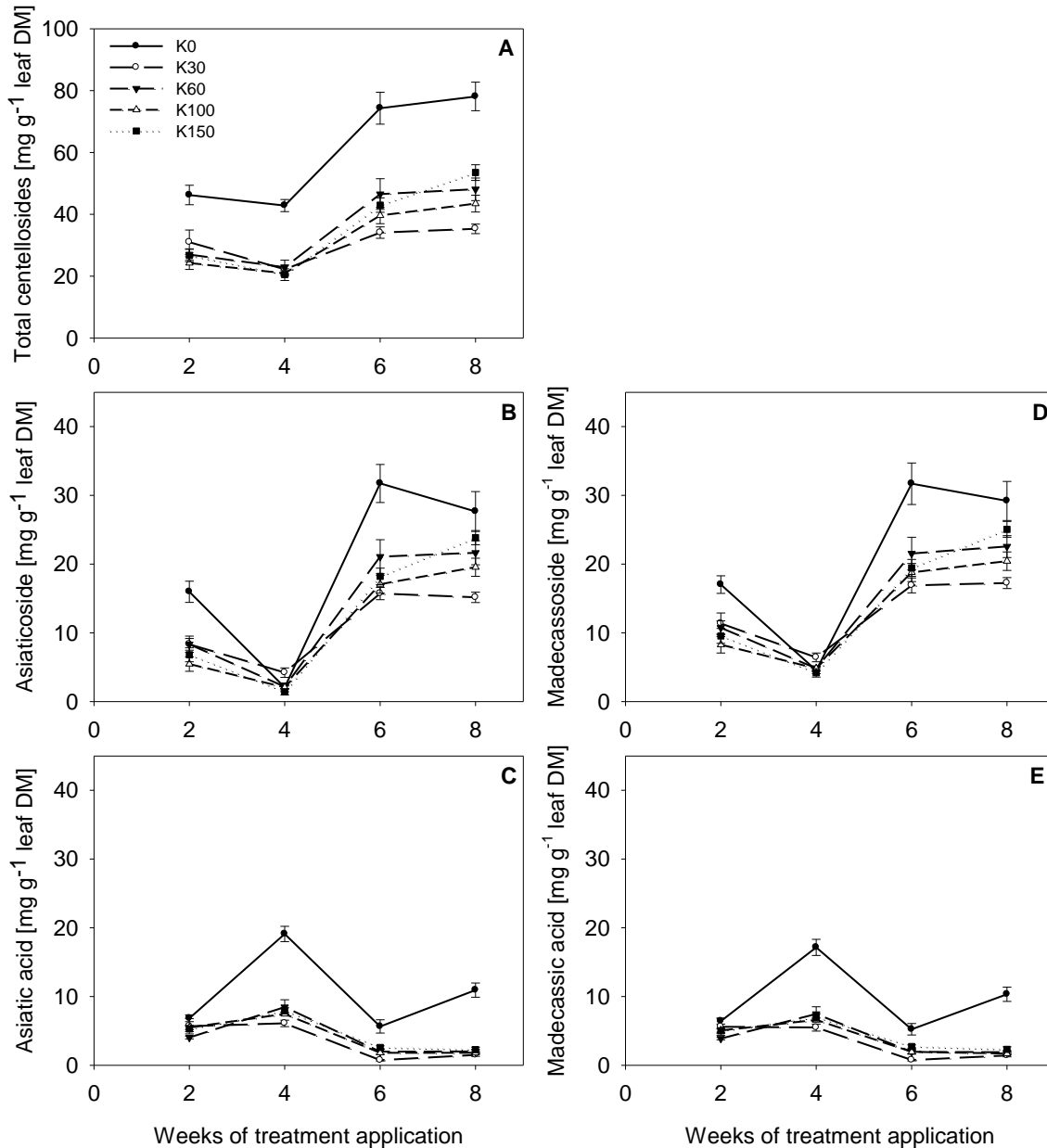
Fig. 4. Effect of K supply levels on total centelloside (A), asiaticoside (B), asiatic acid (C), madecassoside (D), and madecassic acid (E) concentrations in leaves of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (K0), 30% (K30), 60% (K60), 100% (K100), or 150% (K150) of the K amount in the standard Hoagland solution, and evaluated after 2, 4, 6, and 8 weeks of treatment application. Mean ± standard error (n = 10).

4   Discussion

In the present study the significance of N, P, or K supply for herb and leaf yield as well as for centelloside accumulation in leaves of *C. asiatica* was investigated. Thereby, we focussed on the causal relationship among net photosynthesis, leaf N, P, and K concentrations, herb and leaf production, and saponin as well as sapogenin concentrations in leaves. We hypothesized that increasing N, P, or K from 0 to 150% of the amount in a standard Hoagland solution favors herb and leaf yield of *C. asiatica* but decreases saponin and sapogenin concentrations in leaves.

As expected, the supply of N, P, and K had a strong impact on herb and leaf yield (Tables 1, 3, and 5). The highest yields were reached with the treatments N60 and N100 in the N experiment (Table 1), with the treatment P150 in the P experiment (Table 3), and with the treatment K30 in the K experiment (Table 5). Moreover, these treatments presented a high net photosynthesis, which was related to high N concentrations in leaves (Table S1 and Fig. S1A–C). The positive relationship between N concentrations and net photosynthesis is well documented (Evans, 1983; Sage et al., 1987; Lawlor et al., 2001). High leaf N concentrations favor the synthesis of components of the photosynthetic apparatus, especially of the CO_2 assimilating enzyme ribulose-1,5-bisphosphate carboyxlase/oxygenase (Wong, 1979; Ferrar and Osmond, 1986; Evans and Terashima, 1987; Seemann et al., 1987). This presumably led to the improved photosynthetic capacity in the leaves of the treatments N60, P150, and K30, resulting in the higher net photosynthesis and finally in the higher herb and leaf yields (Hirose, 1988). The further increase in N (N150) and especially in K (K60, K100, and K150) supply did not have additional positive effects but even caused a decline in herb and leaf yields. Considering the decrease in leaf N concentrations (Table S1), we assume that the higher N and K supply provoked an imbalance in nutrient uptake, resulting in the lower N concentrations in leaves, lower net photosynthesis (Fig. S1A and C), and consequently a decline in herb and leaf yields.

Independently from N, P, or K supply the accumulation of the two saponins (asiaticoside and madecassoside) and also of the two sapogenins (asiatic acid and madecassic acid) followed the same course throughout the period of each experiment (Figs. 2B–E, 3B–E, and 4B–E). In contrast, the leaf concentrations of saponins and sapogenins developed inversely to each other. Considering the fluctuations throughout the experimental time (Figs. 2B–E, 3B–E, and 4B–E), a metabolic turnover of saponins and sapogenins is suggested. However, we could not assess if this turnover is rather related to the processes of glycosylation and hydrolysis, the degradation and *de novo* synthesis of entire molecules, or both in parallel.

Beyond herb and leaf yield, higher doses of N, P, and K also influenced leaf concentrations of centellosides. The highest total centelloside concentrations were observed in the leaves of those plants which did not receive any N, P, or K *via* fertigation (Figs. 2A, 3A, and 4A). The very high saponin and sapogenin concentrations induced by these treatments are in accordance with previous studies reporting on higher concentrations caused by a lower nutrient availability (Devkota et al., 2010 a, b; Prasad et al., 2012). Plant growth of the treatments N0, P0, and K0 was strongly limited (Table 1, 3, and 5). Considering the assumptions of the CNB and the GDB (Bryant et al., 1983; Herms and Mattson, 1992) it is suggested, that N, P, or K deficiency promoted substrate allocation from primary to secondary metabolism favoring the accumulation of defensive compounds, *i.e.*, of saponins and sapogenins in the leaves of these treatment groups.

The increase in N supply by trend resulted in a decrease in saponin concentrations in leaves (Fig. 2B and D). Analogous findings were also published for other terpenoid compounds (*e.g.*, Mihaliak and Lincoln, 1989; Singh et al., 1989; Van Wassenhove et al., 1990). The negative correlations between saponin concentrations and herb and leaf yield, net photosynthesis, and especially N concentrations in leaves (Table 2) support the idea of a trade-off between primary and secondary metabolism (Coley et al., 1985; Herms and Mattson, 1992). Saponins are generated by the glycosylation of sapogenins, mediated by glycosyltransferases (James and Dubery, 2009; Augustin et al., 2011). Therefore we presume that the accumulation of N in the leaf was allocated to the synthesis of nitrogenous compounds required for photosynthesis and growth processes at the expenses of the synthesis of enzymes required for saponin synthesis, *i.e.*, glycosyltransferases. In contrast to the saponins, leaf concentrations of sapogenins augmented with the level of N fertigation from the treatment N30 up to the treatment N150 (Fig. 2C and E). This augmentation might partly be explained by constrains in sapogenin glycosylation. However, contrary to the treatments N30 and N60, the total centelloside concentrations in the leaves of the treatment groups N100 and especially N150 increased from 2 to 8 WTA (Fig. 2A). This suggests that also *de novo* synthesis of sapogenins should have contributed to the sapogenin concentrations in the leaves of these treatment groups. Nevertheless, it is not clear whether the increase in N supply itself promoted the augmentation in sapogenin concentrations. Considering the lower P and K concentrations in the leaves of the treatment groups N150 and N100 (Table S1), and regarding the negative associations between sapogenins and P and K concentrations in leaves (Table 2), it is suggested that high P and K concentrations in leaves counteracted, rather than high N levels promoted sapogenin accumulation.

During the time course of the P experiment, the increase in saponin concentrations in leaves was accompanied by a decrease in sapogenin concentrations (Fig. 3B–E), which notwithstanding resulted in an increase in total centelloside concentrations (Fig. 3A). This indicates that saponin and sapogenin accumulation occurred concomitantly. Since the increase in total centelloside concentrations was more pronounced in the leaves of the low P supply treatments (Fig. 3A), it is suggested that both glycosylation processes and *de novo* synthesis of sapogenins were promoted. Consequently, the increase in P supply up to the treatment P60 resulted in a decline in leaf concentrations of saponins and sapogenins (Fig. 3B–E). Analogous to the N experiment, strong negative correlations were observed between saponins and herb and leaf yield, net photosynthesis, and N concentrations as well as between sapogenins and K concentrations in leaves (Table 4). Therefore, it is supposed that also the increase in P supply favored the distribution of N towards photosynthesis and growth processes resulting in the lower saponin accumulation in the leaves of the high P supply treatments, while sapogenin synthesis rather was negatively affected by augmenting P and K concentrations in leaves.

In contrast to the N and P experiments, with the exception of K0 the intensification of K fertigation by trend led to an increase in saponin concentrations in leaves (Fig. 4B and D). Nevertheless, saponins were negatively associated with N concentrations. Since the increase in K supply caused a decline in N disposability in leaves (Table S1), accompanied by reductions in growth (Table 5), it is presumed that the decrease in N demands for growth processes favored the distribution of N towards glycosylation processes, leading to higher saponin concentrations in the leaves of the high K supply treatments. Furthermore, in this experiment the sapogenins were stronger negatively correlated with herb and leaf yield and net photosynthesis, than the saponin concentrations in leaves (Table 6). Since the saponin concentrations increased with the level of K fertigation and sapogenin concentrations remained similar for all treatment groups, there should have been a higher rate of *de novo* synthesis of sapogenins in the high K supply treatments. Anyway, the negative association between sapogenin and K concentrations in leaves suggests, that it was not the increase in K supply itself that promoted the synthesis of these compounds. Considering the limited growth and the corresponding decline in resource demand for primary metabolism, the higher rate of *de novo* synthesis of sapogenins in the high K supply treatments can possibly be attributed to higher resource availability for secondary metabolism (Coley et al., 1985; Herms and Mattson, 1992). Moreover, since nutrient deficiency affects growth more than it affects assimilation rate per time unit, it is conceivable that especially the build-up of fixed carbon

promoted the synthesis of carbon-based sapogenins in the high K supply treatments (Watson, 1963; Epstein, 1972; Smith, 1973; McKey, 1979; Bryant et al., 1983).

Accordingly, a number of studies report on the increased accumulation of phenolic compounds in response to nutrient limitation (Muzika, 1993; Haukioja, 1998; Hale et al., 2005). The carbon skeletons required for phenol synthesis are known to be provided either by the Calvin cycle or by the oxidative pentose phosphate pathway (OPPP). Under stress conditions, the activity of the OPPP is enhanced leading to an increased substrate supply for the synthesis of phenolic compounds (Fahrendorf et al., 1995; Hare and Cress, 1997; Lattanzio et al., 2009). However, in case of the synthesis of saponins it is still unclear which path is predominantly affected by the mineral supply, since the source of carbon skeletons for the centelloside synthesis was not investigated in the present study.

In summary, our hypothesis was only partly confirmed. Indeed, the increasing N, P, or K application enhanced herb and leaf yield, but plant growth immediately declined when the applied amount of one single nutrient caused nutrient imbalances accompanied by declining leaf N concentrations. Constraints in growth were paralleled by enhanced saponin concentrations, especially due to N allocation towards secondary metabolism. Sapogenin concentrations increased when leaf K concentrations decreased and when growth was inhibited, thus when substrate was diverted from primary to secondary metabolism.

As indicated above, a number of studies tried to exploit the relevance of the mineral nutrition for the carbon allocation in plants. Nevertheless, frequently divergent findings are reported, *i.e.*, no effects or even an increase in terpenoid accumulation in response to nutrient supply (*e.g.*, Muzika et al., 1993; Björkmann et al., 1991; McCullough and Kulmann, 1991; Cronin and Hay, 1996). These inconsistencies led to a more critical view of the CNB and the GDB hypotheses, and their feasibility to predict the effects of environmental factors on terpenoid accumulation (Muzika, 1993; Gershenzon, 1994; Haukioja, 1998). The steadiness of our results, *i.e.*, the promotion of centelloside accumulation in response to N, P, as well as K limitations, suggests that carbon as well as nutrient availability for secondary metabolism plays a major role in the regulation of saponin and sapogenin synthesis.

In conclusion, our experiments demonstrate for the first time that the accumulation of saponins and sapogenins is strongly affected by resource partitioning between primary and secondary metabolism. This indicates that a well-directed cultivation of *C. asiatica*, and analogously the domestication of other wild medicinal plants, requires a carefully optimized and controlled fertilization to steer the biosynthesis of the desired secondary compounds.

The following pages of this chapter display the supplementary Table S1 and Figure S1.

Table S1. Effect of N, P, and K supply levels on the concentrations of N, P, and K 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 after 8 weeks of treatment application.

Effect of N supply levels			
Treatment	[mg g^{-1} leaf DM]		
	N	P	K
N0	6.93 ± 0.11 a	7.27 ± 0.34 a	45.40 ± 1.03 a
N30	33.50 ± 0.42 b	14.88 ± 0.50 c	107.70 ± 2.45 d
N60	35.70 ± 0.63 c	9.53 ± 0.51 b	65.44 ± 1.90 c
N100	36.25 ± 0.60 c	7.24 ± 0.12 a	53.81 ± 1.27 b
N150	33.10 ± 1.41 b	6.65 ± 0.13 a	52.96 ± 0.95 b
Effect of P supply levels			
Treatment	[mg g^{-1} leaf DM]		
	N	P	K
P0	25.45 ± 0.59 a	4.61 ± 0.01 a	51.80 ± 1.19 a
P30	30.71 ± 0.34 b	5.57 ± 0.06 b	58.21 ± 0.77 b
P60	36.63 ± 0.26 c	9.22 ± 0.10 c	64.76 ± 0.53 c
P100	37.76 ± 0.23 c	11.38 ± 0.28 d	65.90 ± 0.51 c
P150	37.35 ± 0.57 c	11.38 ± 0.30 d	65.31 ± 0.74 c
Effect of K supply levels			
Treatment	[mg g^{-1} leaf DM]		
	N	P	K
K0	29.33 ± 1.07 b	12.31 ± 0.47 b	10.95 ± 0.79 a
K30	36.60 ± 1.02 c	11.47 ± 0.68 b	30.77 ± 0.65 b
K60	30.57 ± 1.22 b	7.15 ± 0.19 a	49.45 ± 0.62 c
K100	30.09 ± 0.76 b	7.33 ± 0.11 a	60.13 ± 1.42 d
K150	24.33 ± 0.84 a	6.91 ± 0.17 a	63.71 ± 1.58 e

Mean ± standard error ($n = 10$). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).
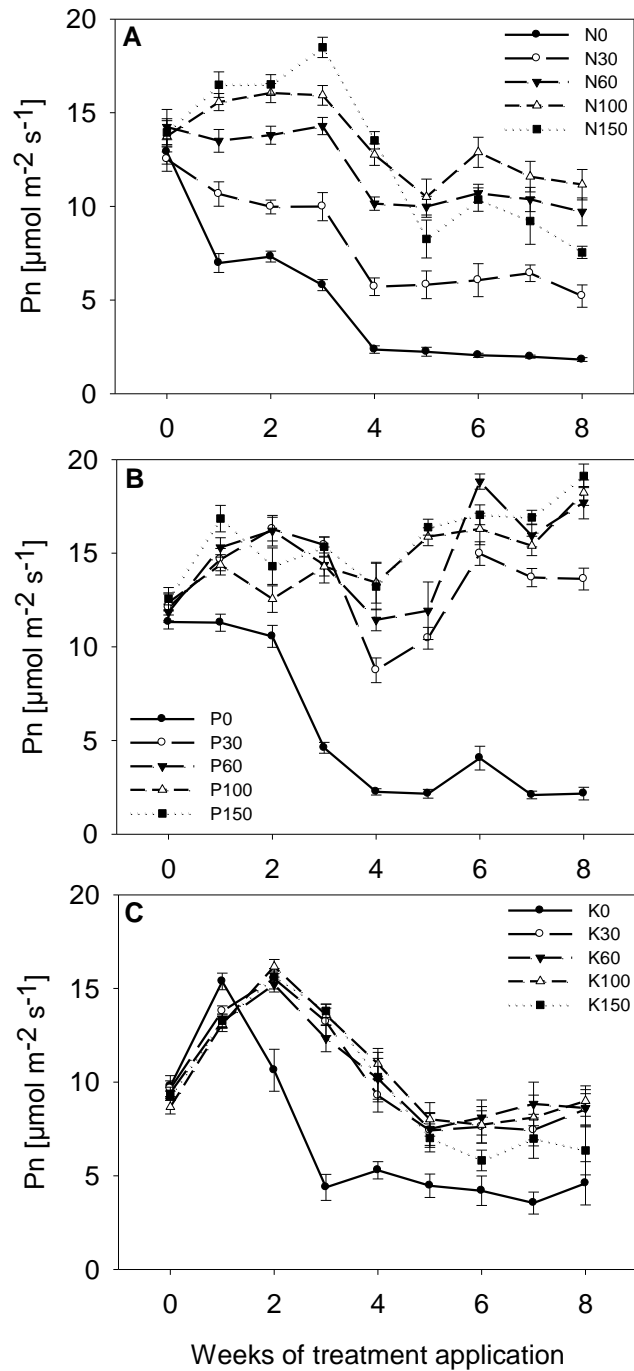
Fig. S1. Effect of N (A), P (B), and K (C) supply levels on the net photosynthesis of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount, 0% (P0), 30% (P30), 60% (P60), 100% (P100), or 150% (P150) of the P amount, and 0% (K0), 30% (K30), 60% (K60), 100% (K100), or 150% (K150) of the K amount in the standard Hoagland solution. Measurements were performed weekly during a period of 8 weeks of treatment application. Mean ± standard error ($n = 10$).

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C Estimation of flavonoid and centelloside accumulation in leaves of *Centella asiatica* L. Urban by multiparametric fluorescence measurements[2]

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 nutritionals, such as fiber, protein, calcium, and beta-carotin (Sritongkul et al., 2009). Moreover, the presence of various flavonoid derivatives, *e.g.*, quercetin and kaempferol (Zheng 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 flavonols and anthocyanins (Dixon and Paiva, 1995; Winkel-Shirley, 1999; Winkel-Shirley, 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; McKey, 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-phenolic competition for the limiting precursor phenylalanine, rather than by the availability of carbohydrates (Jones and Hartley, 1999). Apart from the specific assumptions, the models assume 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 concentrations 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 eight 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 fertigation 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* plants were conducted consecutively in a greenhouse. Each experiment consisted of five treatments, which were applied to forty plants, respectively, for a period of eight 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, N30, 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 petioles 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 flavonols 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 (IOM GmbH, Berlin, Germany), as described elsewhere (Bürling et al., 2011). The detailed results of the measurements are presented in Fig. A.1.

2.3 Determination of flavonoid and anthocyanin concentrations

The flavonoid and anthocyanin concentrations in leaves were determined by wet chemical analysis after eight 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 of 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 was 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 × 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, Missouri, USA). The isomers madecassoside and

asiaticoside B as well as madecassic acid and terminolic acid were considered as one compound, respectively. Total centellosides 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 treatment 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 the 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.
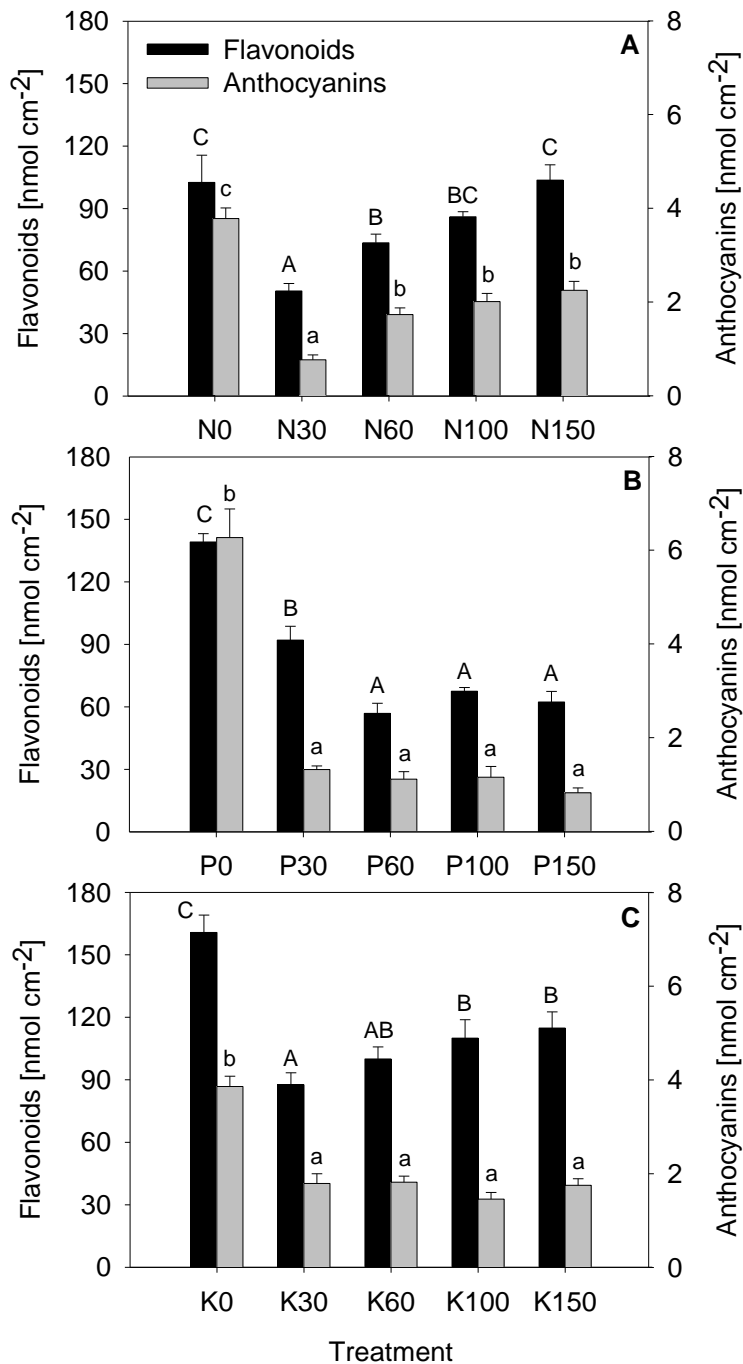
Fig. 1. Effect of N (A), P (B), and K (C) supply levels on flavonoid and anthocyanin concentrations in leaves of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount, 0% (P0), 30% (P30), 60% (P60), 100% (P100), 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 after 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 ($p \leq 0.05$).

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 ($p \leq 0.05$). 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 ($p \leq 0.05$). 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, $p \leq 0.05$), followed by the N30 treatment (2–8 WTA, $p \leq 0.05$) (Fig. 2D). At specific dates (2–5 WTA and 7 WTA, $p \leq 0.05$), 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, $p \leq 0.05$) and ANTH_RG (2–3 WTA and 5–7 WTA, $p \leq 0.05$) fluorescence-based indices. The FLAV index obtained from the treatment P30 by trend increased from 4 until 8 WTA, while it slightly decreased in the treatments P100 and P150 from 5 until 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, $p \leq 0.05$) (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.
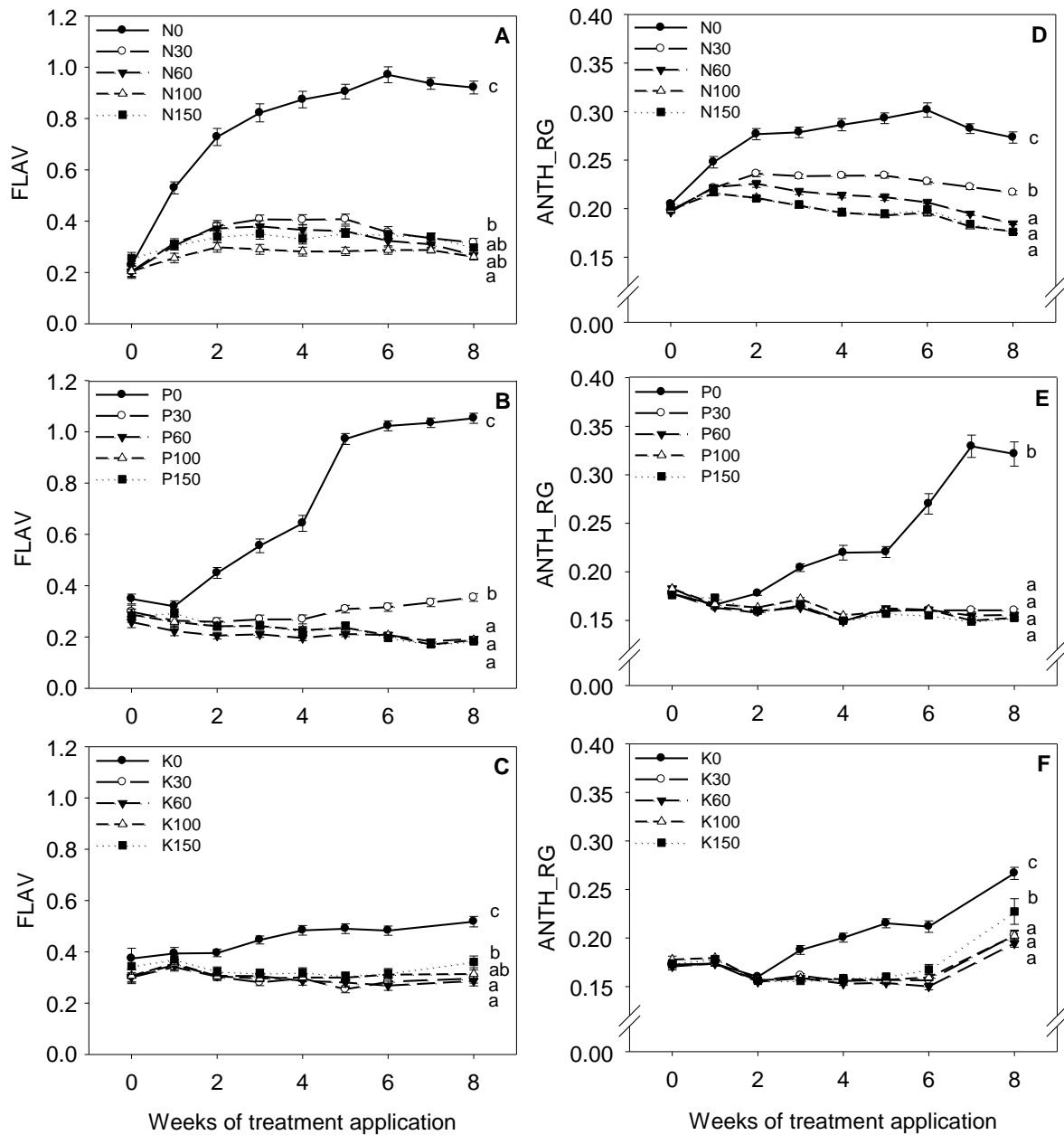
Fig. 2. Effect of N, P, and K supply levels on the FLAV (A, B, C) and ANTH_RG (D, E, F) fluorescence-based indices recorded from leaves of *C. asiatica*. Plants were fertigated with treatment solutions containing 0% (N0), 30% (N30), 60% (N60), 100% (N100), or 150% (N150) of the N amount, 0% (P0), 30% (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. Recordings were taken weekly. Mean ± standard error ($n =$ 20). Means at 8 weeks of treatment application followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

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).

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 after 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.

Factor	Fluorescence index	Flavonoids [nmol cm^{-2}]	Anthocyanins [nmol cm^{-2}]
N supply	**FLAV**	0.31	0.73**
	ANTH_RG	0.02	0.44**
	BFRR_UV	0.39*	0.68**
P supply	**FLAV**	0.89**	0.89**
	ANTH_RG	0.83**	0.93**
	BFRR_UV	0.84**	0.95**
K supply	**FLAV**	0.61**	0.75**
	ANTH_RG	0.58**	0.74**
	BFRR_UV	0.53**	0.84**

*Level of significance: $p \leq 0.05$. **Level of significance: $p \leq 0.01$.

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 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 centelloside 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 (Table 2 and 3).

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 after 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.

Parameters	Centellosides [mg g^{-1} DM]				
	Asiatico-side	Madecasso-side	Asiatic acid	Madecassic acid	Total
Flavonoids [nmol cm^{-2}]	0.28	0.30	0.57**	0.64**	0.41**
Anthocyanins [nmol cm^{-2}]	0.74**	0.77**	0.51**	0.65**	0.84**
FLAV	0.95**	0.95**	0.22	0.39*	0.96**
ANTH_RG	0.88**	0.87**	–0.01	0.14	0.84**
BFRR_UV	0.93**	0.94**	0.17	0.35*	0.92**

*Level of significance: $p \leq 0.05$. ** Level of significance: $p \leq 0.01$.

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 after 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.

Parameters	Centellosides [mg g^{-1} DM]				
	Asiatico-side	Madecasso-side	Asiatic acid	Madecassic acid	Total
Flavonoids [nmol cm^{-2}]	0.89**	0.89**	0.57**	0.67**	0.91**
Anthocyanins [nmol cm^{-2}]	0.81**	0.84**	0.51**	0.59**	0.84**
FLAV	0.90**	0.92**	0.75**	0.76**	0.95**
ANTH_RG	0.85**	0.86**	0.68**	0.67**	0.88**
BFRR_UV	0.84**	0.86**	0.72**	0.71**	0.89**

*Level of significance: $p \leq 0.05$. **Level of significance: $p \leq 0.01$.

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 after 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.

Parameters	Centellosides [mg g^{-1} DM]				
	Asiatico-side	Madecasso-side	Asiatic acid	Madecassic acid	Total
Flavonoids [nmol cm^{-2}]	0.65**	0.65**	0.78**	0.77**	0.85**
Anthocyanins [nmol cm^{-2}]	0.46*	0.51**	0.87**	0.86**	0.77**
FLAV	0.51**	0.55**	0.79**	0.78**	0.74**
ANTH_RG	0.45**	0.47**	0.76**	0.74**	0.68**
BFRR_UV	0.51**	0.57**	0.81**	0.81**	0.76**

*Level of significance: $p \leq 0.05$. **Level of significance: $p \leq 0.01$.

4 Discussion

The present study aimed to investigate the accumulation of centellosides 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 fertigation levels. Thereby, we hypothesized that the accumulation of centellosides is affected by N, P, and K fertigation in the same extent as the accumulation of flavonoids and anthocyanins, and that centelloside 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 fertigation, although the effects were more pronounced for the flavonoids. In accordance with the centelloside 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 fertigation (Fig. 1A–C). These findings agree with a number of studies reporting on the increase in 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 at el., 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 two 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; Herms and Mattson, 1992; Jones and Hartley, 1999) and illustrated for the centellosides (Müller et al., 2013), flavonoid and anthocyanin concentrations were inversely related to plant growth, irrespective of N, P, and K supply.

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 two 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 Gülz, 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 suggest 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_RG, and BFRR_UV (Table 2–4). Screenings of the asiaticoside and asiatic 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 region (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 flavonoid (P supply) and anthocyanin (N and P supply) concentrations (Table 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 (James and Dubery, 2009; Augustin et al., 2011) and flavonoids *via* the phenylpropanoid pathway (Dixon and Paiva, 1995; Winkel-Shirley, 1999; Winkel-Shirley, 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, Bürling et al., 2013), and the leaf flavonoid, anthocyanin, as well as centelloside concentrations (Table 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 fertigation 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. Among 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.

The following pages of this chapter display the supplementary Table A.1 and Figure A.1.

Table A.1. Effect of N, P, and K supply levels on growth rate (GR) of *C. asiatica* during a period of 8 weeks of treatment application. 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 biweekly.

		Effect of N supply levels								
	Treat-ment	Weeks of treatment application								
		2		4		6			8	
GR [mg DM]	N0	0.54 ± 0.03	a B	0.28 ± 0.05	a A	0.21 ± 0.07	a	A	0.16 ± 0.04	a A
	N30	0.79 ± 0.04	b AB	0.46 ± 0.08	a A	1.53 ± 0.34	b	B	2.53 ± 0.55	b C
	N60	1.10 ± 0.08	c A	1.50 ± 0.13	b A	4.38 ± 0.30	cd	B	6.97 ± 0.60	d C
	N100	1.28 ± 0.10	c A	1.89 ± 0.14	c C	5.39 ± 0.32	d	C	5.18 ± 0.61	c B
	N150	1.14 ± 0.07	c A	1.96 ± 0.14	c A	4.16 ± 0.58	c	B	3.22 ± 0.61	b B

		Effect of P supply levels								
	Treat-ment	Weeks of treatment application								
		2		4		6			8	
GR [mg DM]	P0	0.63 ± 0.05	a N.S.	0.37 ± 0.10	a	0.32 ± 0.12	a		0.30 ± 0.11	a
	P30	0.83 ± 0.06	b A	1.39 ± 0.16	b A	3.57 ± 0.28	b	B	2.53 ± 0.27	b B
	P60	0.89 ± 0.07	b A	1.66 ± 0.13	bc B	4.22 ± 0.20	c	D	3.58 ± 0.16	cd C
	P100	0.98 ± 0.04	b A	1.79 ± 0.17	bc B	4.47 ± 0.16	c	D	3.41 ± 0.29	c C
	P150	0.96 ± 0.05	b A	2.11 ± 0.12	c B	4.23 ± 0.19	bc	C	4.16 ± 0.27	d C

		Effect of K supply levels								
	Treat-ment	Weeks of treatment application								
		2		4		6			8	
GR [mg DM]	K0	0.70 ± 0.05	n.s. N.S.	0.83 ± 0.20	a	0.74 ± 0.23	a		0.99 ± 0.40	a
	K30	0.59 ± 0.07	A	2.90 ± 0.21	b B	6.68 ± 0.34	c	C	9.23 ± 0.64	d D
	K60	0.73 ± 0.07	A	2.44 ± 0.19	b B	5.98 ± 0.43	c	C	6.73 ± 0.91	c C
	K100	0.62 ± 0.04	A	2.34 ± 0.16	b B	6.59 ± 0.26	c	C	6.41 ± 0.50	bc C
	K150	0.62 ± 0.05	A	2.31 ± 0.23	b B	4.71 ± 0.68	b	C	4.57 ± 0.75	b C

Mean ± standard error ($n = 10$). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$). Differences among the treatment groups at specific sampling dates are indicated by small letters in the column; differences among harvesting dates (weeks of treatment application) are indicated by capital letters in the row; n.s., N.S., not significant.
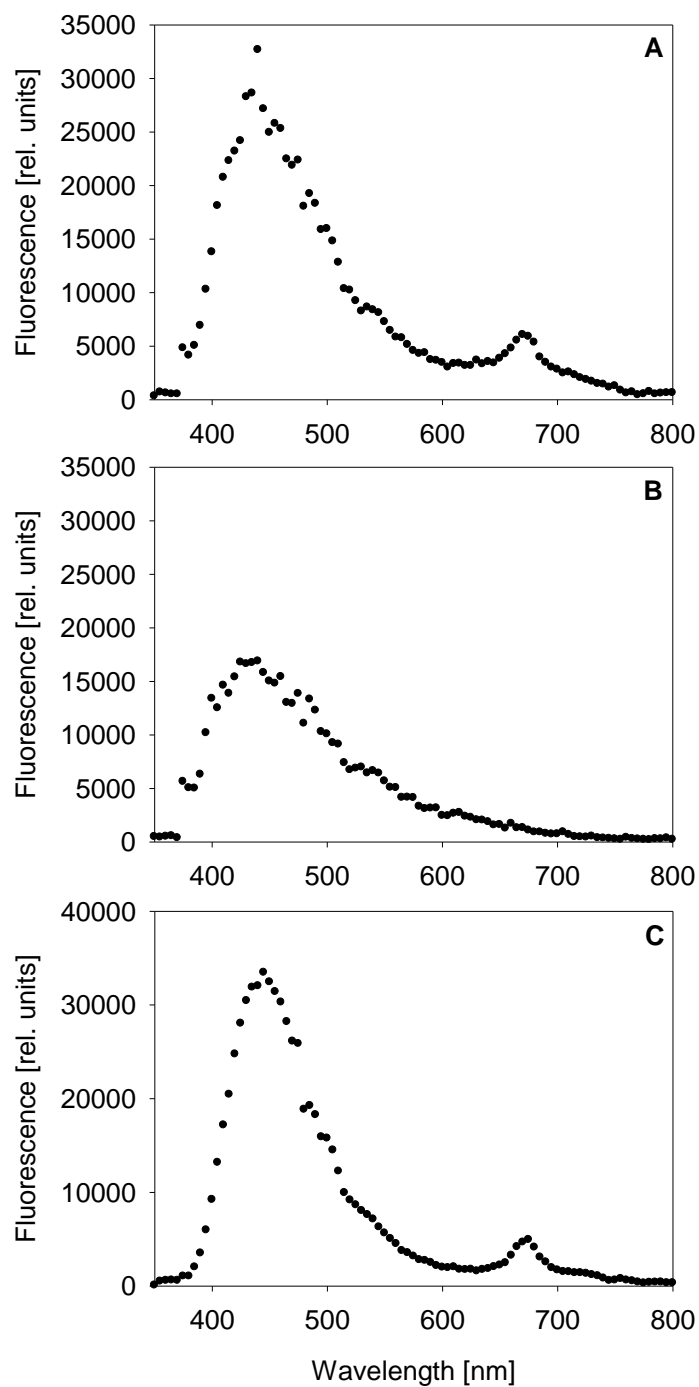
Fig. A.1. Fluorescence spectra (370–800 nm) recorded from asiaticoside (A, 1 mg mL^{-1} methanol/water, 9/1, v/v) and asiatic acid (B, 1 mg mL^{-1} methanol/water, 9/1, v/v) reference compounds (Sigma Aldrich, St. Louis, Missouri, USA), and the methanolic leaf extract of *C. asiatica* (C).

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D Ecologically relevant UV-B dose combined with high PAR intensity distinctly affect plant growth and accumulation of secondary metabolites in leaves of *Centella asiatica* L. Urban[3]

1 Introduction

Ultraviolet (UV)-B radiation (280–315 nm) is a natural component of sunlight. Due to ozone depletion, the amount of UV-B radiation reaching the Earth's surface has increased over the last decades. Therefore, many of the early (1980s–2000s) studies focused on the impact of above-ambient UV-B levels on plants. In this context, damaging effects of UV-B radiation on DNA, as well as alterations in photosynthesis and retardations in growth and development, have frequently been reported (Caldwell et al., 1995; Caldwell et al., 1998; Searles et al., 2001).

Since ozone depletion has been reduced significantly, recent experiments progressively deal with low, environmentally relevant UV-B levels. These studies reveal that damaging effects on plants are predominantly induced by above-ambient UV-B levels, which trigger non-specific signaling pathways resulting in a general stress response. In contrast, low UV-B levels rather act as informational signal (Jansen et al., 1998; Jansen et al., 2008), stimulating the expression of genes involved in the UV-protection of plants, many of them mediated by UV-B specific photomorphogenic signaling pathways (Jenkins and Brown, 2007; Jenkins, 2009). In this context, the up-regulation of the phenylpropanoid metabolism and the increased accumulation of flavonoids have been explored thoroughly (*e.g.*, Li et al., 1993; Frohnmeyer and Staiger, 2003). Nevertheless, plants are naturally exposed to a multitude of environmental conditions. Thus, the effect of UV-B radiation on plants and its interaction with other factors, including PAR, has to be evaluated. In this regard, it was shown that flavonoid synthesis is induced by high PAR intensity, even in the absence of UV-B radiation; though, its accumulation is additively enhanced in the presence of UV-B radiation (Nitz et al., 2004; Götz et al., 2010; Agati et al., 2011).

Besides flavonoids, the accumulation of other secondary compounds in response to UV-B exposure, some of them probably acting as UV-protectants (Zhang and Björn, 2009), has been recorded. Accordingly, it was proposed that the synthesis of saponins is triggered by UV-B radiation (Afreen et al., 2005). Furthermore, a few studies indicated a promoting effect of

higher light intensities on saponin concentrations in plants (Mathur et al., 2000; Devkota et al., 2010; Maulidiani et al., 2012). However, findings are scarce and even contradictory (Szakiel et al., 2010 and references therein). Moreover, the combination of UV-B and PAR, and their impact on the accumulation of saponins, has not been investigated, yet. In the present study we aimed to examine the effects of environmentally relevant UV-B dose combined with different PAR intensities on saponin accumulation in *Centella asiatica* L. Urban (Apiaceae). *C. asiatica* accumulates both pentacyclic triterpene saponins and flavonoids in its leaves, and is thus an excellent model to study changes in the accumulation pattern of secondary metabolites in the plant tissue in response to different light regimes. Further, as reviewed in our previous work (Müller et al., 2013a), the saponins of *C. asiatica*, known as centellosides, are widely used in the pharmaceutical, food, and cosmetic industries. Thus, fundamental knowledge on the potential promotion of centelloside accumulation by light regimes will encourage more practical investigations aiming the target-oriented increase in saponin concentrations.

Saponins are synthesized *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. The latter are modified by oxidation, hydroxylation, and other substitutions, generating the *C. asiatica* sapogenins. Finally, the sapogenins are transformed into saponins by glycosylation processes (James and Dubery, 2009; Augustin et al., 2011). On the contrary, the biosynthesis of flavonoids starts with the deamination of the amino acid phenylalanine, which is then 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, including flavonols and anthocyanins (Dixon and Paiva, 1995; Winkel-Shirley, 1999; Winkel-Shirley, 2001).

In our work we hypothesized that ambient dose of UV-B radiation and high PAR intensity additively promote the accumulation of saponins and their respective genins in leaves of *C. asiatica*. For this purpose, *C. asiatica* plants were exposed to one of four light regimes, *i.e.*, two PAR intensities with or without UV-B radiation. The five-week experiment was conducted in technically complex sun simulators, providing an irradiance spectrum which is very similar to the natural solar radiation (Thiel et al., 1996). Moreover, the PAR and UV-B intensities followed the natural diurnal variations of the solar irradiance, and the climatic conditions were controlled precisely. The determination of saponin and sapogenin concentrations was realized weekly. In order to elucidate whether the effects of UV-B and

PAR on centelloside accumulation depend on the physiological age of the plant tissue and the duration of exposure (Schreiner et al., 2009; Schreiner et al., 2012 and references therein), we compared the saponin and sapogenin concentrations in the leaves which had emerged before treatment initiation and in those which emerged during the experiment. Furthermore, we investigated the causal relationship among the accumulation of centellosides in leaves, photosynthesis, as well as herb and leaf yield of *C. asiatica*. Aiming a monitoring of the specific UV-B response of the plants, we additionally recorded the accumulation of epidermal flavonols and anthocyanins *in vivo* by multiparametric fluorescence measurements. In comparison with traditional wet chemical analysis the non-destructive estimation of flavonoid amounts is less costly and enables repeated measurements of the same leaf during the whole experimental period.

2 Materials and methods

2.1 *Plant material*

Stock plants of *Centella asiatica* L. Urban were provided by D. Randriamampionona (Laboratoire de Biotechnologie Végétale, Université Libre de Bruxelles, Gosselies, Belgium/Institut Malgache de Recherches Appliquées, Antananarivo, Madagascar). The genome-based identification of the species was carried out by A. N. Nicolas (Institute of Systematic Botany, The New York Botanical Garden, NY, USA). Four weeks before starting the experiment, the plantlets were propagated vegetatively by cuttings obtained from the stock plant stolons. Nodules having one expanded leaf were cut off and stuck into propagation boxes filled with a mixture of peat soil, sand, and perlite (3:2:1) as rooting medium. As soon as the cuttings had rooted they were transplanted into cultivation pots containing the same mixture as substrate. The plantlets were raised in a greenhouse, and 180 homogeneous plants having four to six completely expanded leaves were selected for the experiment.

2.2 *Treatments and growth conditions*

The five-week experiment was conducted in two sun simulators of the Helmholtz Zentrum München (Neuherberg, Germany) in June/July 2011. The sun simulators provided an irradiance spectrum which is very similar to the natural solar radiation (Fig. S1). The simulation of the spectrum from 280 to 850 nm was achieved by a combination of metal halide lamps (HQI/D, 400 W, Osram, München, Germany), quartz halogen lamps (Halostar, 300 W and 500 W, Osram, München, Germany), blue fluorescent (TLD 18, 36 W, Philips, Amsterdam, Netherlands), and UV-B fluorescent (TL 12, 40 W, Philips, Amsterdam,

Netherlands) tubes. Oversupplied infrared radiation was reduced by a layer of water. The wavelengths below 280 nm were blocked efficiently using selected borosilicate and lime glass filters. A suitable combination of these glasses allowed us the simulation of different UV-B scenarios. The natural diurnal variations of the solar irradiance were realized by switching appropriate groups of lamps on and off (Thiel et al., 1996; Behn et al., 2010). The photoperiod was 12 h with day/night temperatures of 25 °C/15 °C and relative humidity of 60%/80%. Each sun simulator was subdivided into two compartments enabling four experimental treatments with 45 plants at random placed in each compartment. After an acclimatization phase of four days, the PAR intensity was raised from 150 µmol m^{-2} s^{-1} to 455 µmol m^{-2} s^{-1}. Starting the experiment, the PAR intensity was maintained at 455 µmol m^{-2} s^{-1} in the first sun simulator, while it was adjusted to 835 µmol m^{-2} s^{-1} in the second one. Moreover, using UV-B absorbing acrylic glass the UV-B radiation was set at 0 W m^{-2} in one compartment and at 0.3 W m^{-2} in the other compartment of each sun simulator. Thus, the treatments will be referred to as follows: high PAR/–UV-B, high PAR/+UV-B, low PAR/–UV-B, and low PAR/+UV-B. The plants were watered daily, while fertigation (EC 2.0 mS cm^{-1}, Hakaphos Blau containing 15% N, 10% P_2O_5, 15% K_2O, and 2% MgO, Compo, Münster, Germany) was realized every second day.

2.3 Multiparametric fluorescence measurements

For the *in vivo* monitoring of the UV-B specific response of the *C. asiatica* plants during the whole experimental period, the accumulation of epidermal flavonoids was recorded by non-destructive fluorescence measurements using a multiparametric portable optical sensor (Multiplex® Research, FORCE-A, Orsay, France). The accumulation of flavonols was ascertained considering the decadic logarithm of the red to UV excitation ratio of far-red chlorophyll fluorescence (FLAV index), which is proportional to the flavonol content of the leaves (Cerovic et al., 2002) Moreover, the anthocyanins were evaluated by 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). The suitability of the fluorescence-based FLAV and ANTH_RG indices for prediction of flavonol and anthocyanin accumulation in *C. asiatica* leaves was validated before (Müller et al., 2013b). Further, the blue and the far-red fluorescence, both excited with UV light, were used to calculate the blue-to-far red ratio (BFRR_UV index). The BFRR_UV index may provide additional information on the stress-induced accumulation of blue-fluorescing compounds in the epidermis (Cerovic et al., 1999) and is a robust 'stress indicator' (Lichtenthaler and

Miehé, 1997) as shown, *e.g.*, for the early detection of nutrient deficiency (Chappelle et al., 1984), drought (Bürling et al., 2013; Leufen et al., 2013), and pathogen infection (Lüdeker et al., 1996).

The fluorescence-based indices were recorded twice a week from 18 leaves each treatment group (nine plants and two leaves per plant) under standardized conditions. The petioles of the leaves were labelled at the onset of the experiment (leaf type I; see section *2.5*) ensuring measurements on the same ontogenetic stage of the leaves in each treatment group and during the whole experimental period.

2.4 Gas-exchange measurements

The net photosynthesis (Pn), internal CO_2 concentration, stomatal conductance, and transpiration rate were recorded after five weeks of treatment application (WTA) using a portable gas exchange and fluorescence system (GFS3000, Heinz-Walz GmbH, Effeltrich, Germany). Measurements were performed at ten leaves each treatment group, randomly chosen from those leaves which were also used for the multiparametric fluorescence measurements. The leaves were fixed in a leaf chamber with a cuvette area of 4 cm^2. Gas exchange was measured at a PAR intensity of 200, 500, and 1000 µmol m^{-2} s^{-1} provided by an artificial light source (light emitting diode, 92% red (650 nm) and 8% blue light (470 nm)). The leaf cuvette environment was adjusted to a relative humidity of 55%, a cuvette temperature of 25 °C, and a CO_2 concentration of 390 ppm. The air flow rate was 700 µmol s^{-1}.

2.5 Sampling and sample preparation

The leaves and stems of nine experimental plants of each treatment group were harvested separately once a week. Labelling of the samples took into consideration whether the leaves had emerged before treatment initiation (leaf type I; leaves labeled at the onset of the experiment; at 5 WTA these leaves were at the age of 5–9 weeks) or during the experimental period (leaf type II; at 5 WTA these leaves were at the age of 1–5 weeks). At harvest, the samples were immediately shock-frozen in liquid nitrogen and stored at −25 °C. Thereafter they were lyophilized and the dry weight of every sampling group was determined using laboratory scales. Herb yield was calculated by summing up leaf and stem dry weight. Subsequently, the samples were ground with a mixer mill (MM 2000, Retsch, Haan, Germany) obtaining a fine powder.

2.6 Determination of saponin, sapogenin, and total centelloside concentrations in leaves

Extraction procedure and analysis were performed according to Müller et al. (2013a). Briefly, 50 mg of leaf powder of each plant (n = 9) was extracted under sonication with MeOH-H2O (9/1, v/v). Every sample was assayed in duplicate. The saponin (asiaticoside, madecassoside) and sapogenin (asiatic acid, madecassic acid) concentrations were assessed using an HPLC device (Series 1100, Agilent, Waldbronn, Germany) equipped with a micro vacuum degasser (G1379A), a binary gradient pump (G1312A), an autosampler (G1313A), a thermostated column compartment (G1316A), and a multiwavelength-detector (G1365A, 1024-element photodiode array). A Nucleodur C18 reversed phase column (250 × 4 mm, 5 μm; Macherey & Nagel, Düren, Germany) was chosen as stationary phase. A gradient program was established using water and acetonitrile, both acidified with 0.01% formic acid (VWR, West Chester, USA), as mobile phase. Separation was performed at 30 °C and a flow rate of 1 mL min^{-1}. The saponins and sapogenins were detected at 206 nm. Reference compounds were used to set an external calibration curve for asiaticoside and asiatic acid. As proposed by Rafamantanana et al. (2009), madecassoside and madecassic acid were quantified using the reference substances of asiaticoside and asiatic acid. The isomers madecassoside and asiaticoside B as well as madecassic acid and terminolic acid were considered as one compound, respectively. Total centellosides were defined as the summation of the determined asiaticoside, madecassoside, asiatic acid, and madecassic acid concentrations.

2.7 Statistics

For statistical evaluation data were subjected to the analysis of variance (one-way ANOVA), and differences among the treatments were assessed by Duncan's multiple range test. One exception is found in the comparison of the leaf types in Table 1, where significant differences were determined by Student's *t*-test. All statistical analyses were conducted with PASW statistics software (Version 20.0, SPSS Inc., Chicago, USA), while graphs were drawn with Sigma Plot 11.0 (Systat Software Inc., Richmond, CA, USA).

3 Results

3.1 Vegetative growth and net photosynthesis

During the first 3 WTA there were no significant differences among the treatment groups concerning herb and leaf yield of the plants. At 4 WTA the highest leaf yield was obtained from the treatment groups high PAR/+UV-B and low PAR/+UV-B ($p \leq 0.05$) (Fig. 1A and

B). At 5 WTA this was also the case for the herb yield ($p \leq 0.05$). However, the PAR intensity did not significantly affect herb and leaf yield ($p \geq 0.05$). Similarly, we observed no significant influence of the light regimes on the net photosynthesis ($p \geq 0.05$) (Fig. S2), internal CO_2 concentration, stomatal conductance, and transpiration rate ($p \geq 0.05$) (*data not shown*), all recorded at 200, 500, and 1000 µmol m^{-2} s^{-1} PAR.
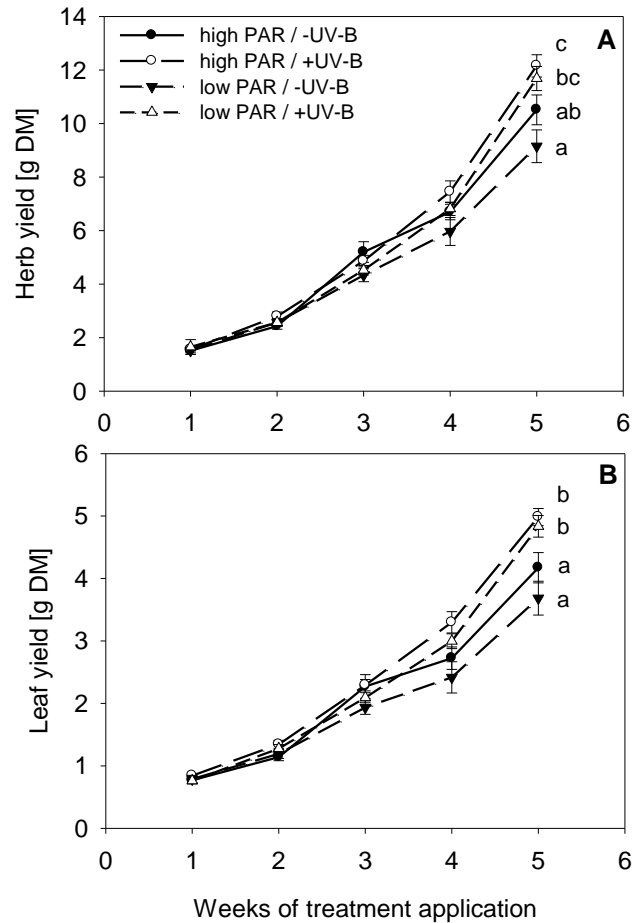
Fig. 1. Effect of PAR and UV-B radiation on herb and leaf yield of *C. asiatica*. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation. Evaluation of herb yield (A) and leaf yield (B) was done weekly during a period of 5 weeks of treatment application (WTA). Mean ± standard error (*n* = 9). Means at 5 WTA followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

3.2 *Fluorescence-based indices*

The fluorescence-based flavonol (FLAV) and anthocyanin (ANTH_RG) indices raised in all treatment groups, particularly during the first three experimental weeks (Fig. 2A and B).
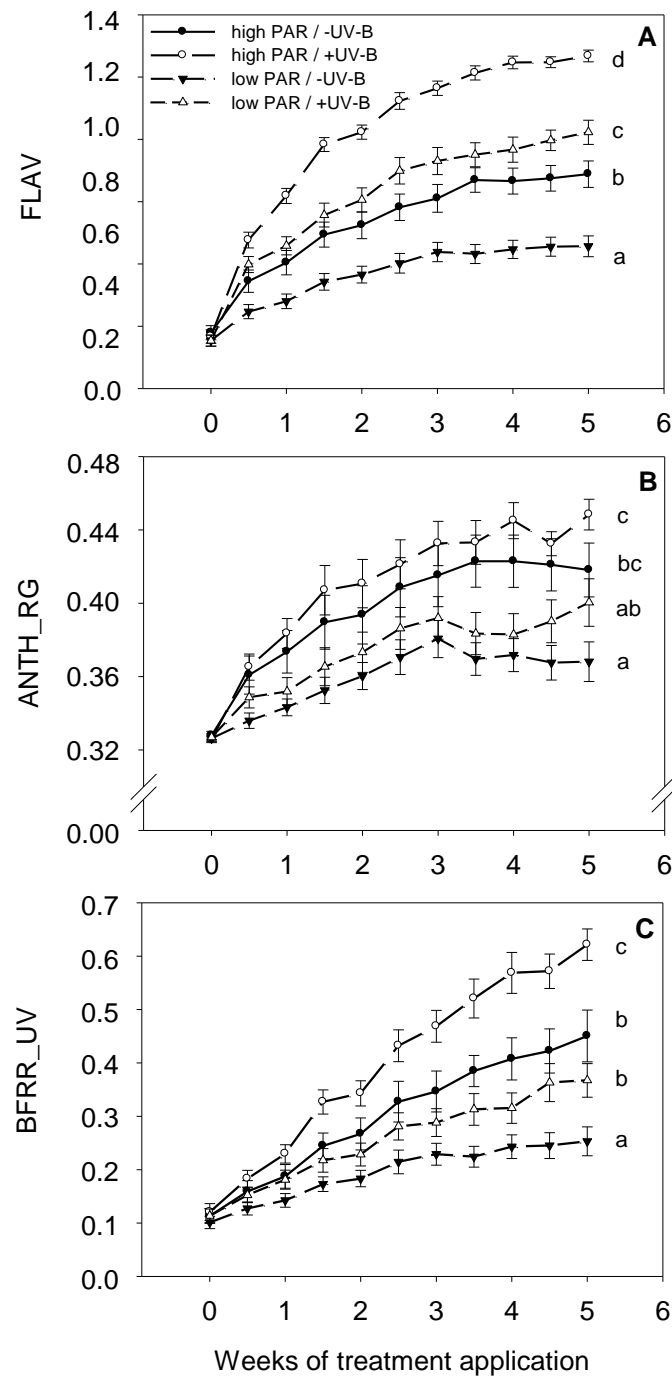
Fig. 2. Effect of PAR and UV-B radiation on the FLAV (A), ANTH_RG (B), and BFRR_UV (C) fluorescence-based indices recorded from leaves of *C. asiatica*. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation. Recordings were taken twice a week during a period of 5 weeks of treatment application (WTA). Mean ± standard error ($n = 18$). Means at 5 WTA followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

The increase in the FLAV index was more pronounced in the treatment groups high PAR/+UV-B and low PAR/+UV-B (Fig. 2A). In contrast, the ANTH_RG values particularly augmented in the treatment groups high PAR/+UV-B and high PAR/–UV-B (Fig. 2B). In the period of 3–5 WTA, the FLAV index kept increasing at a steady pace in the +UV-B groups (high PAR/+UV-B and low PAR/+UV-B), while saturation was observed in the –UV-B groups (high PAR/–UV-B and low PAR/–UV-B). The ANTH_RG values suffered oscillation from 3–5 WTA with decreases and subsequent rises in both +UV-B treatment groups; the values in both –UV-B treatment groups decreased consistently. Both the lowest FLAV and the lowest ANTH_RG values were observed in the treatment low PAR/–UV-B ($p \leq 0.05$).

The BFRR_UV index increased in all treatment groups during the whole experimental period (Fig. 2C). The increase was most pronounced in the treatment high PAR/+UV-B, while it was least evident in the treatment low PAR/–UV-B. At 5 WTA, the highest BFRR_UV index was recorded from the leaves of the treatment high PAR/+UV-B, followed by high PAR/–UV-B ($p \leq 0.05$).

3.3 Concentration of centellosides

Considering the entire leaf mass of the plants, we observed increasing concentrations of the two saponins (asiaticoside and madecassoside) and of the total centellosides during the whole experimental period (Fig. 3A, B, and D). From 3 WTA until the end of the study, higher concentrations were determined in the high PAR treatments as compared to the low PAR treatment groups ($p \leq 0.05$). In contrast, UV-B radiation did neither significantly influence saponin nor total centelloside concentrations ($p \geq 0.05$), except at 4 WTA, when the lowest concentrations were observed in the treatment low PAR/+UV-B ($p \leq 0.05$).

The concentrations of the two sapogenins (asiatic acid and madecassic acid) slightly decreased from 0 until 3 WTA in the treatment groups high PAR/–UV-B and low PAR/+UV-B, and from 0 until 4 WTA in the treatment groups high PAR/+UV-B and low PAR/–UV-B (Fig. 3C and E). Subsequently, the concentrations increased and at 5 WTA they had reached the initial values again. However, the standard errors were considerably high, and consequently there were no significant differences among the treatment groups concerning the sapogenin concentrations in the entire leaf mass of the plants during the whole experimental period ($p \geq 0.05$) (Fig. 3C and E).
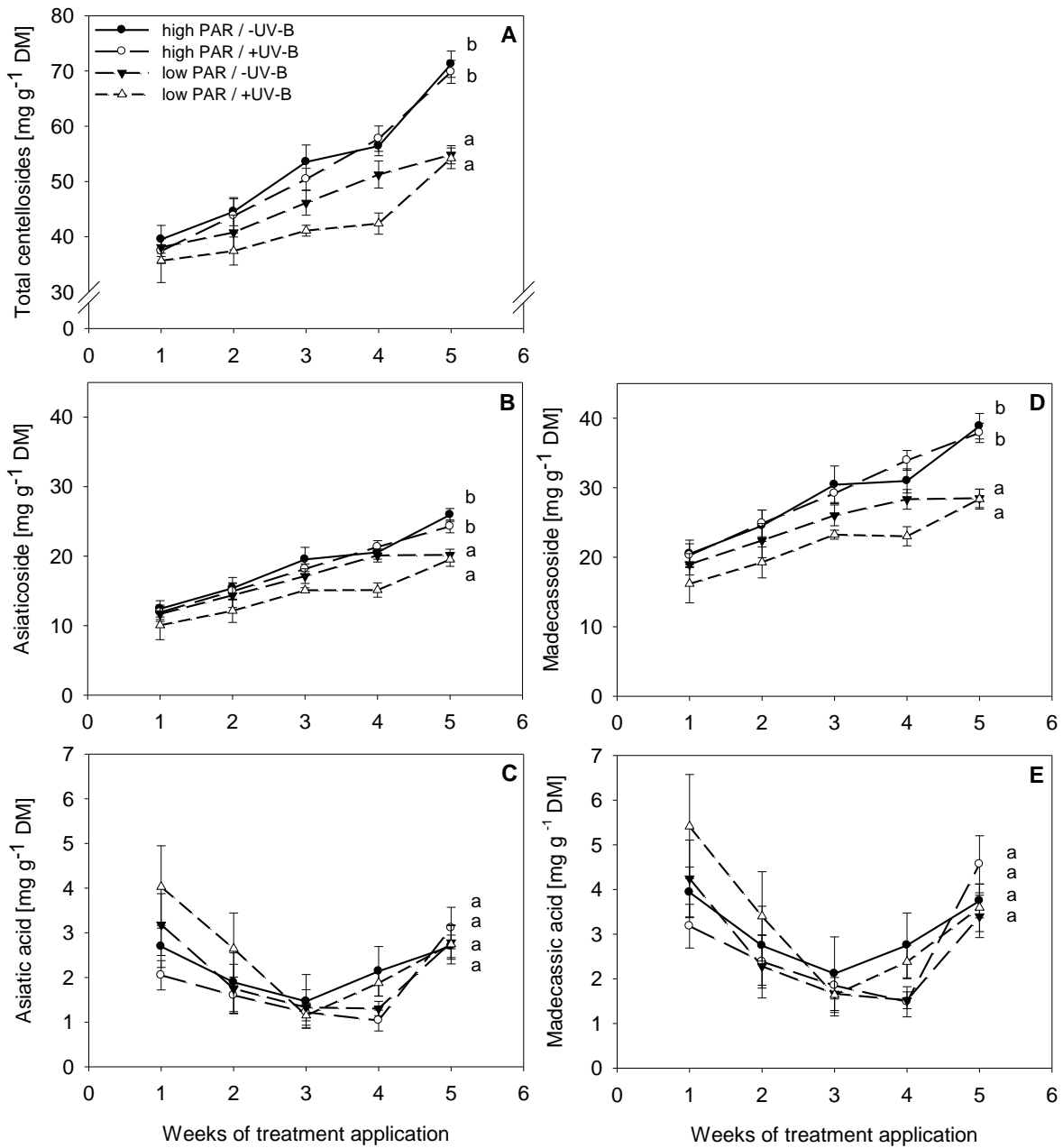
Fig. 3. Effect of PAR and UV-B radiation on centelloside accumulation in leaves of *C. asiatica*. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation. Concentrations of centellosides (total centellosides (A), asiaticoside (B), madecassoside (D), asiatic acid (C), and madecassic acid (E)) were determined weekly during a period of 5 weeks of treatment application (WTA). Mean ± standard error ($n = 9$). Means at 5 WTA followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

Table 1. Effect of PAR and UV-B radiation on centelloside accumulation in leaves of *C. asiatica*. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation. Concentrations of centellosides (asiaticoside, madecassoside, asiatic acid, madecassic acid, and total centellosides) were determined at 5 weeks of treatment application. Leaf type I = leaves which had emerged before treatment initiation; leaf type II = leaves which emerged during the experiment.

Compound [mg g^{-1} DM]		leaf type	high PAR −UV-B			high PAR +UV-B			low PAR −UV-B			low PAR +UV-B		
Saponins	**Asiaticoside**	**I**	22.02 ± 1.40	b	A	18.39 ± 1.44	ab	A	14.87 ± 1.14	a	A	15.18 ± 1.17	a	A
		II	27.63 ± 0.96	b	B	26.00 ± 0.90	b	B	22.19 ± 1.23	a	B	20.82 ± 1.21	a	B
	Madecasso-side	**I**	34.41 ± 2.20	b	A	30.22 ± 2.08	b	A	22.11 ± 1.35	a	A	22.38 ± 1.47	a	A
		II	40.77 ± 1.94	b	B	40.13 ± 1.36	b	B	30.88 ± 1.80	a	B	30.16 ± 1.80	a	B
Sapogenins	**Asiatic acid**	**I**	3.99 ± 0.54	a	B	6.87 ± 0.78	b	B	3.81 ± 0.36	a	B	4.26 ± 0.51	a	B
		II	2.21 ± 0.35	a	A	2.00 ± 0.40	a	A	2.40 ± 0.50	a	A	2.29 ± 0.58	a	A
	Madecassic acid	**I**	5.55 ± 0.71	a	B	9.81 ± 1.06	b	B	4.66 ± 0.47	a	B	5.49 ± 0.60	a	B
		II	3.05 ± 0.51	a	A	3.03 ± 0.58	a	A	2.92 ± 0.59	a	A	3.04 ± 0.72	a	A
Total Centellosides		**I**	65.97 ± 2.71	b	A	65.28 ± 2.53	b	A	45.45 ± 1.95	a	A	47.31 ± 1.75	a	A
		II	73.29 ± 2.69	b	A	71.16 ± 2.06	b	A	58.39 ± 2.37	a	B	56.32 ± 2.20	a	B

Mean ± standard error ($n = 9$). Values followed by the same letter do not differ statistically; small letters in the row indicate significant differences among the treatment groups for each compound according to Duncan's multiple range test ($p \leq 0.05$), capital letters in the column indicate significant differences between the leaf types for each compound according to Student's *t*-test ($p \leq 0.05$).

Separating the leaves into two groups according to the time of their emergence (before or after treatment initiation), we detected that the sapogenin concentrations were similar only for leaf type II ($p \geq 0.05$) (Table 1). In contrast, leaf type I contained significantly higher sapogenin concentrations when exposed to high PAR/+UV-B ($p \leq 0.05$). The saponin and total centelloside concentrations were higher in the high PAR treatments, irrespective of the time of leaf emergence ($p \leq 0.05$) (Table 1). Contrary to that, UV-B radiation did not significantly influence the saponin and total centelloside concentrations, although the trend indicates lower asiaticoside concentrations in leaf type I when exposed to high PAR/+UV-B as compared to high PAR/–UV-B ($p \geq 0.05$). In general, leaf type II contained higher saponin but lower sapogenin concentrations than leaf type I ($p \leq 0.05$) (Table 1). Total centelloside concentrations were similar for both leaf type I and II in the high PAR treatments ($p \geq 0.05$), while they were higher in leaf type II in the low PAR treatments ($p \leq 0.05$).

4   Discussion

In the present paper we studied the relevance of PAR and UV-B, in four combinations, on saponin accumulation in leaves of *C. asiatica*. The specific UV-B response in terms of flavonol and anthocyanin accumulation in the leaves was monitored *in vivo* during the whole experimental period by multiparametric fluorescence measurements. Further, we elucidated the causal relationship among centelloside concentration, photosynthesis, and herb and leaf yield. We hypothesized that high PAR intensity and ambient level of UV-B radiation additively favor the accumulation of saponins and their respective genins in leaves of *C. asiatica*.

4.1 PAR and UV-B have distinct impact on plant growth and accumulation of secondary metabolites

Previous studies report on growth reducing effects of above ambient UV-B levels on plants (Caldwell et al., 1995; Caldwell et al., 1998; Searles et al., 2001). Our results proof that the application of ambient UV-B doses even favor herb and leaf production of *C. asiatica* (Fig. 1A and B). Corresponding findings were recently published for *Nepeta cataria* L. *f. citriodora*, *Salvia officinalis* L., and *Melissa officinalis* L., which produced higher herb yields in the presence of UV-B radiation (Manukyan, 2013). The same author observed an increase in plant growth under higher PAR intensity (Manukyan, 2013). In our experiment, the PAR intensity did not significantly influence herb and leaf yield (Fig. 1A and B). Further, the net photosynthesis of the plants grown under different UV-B and PAR combinations was similar for all treatment groups (Fig. S2). This indicates that the photosynthetic apparatus was either

not affected, or that it underwent adjustments to the PAR/UV-B combinations. Thus, neither the photosynthetic rate was a limiting factor for growth nor the applied UV-B level was harmful to the plant.

The adjustment of the photosynthetic apparatus to high irradiance in a time scale of days comprises a decrease in the level of light-harvesting complexes of photosystem II, resulting in a reduction of the photosystem II absorption cross-section (Walters, 2005). Concomitantly, an enhanced content of carotenoids, particularly xanthophyll cycle pigments, usually increases the capacity of thermal energy dissipation (Demming-Adams, 1998; Bailey et al., 2004). Further, the carotenoid-mediated photoprotection is amended by the accumulation of epidermal shielding compounds, such as flavonoids, in this way efficiently protecting the photosynthetic apparatus from high PAR and UV radiation (Štroch et al., 2008). Tracking the content of epidermal flavonoids *in vivo* with the fluorescence-based flavonol (FLAV) and anthocyanin (ANTH_RG) indices, we demonstrate the accumulation of these compounds in all treatment groups during the whole experimental period (Fig. 2A and B). As expected, the FLAV index was higher in the presence of UV-B radiation. Moreover, in accordance with recent literature (Nitz et al., 2004; Götz et al., 2010; Agati et al., 2011), our results substantiate the increase in the FLAV index at high PAR even in the absence of UV-B radiation (Fig. 2A). Considering the individual flavonols, it was shown before that in particular the accumulation of *ortho*-dihydroxy B-ring substituted compounds is affected by different light regimes (Markham et al., 1998; Agati et al., 2011). As compared to their *ortho*-monohydroxy B-ring substituted counterparts, *ortho*-dihydroxy B-ring substituted flavonols possess a greater ability to scavenge light-induced reactive oxygen species (ROS) (Agati et al., 2011). Therefore, besides its role in UV-shielding (Li et al., 1993), the function of flavonols as ROS detoxifying agents has increasingly gained attention (Agati et al., 2012; Agati et al., 2013). Since the FLAV index is closely related to the concentration of *ortho*-dihydroxy B-ring substituted flavonols (Agati et al., 2011), we assume that corresponding compounds were accumulated in the *C. asiatica* leaves in our experiment, presumably protecting the plants from oxidative damage.

Analogous to the FLAV index, the higher ANTH_RG values in response to high PAR were expected. Due to their characteristic absorption spectrum, anthocyanins are supposed to provide protection by attenuation of high fluxes of visible radiation (Steyn et al., 2002; Solovchenko and Merzylak, 2008). In addition, its accumulation was shown to be induced by UV-B radiation as well, and particularly acylated anthocyanins may even contribute to protection in this spectral region (Steyn et al., 2002; Hada et al., 2003; Gould, 2004;

Solovchenko and Merzylak, 2008). Similar to the flavonols, also anthocyanins are effective ROS scavengers (Gould, 2004). Nevertheless, since a structural classification of the non-destructively measured anthocyanins is not possible at this point, the specific function of the compounds in the *C. asiatica* leaves remains unclear. Besides that, the strongest accumulation of both flavonols and anthocyanins under high PAR/+UV-B corroborates the protective function of these constituents in *C. asiatica* leaves. As shown before, light sensing in plants occurs *via* different photoreceptors, which absorb specific wavelengths of solar irradiance and initiate physiological and developmental changes enabling the plant to adapt to the environment (Möglich et al., 2010; Heidje and Ulm, 2012). Correspondingly, the specific UV-B receptor UVR8 mediates the expression of genes involved in UV acclimation and tolerance, including flavonoid formation (Jenkins, 2009; Möglich et al., 2010). Recent publications strongly suggest the existence of a crosstalk between the UVR8 pathway and the visible light photoreceptor pathways (Boccalandro et al., 2001; Wade et al., 2001; Möglich et al., 2010). This might explain the additive effects of UV-B combined with high PAR on flavonol and anthocyanin accumulation in leaves of *C. asiatica*.

Following the pattern of plant growth, the FLAV and ANTH_RG indices increased consistently in the +UV-B treatment groups. On the other hand, the decreasing FLAV and ANTH_RG values in the −UV-B treatments from 3 until 5 WTA suggest that the absence UV-B radiation was rather unfavorable for the plant, indicating that the UV-B radiation was necessary for appropriate growth.

Similar to the FLAV and ANTH_RG indices, the BFRR_UV index was higher in the high PAR/+UV-B treatment, followed by high PAR/−UV-B. The BFRR_UV index is generally used as a robust indicator of plant stress (Chappelle et al., 1984; Lüdeker et al., 1996; Lichtenthaler and Miehé, 1997; Bürling et al., 2013; Leufen et al., 2013) since it might result from changes in the emission of blue fluorescence caused by the accumulation of blue-fluorescence emitting fluorophores, in particular hydroxycinnamic acids (reviewed in Buschmann and Lichtenthaler, 1998; Cerovic et al., 1999). Further, the fluorescence emitted by chlorophyll molecules (FRF) might be influenced: either the FRF increases due to the impact of light on the photosynthetic apparatus, or it decreases under long-lasting or stronger stress situations either due to chlorophyll degradation or because the UV excitation light is mainly absorbed by epidermal pigments (Buschmann and Lichtenthaler, 1998). Thus, in our study, the clear increase in BFRR_UV explicitly reflects the biochemical and physiological adaptations in the plant tissue to the irradiation regimes.

As shown for the flavonols and anthocyanins, also the leaf saponin and total centelloside concentrations augmented during the experimental period (Fig. 3A, B, and D). At 5 WTA, higher concentrations were assessed in the high PAR treatments. Higher saponin concentrations induced by high light intensity are in agreement with previous findings on *C. asiatica* as well as on other saponin accumulating plant species (Fournier et al., 2003; Devkota et al., 2010; Maulidiani et al., 2012). Studies on the exposure of plants or cell cultures to abiotic or biotic elicitors revealed that saponin synthesis is induced by oxidative stress (Yendo et al., 2010). Therefore, it is conceivable that the high PAR intensity provoked some kind of oxidative stress, triggering the formation of saponins in these treatment groups. Besides, it was proposed earlier that saponins possess radical scavenging activity (Tung et al., 2010). Thus, in addition to the flavonoids, the high saponin concentrations in the high PAR treatments might have contributed to the protection of the plant from oxidative damage. Contrary to the PAR intensity and in contrast to existing literature (Afreen et al., 2005), the supply of UV-B radiation did neither influence saponin nor total centelloside accumulation (Fig. 3A, B, and D). Since the sapogenin concentrations were similar in all treatment groups (Fig. 3C and E), the higher rate of sapogenin glycosylation, leading to the higher saponin concentrations under high PAR, must have been accompanied by an enhanced *de novo* synthesis of sapogenins in these treatment groups. Moreover, our findings indicate the particular role of glycosylation under high PAR. In general, glycosylation affects the stability, bioactivity, solubility, and signalling for storage or intra- and intercellular transport of the compounds (Wang and Hou, 2009; Augustin et al., 2011). Thus, on the one hand glycosylation might have been essential for the protective function of the constituents; on the other hand, analogous to the flavonols (Klein et al., 1996; Olsson et al., 1998), it cannot be excluded that the glycosylation is necessary for transport, *e.g.*, from the cytosol into the vacuole, and finally for compartmentalization of the saponins. Nevertheless, further research on the light induction, localization, and possible roles of saponins in plant protection to environmental stresses is needed.

4.2 *Relevance of the age of the tissue*

Our comparisons indicate that leaf type II generally contained higher saponin but lower sapogenin concentrations than leaf type I. Since younger leaves are assumed to be photosynthetically more valuable to plants than older leaves (Iwasa et al., 1996; Lambdon et al., 2003), we hypothesize that the saponins were particularly accumulated in leaf type II protecting the younger tissue from abiotic factors. Since the total centelloside concentrations

were similar for both leaf type I and II in the high PAR treatments, there must have been a higher rate of glycosylation in leaf type II as compared to leaf type I in these treatment groups. In contrast, in both low PAR groups leaf type II contained higher total centelloside concentrations than leaf type I indicating a higher glycosylation rate, accompanied by a higher *de novo* synthesis of sapogenins.

In general, higher saponin and higher total centelloside concentrations were observed under high PAR intensity, irrespective of the leaf type (Table 1). This supports our assumption that glycosylation is important under conditions of high light intensity. The sapogenin concentrations were not affected by the light regimes in leaf type II, while leaf type I contained significantly higher sapogenin concentrations when exposed to high PAR/+UV-B. By trend (no statistical significance), the higher sapogenin concentrations were accompanied by a lower accumulation of asiaticoside (Table 1). Over all, the results indicate that besides flavonoids, the centellosides were accumulated in the *C. asiatica* leaves enabling the plant to adapt to the different PAR intensities. We propose that glycosylation of the sapogenins is relevant for effective protection, and that the dimension of glycosylation processes is dependent on the PAR intensity and on the value of the plant tissue. Moreover, the data suggest that there might be a feedback mechanism, which regulates the *de novo* synthesis of sapogenins. Besides, it still has to be clarified whether *de novo* synthesis and glycosylation occur in the same plant organ or whether translocation processes are involved in the accumulation of the respective compounds.

In summary, our hypothesis that high PAR and ambient UV-B doses additively favor the accumulation of saponins and sapogenins in leaves of *C. asiatica* was not confirmed. Indeed, the concentrations of saponins and total centellosides and even the *de novo* synthesis of sapogenins were enhanced under high PAR, possibly due to their ability to protect the plant from oxidative damage. However, in contrast to the flavonoids, the exposure to UV-B radiation had no distinct effects on the accumulation of the centellosides. The reason for the different response pattern of the two classes of constituents might conceivably be found in their protective function. In addition to their role as radical scavengers, epidermal flavonoids are considered to act as UV-shielding pigments. By contrast, regarding the absorption spectrum of saponins, an UV-attenuating function of these compounds can be excluded. Our results further suggest that the increased accumulation of flavonoids in the presence of UV-B radiation may have contributed to the preservation of the photosynthetic performance of the plants and assured the function of cellular metabolism, leading to higher growth rates. In

contrast, the lower herb and leaf yield in the absence of UV-B radiation showed that these conditions rather constrained plant development.

Conclusively, ecologically relevant level of UV-B and high PAR intensity distinctly affected the accumulation flavonoids and centellosides in leaves of *C. asiatica,* enabling the efficient adaptation of the plants to the light regimes. Further, our study raises a number of questions as related to the induction (regulation) of the synthesis, location, and function of centellosides particularly in the context of plant protection to high PAR intensity. Some of the information gaps could be closed by using molecular approached elucidating a possible relationship and/or complementary function of flavonoids and saponins in plant protection.

The following pages of this chapter display the supplementary Figures S1 and S2.
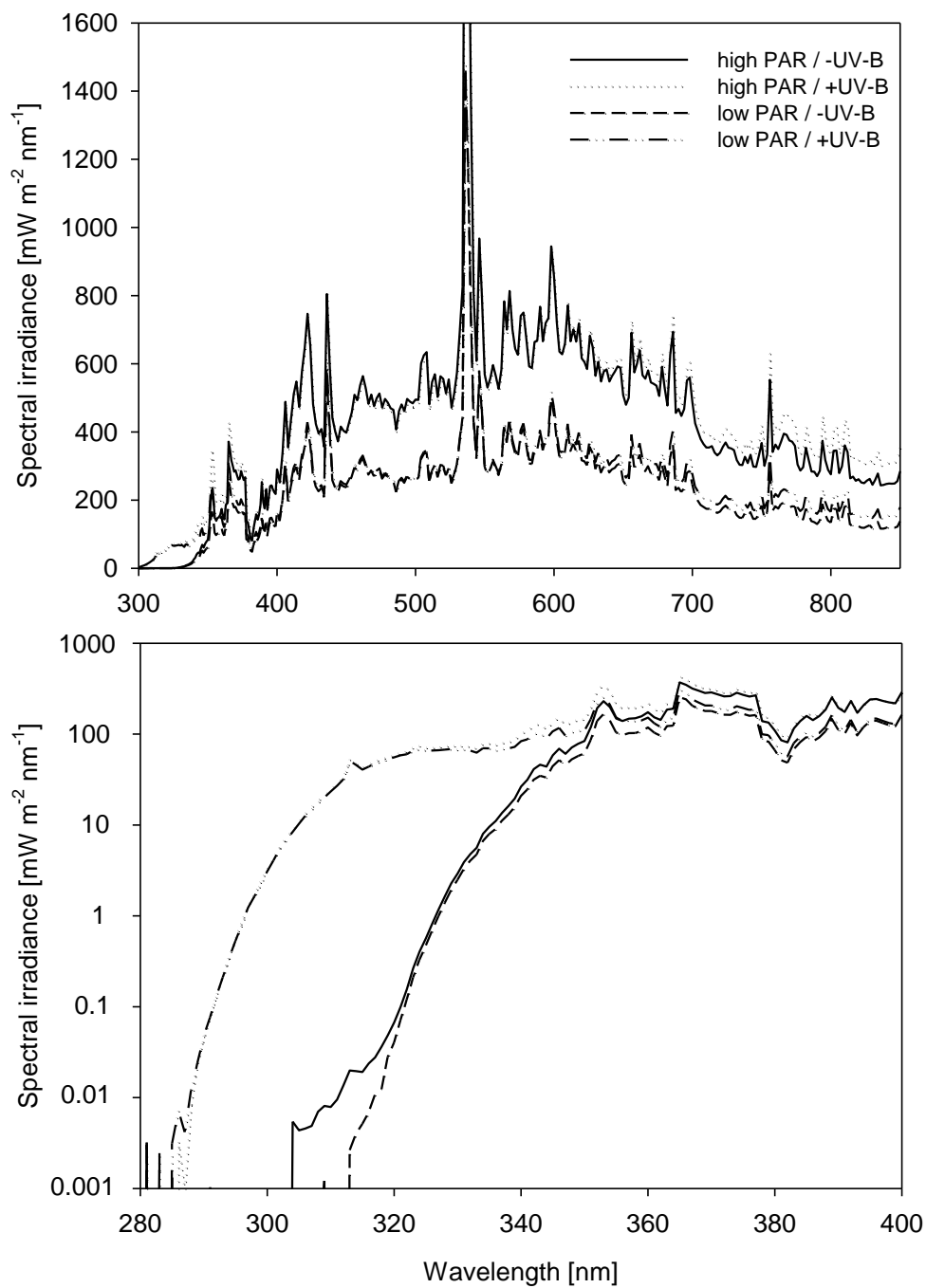
Fig. S1. Simulated irradiance spectra of the four treatments on a linear scale from 300 to 850 nm (A) and on a logarithmic scale showing the UV range from 280 to 400 nm (B).
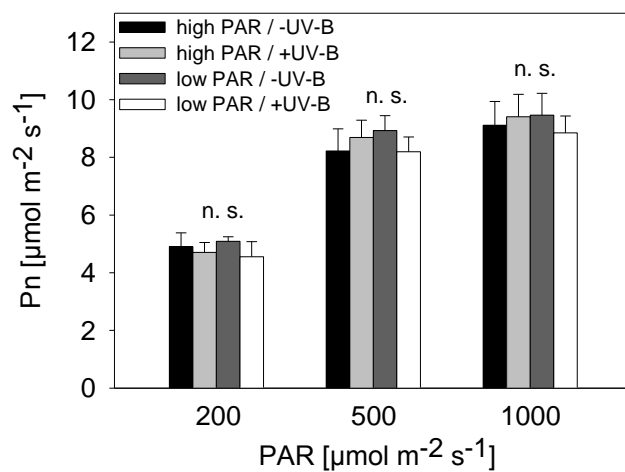
Fig. S2. Effect of PAR and UV-B radiation on the net photosynthesis of *C. asiatica* plants measured after 5 weeks of treatment application. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation. Measurements were performed at PAR of 200, 500, and 1000 μmol m^{-2} s^{-1} provided by an artificial light source (light emitting diode). Mean $\pm$ standard error ($n = 10$). Significant differences among the treatments were determined by Duncan's multiple range test ($p \leq 0.05$); n.s.: not significant.

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E   Distribution pattern and concentration of phenolic acids, flavonols, and hinokinin in *Hydrocotyle leucocephala* is differently influenced by PAR and ecologically relevant UV-B level

1   Introduction

The genus *Hydrocotyle* L. (Araliaceae) comprises more than 130 species, which are distributed throughout temperate and tropical regions of the world (Plunkett et al., 2004; Menglan et al., 2005; Nicolas and Plunkett, 2009). *Hydrocotyle leucocephala* Cham. & Schlecht. is an aquatic species with its origin in South America (Alvarez, 2001; Ramos et al., 2006). Among other substances, several flavonoids, such as kaempferol-3-O-α-L-arabinopyranosid, quercetin-3-O-α-L-arabinopyranosid, quercetin-3-O-β-D-galactopyranosid, and kaempferol-3-O-β-D-galactopyranosid, as well as the dibenzylbutyrolactone lignan (–)-hinokinin have been isolated from its extracts (Ramos et al., 2006). Flavonoids are known to possess antioxidant, antiestrogenic, and antiproliferative properties and are generally considered to promote human health and to prevent cardiovascular diseases and cancer (Ross and Kasum, 2002; Fraga et al., 2010). Correspondingly, hinokinin was shown to have immunomodulatory effects and to possess anti-inflammatory, anti-genotoxic, as well as trypanocidal activity (e Silva et al., 2004; da Silva et al., 2005; Ramos et al., 2006; Medola et al., 2007; Saraiva et al., 2007). Thus, *H. leucocephala* is a promising source for several secondary metabolites, which potentially might be considered for the development of new drugs.

Both flavonoids and lignans belong to the group of phenylpropanoids. The respective precursors *p*-coumaroyl-CoA and coniferyl alcohol are generated in the general phenylpropanoid and the cinnamate/monolignol pathway. At first, the deamination of the aromatic amino acid phenylalanine leads to cinnamic acid, which is then hydroxylized to *p*-coumaric acid. Subsequently, *p*-coumaric acid is enzymatically transformed either into *p*-coumaroyl-CoA in case of flavonoids or, *via* several intermediates, into coniferyl alcohol in case of lignans (Winkel-Shirley, 2001; Sakakibara et al., 2007; Suzuki and Umezawa, 2007).

Phenylpropanoids are generally known to be involved in plant defense (Dixon et al., 2002). Flavonoid biosynthesis is triggered by both biotic and abiotic factors. Among others, flavonoids play a major role in photoprotection of plants (Treutter, 2006; Agati and Tattini, 2010). In this regard, the enhanced accumulation of flavonoids in response to ecologically relevant ultraviolet-B levels (UV-B radiation, 280–315 nm) mediated by specific signaling pathways is well documented (*e.g.*, Li et al., 1993; Frohnmeyer and Staiger, 2003; Jenkins,

2009). Beyond, flavonoid synthesis was also shown to be induced by high intensity of photosynthetically active radiation (PAR, 400–700 nm), even in the absence of UV-B; although the presence of UV-B radiation additively promoted flavonoid accumulation in the plant tissue (Nitz et al., 2004; Götz et al., 2010; Agati et al., 2011; Müller et al., 2013).

In contrast, lignans are thought to be primarily involved in plant protection from herbivory and pathogen attack (Gang et al., 1999; Harmatha and Nawrot, 2002). So far, there is no information on the accumulation of lignans in response to abiotic factors. However, since the expression of key-enzymes of the general phenylpropanoid pathway, *e.g.*, phenylalanine ammoniumlyase, is known to be stimulated by UV radiation (Chappell and Hahlbrock, 1984; Strid et al., 1994; Jenkins et al., 2001), it is conceivable that also lignan synthesis is influenced by different light regimes.

The objective of our study was to examine the effects of ecologically relevant UV-B and PAR doses on the accumulation of hinokinin in *H. leucocephala* plants. Thereby, we aimed to elucidate its possible role in the acclimation of the plants to different light regimes and its interplay with other phenylpropanoid compounds, *e.g.*, flavonoids. For this purpose, *H. leucocephala* was cultivated under one of four light regimes, *i.e.*, two PAR levels including or excluding UV-B radiation. The experiment was conducted in special sun simulators at the Research Unit Environmental Simulation, Helmholtz Zentrum München, providing an irradiance spectrum and a diurnal variation of the photons flux rate according to natural solar radiation. We hypothesized that the accumulation of hinokinin is enhanced under ambient level of UV-B and high intensity of PAR. Among hinokinin, the most abundant phenylpropanoids, including flavonoids, were quantified after two and four weeks of treatment application. Moreover, to proof the effects of UV-B and PAR on compound accumulation in dependence on the developmental stage of the tissue and the duration of exposure, we compared the concentrations in leaves which had emerged before treatment initiation and in those ones which emerged during the experiment. Furthermore, since hinokinin was previously reported to preferentially occur in the heartwood region of trees (Erdtman and Harmatha, 1979; Takaku et al., 2001; Suzuki and Umezawa, 2007), we extended our research by additional consideration of the stems.

2 Materials and methods

2.1 Plant material

Stock plants of *H. leucocephala* Cham. & Schlecht. were obtained from the Botanical Gardens of the University of Bonn, Germany. The genome-based identification of the species was performed by A. N. Nicolas (Institute of Systematic Botany, The New York Botanical Garden, NY, USA). Four weeks before treatment initiation, plantlets were propagated vegetatively thus ensuring genetically identical plants. Nodules were cut from the stock plant stolons and stuck into Jiffy pots (Jiffy-7, Jiffy Products, Germany). Immediately after root formation, the cuttings were planted into standard cultivation pots containing a mixture of peat soil, sand, and perlite (3:2:1) as substrate. 180 homogeneous plants having six completely expanded leaves were chosen for the experiment.

2.2 Irradiation regimes and growth conditions

The four-week experiment was conducted in two sun simulators at the Helmholtz Zentrum München (Neuherberg, Germany) in October/November 2010. In each sun simulator, a combination of four different lamp types, *i.e.*, metal halide lamps, quartz halogen, blue fluorescent, and UV-B fluorescent tubes, provided an irradiance spectrum (280–850 nm) very similar to the natural solar radiation (Fig. S1). The short-wave cut-off was achieved using appropriate borosilicate and lime glass filters. The arrangement of the different lamp types in several groups enabled the simulation of natural diurnal variations of solar irradiance by switching appropriate groups on and off (Thiel et al., 1996). In order to ensure the comparability to the study published by Ramos et al. (2006), the climatic conditions were adjusted to those predominating in October in Manizales, Colombia, *i.e.*, the photoperiod was 12 h with day/night temperatures of 22/12 °C and a constant relative humidity of 80%. Fertigation was realized daily with a nutrient solution containing 19% N, 6% P_2O_5, 20% K_2O, 3% MgO, and 3% S (EC 1.35 mS cm^{-1}, Kristalon-Blaumarke, Yara GmbH & Co. KG, Germany). Each sun simulator was subdivided into two compartments, a pre-requisite to implement the four experimental treatments. 45 plants were randomly distributed in each compartment. After a one-week-period of acclimatization, the PAR intensity was increased from 200 µmol m^{-2} s^{-1} to 516 µmol m^{-2} s^{-1}. At the onset of the experiment, the PAR intensity was maintained at 516 µmol m^{-2} s^{-1} in the first sun simulator, while it was adjusted to 906 µmol m^{-2} s^{-1} in the second one. Moreover, using UV-B absorbing acrylic glass the UV-B radiation was completely excluded in one compartment, while it was set to 0.4 W m^{-2} in the

second compartment of each sun simulator. Hereinafter, the treatments will be referred to as high PAR/–UV-B, high PAR/+UV-B, low PAR/–UV-B, and low PAR/+UV-B.

2.3 Sampling and sample preparation

Six experimental plants of each treatment group were harvested after 2 and 4 weeks of treatment application (WTA). The sampled material was separated into three groups: (i) leaves that had emerged before treatment initiation (LT 1; leaves labeled at the onset of the experiment), (ii) leaves that emerged during the experiment (LT 2), and (iii) stems. At harvest, the samples were immediately shock-frozen in liquid nitrogen and stored at –25 °C. After lyophilization, the samples were ground to fine powder with a mixer mill (MM 2000, Retsch, Haan, Germany).

2.4 Identification and quantification of phenylpropanoid compounds

In order to maximize the extraction yield of the main phenylpropanoid compounds from the *H. leucocephala* samples, at first we established an appropriate extraction method. For this purpose, different solvent mixtures, the extraction time, the extraction temperature, and the number of repetitions were tested. The extraction efficiency was evaluated comparing the peak area (UHPLC-UV) of each compound and the most appropriate method was determined based on these data (*data not shown*). The method found as optimal was as follows: for each sample, 50 mg of the powdered plant material was weighed into centrifuge tubes and extracted twice in 1 mL of acetone and then twice in 1 mL of a mixture of acetone-water (7/3, v/v) under sonication for 5 min. After centrifugation at 3,000 rpm and 5 °C (Heraeus Varifuge 3.0 R, Thermo Fisher Scientific, Waltham, USA) for 15 min, the supernatants were collected in 5 mL-flasks and filled up with acetone. Every sample was assayed in duplicate. Before injection in the ultra-high-performance liquid chromatograph (UHPLC) the extracts were filtered through a 0.45 µm regenerated cellulose syringe filter (Chromafil®RC-45/15 MS, Macherey-Nagel, Düren, Germany).

The main phenylpropanoid compounds in the *H. leucocephala* leaves and stems were identified and quantified using an Acquity UHPLC system by Waters (Milford, MA, USA) consisting of a binary pump (BSM), an autosampler (SM; cooled to 10 °C; injected volume: 2 µL), a column oven (CM) set at 40 °C, a diode array detector (PDA) scanning from 190 to 400 nm, and a triple quadrupole mass spectrometer (Acquity TQD) with electrospray interface operating in negative mode. An Acquity BEH Shield RP18 column (150 mm × 2.1 mm, 1.7 µm; Waters) was used for separation with water (A) and acetonitrile (B) as eluents, both acidified with 0.1% (v/v) formic acid, at a flow rate of 0.4 mL min^{-1} with gradient

elution: 10–24% B (0–10 min), 24–60% B (10–14 min), 60–90% B (14–17 min), 90–100% B (17–17.1 min), 100% B (17.1–18.5 min), 100–10% B (18.5–19.5 min), and 10% B (19.5–22 min) (linear solvent composition change between the indicated points). The mass spectrometer was set as follows: capillary voltage –2.0 kV; cone voltage 55 V; extractor voltage 1.0 V; RF voltage 0.10 V; source temperature 150 °C; desolvation temperature 400 °C; cone gas (nitrogen) flow 50 L h^{-1}; desolvation gas (nitrogen) flow 800 L h^{-1}. The collision gas (argon) flow in tandem mass spectrometry mode was 0.3 mL min^{-1}. The whole system was controlled by MassLynx 4.1 software. Identification of the phenylpropanoid compounds was done comparing retention time, UV spectra, and mass spectra to an authentic reference and/or published data. The chlorogenic acid isomers and the unidentified phenolic acid were quantified using chlorogenic acid at 325 nm; the quercetin and kaempferol derivatives were quantified using rutin at 350 nm; the concentrations were corrected by the molecular mass of the analyte as done in previous works (Zimmermann et al., 2011; Jungfer et al., 2012). Accordingly, hinokinin was quantified using an authentic reference. The total concentrations of chlorogenic acid, quercetin, and kaempferol were calculated as the sum of the tentatively identified chlorogenic acid isomers, quercetin, and keampferol derivatives, respectively.

2.5 Statistics

All statistical analyses were carried out using PASW statistics software (Version 20.0, SPSS Inc., Chicago, USA). Means were compared by analysis of variance (One-way ANOVA). Significant differences among the treatment groups were assessed with Duncan's multiple range test ($p \leq 0.05$); differences between the harvesting dates (weeks of treatment application) were identified with Student's *t*-test (* = $p \leq 0.05$, ** = $p \leq 0.01$). The principal component analysis was performed with the freeware Ginkgo Multivariate Analysis System (University of Barcelona, Spain; http://biodiver.bio.ub.es/ginkgo/).

3 Results

3.1 Chromatography and peak identity

As shown in the UV chromatograms (Fig. S2), the phenolic acids and the flavonols of the *H. leucocephala* leaf and stem extracts were separated within 12 min; then, at about 15 min hinokinin was eluted. The detected peaks are listed in Table 1.

Table 1. List of tentatively identified compounds in the leaf and stem extracts of *H. leucocephala*. K: kaempferol; Q: quercetin; hex: hexoside; pen: pentoside; ac: acetyl; ma: malonyl; Me: methyl; x: unknown moiety; n.m.: not measured.

RT/min	[M–H]⁻	Main Fragment(s)[a]	Tentative Identification	UV max	Co-eluting Peaks
2.07	353	135, 191, 127	Chlorogenic acid isomer 1	325	
3.09	353	191, 127, 135	Chlorogenic acid isomer 2	325	
3.40	353	191,127, 135	Chlorogenic acid[c,f]	325	
5.45	367	323	Phenolic acid	326	
6.52	625	300	Q-dihex	350	
6.80	741	300	Q-x680	350	
7.42	595	300	Q-hexpen	354	
7.98	609	300	Q-hexdeshex	350	
8.58	463	300	Q-hex	354	
8.78	637	300	Q-x878	352	
8.91	579	284	K-hexpen	348	Group 891
8.97	565	300, 314	MeQ-x	351	Group 891
9.71	447	284, 300	Q-deshex	334	
9.98	621	284	K-x998	351	Group 1003
10.03	433	300	Q-pen	328	Group 1003
10.14	505	300	Q-ac-hex	350	Group 1003
10.22	621	284	K-x1022	350	Group 1003
10.57	549	300, 284	Q-ma-hex	350	
10.91[b]	505	300, 323	Q-ac-hex	n.m.	
11.72	623	284	K-x1172	329	
11.86	623	284	K-x1186	329	
15.02	355[d], 337[e]	337, 135	Hinokinin[f]	224	

[a]) Listed in order of intensity; [b]) not measured due to small peak intensity; [c]) 3-O-Caffeoylquinic acid [d]) [M+H]⁺; [e]) [M+H–H₂O]⁺ ; [f]) identity confirmed by authentic reference.

We identified three peaks having UV spectra and mass spectra similar to those of chlorogenic acid. The highest peak (3.40 min) of this triplet was identified as 3-O-caffeoylquinic acid by using the authentic reference compound. The following peak (at 5.45 min) had a maximum at 326 nm just as phenolic acids and a neutral loss of 44 in the fragment ion scan suggesting a carboxyl group. In addition, there were several peaks with a maximum around 350 nm and a dominant fragment with m/z 300 or 284 in the fragment ion scan, which

were therefore identified as quercetin and kaempferol derivatives, respectively. In some cases the neutral losses suggested the presence of a hexose, pentose, desoxyhexose, malonyl or acetyl moiety or combinations of those. So, the respective structure was tentatively assigned to the peaks. In other cases, the neutral loss could not be interpreted in that way. Some compounds could not be baseline separated and are therefore quantified as two peak groups denominated 891 and 1003 (referring to their retention times). Hinokinin was identified by comparing retention time, UV spectra, and mass spectra to an authentic reference and published data (da Silva et al., 2005).

3.2 Impact of the experimental factors on the accumulation of phenylpropanoids: an overview

In order to get a first impression on the effects of the experimental factors (light regimes, plant organs, and harvesting dates) on the accumulation of the selected phenylpropanoids in the *H. leucocephala* plants, we conducted a principal component analysis (PCA). Based on the concentrations of the individual compounds in LT 1, LT 2, and in stems, we found that the first two components PC 1 and PC 2 explained 89.67% of the variance of the data. Considering the score plot of PC 1 and PC 2, a clear separation of the leaves and the stems by PC 1 became obvious (Fig. 1A). The two leaf types, LT 1 and LT 2, were rather discriminated by PC 2. Moreover, in case of the leaves an additional clustering was observed for the two harvesting dates, 2 WTA and 4 WTA (Fig. 1A). In contrast, there was no clear discrimination of the treatment groups.

The loading plot indicates that the kaempferol derivatives, especially K-x1172, and hinokinin were the compounds responsible for the separation of the leaves and the stems (Fig. 1B). The discrimination of LT 1 and LT 2 was particularly determined by chlorogenic acid isomer 1 and phenolic acid. In contrast to the score plot, the loading plot did not reveal any clear clustering with respect to the two harvesting dates.

3.3 Distribution pattern of phenylpropanoids in leaves and stems

We investigated the distribution pattern of the phenylpropanoid compounds in different tissues and organs of *H. leucocephala* taking total chlorogenic acid, phenolic acid, total quercetin, total kaemfperol, and hinokinin as example (Table 2). Thereby, we considered the light regimes as well as the initiation and the duration of exposure. In order to elucidate possible divergences within each phenylpropanoid class, we also considered the concentrations of the individual compounds, *i.e.*, the individual chlorogenic acid isomers and the quercetin and kaempferol derivatives, in the three sample groups (Tables S1–S3).
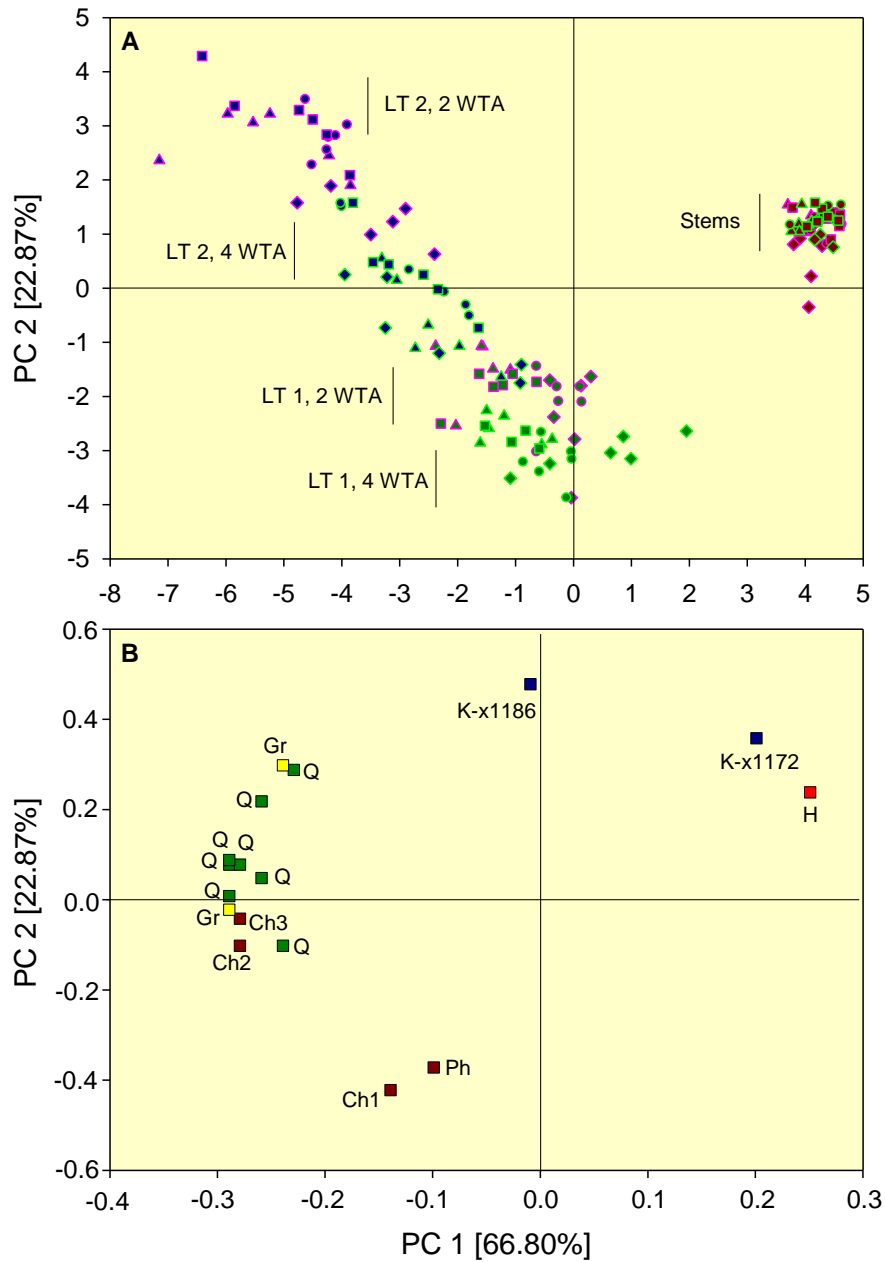
Fig. 1. Two-dimensional score and loading plots of the PCA separated by PC 1 and PC 2 of the *H. leucocephala* extracts. The score plot A) illustrates the discrimination of the plant organs (fill: green = LT 1; blue = LT 2; red = stems), the harvesting dates (frame: pink = 2 WTA; green = 4 WTA) and the treatments (♦ = high PAR/–UV-B; ▲ = high PAR/+UV-B; ● = low PAR/–UV-B; ■ = low PAR/+UV-B). The loading plot B) shows the individual compounds, which contributed to the separation of the groups (Ch = chlorogenic acid; Ph = phenolic acid; Q = quercetin; Gr = group 891, 1003; K = kaempferol; H = hinokinin).

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Table 2. Effect of PAR and UV-B level on the distribution pattern of phenylpropanoid compounds in *H. leucocephala*. Plants were exposed to four irradiation regimes (T 1: high PAR/–UV-B, T 2: high PAR/+UV-B, T 3: low PAR /–UV-B, T 4: low PAR/+UV-B) and evaluated after 2 and 4 weeks of treatment application (WTA). LT 1: leaves which had emerged before tretatment initiation; LT 2: leaves which emerged during the experiment; +: highest concentration; −: lowest concentration of the compounds (within the treatment and sampling date) according to Duncan's multiple range test ($p \leq 0.05$); n.s.: not significant.

Compound	T	2 WTA			4 WTA		
		LT 1	LT 2	Stems	LT 1	LT 2	Stems
Chlorogenic acid[a]	1		+	−		+	−
	2		+	−		+	−
	3		+	−	+	+	−
	4		+	−		+	−
Phenolic acid	1	n.s.			+	+	−
	2	n.s.			+		−
	3	+	−	−	+	−	−
	4	+	−	−	+		−
Quercetin[b]	1		+	−		+	−
	2		+	−		+	−
	3		+	−		+	−
	4		+	−		+	−
Group 891	1		+	−		+	−
	2		+	−	+	+	−
	3		+	−		+	−
	4		+	−		+	−
Group 1003	1	−	+	−	−	+	−
	2		+	−	−	+	−
	3	−	+	−	−	+	−
	4	−	+	−	−	+	−
Kaempferol[c]	1	−		+	−		+
	2	−		+	−		+
	3	−		+	−		+
	4	−		+	−		+
Hinokinin	1	−	−	+	−	−	+
	2	−	−	+	−	−	+
	3	−	−	+	−	−	+
	4	−	−	+	−	−	+

[a]) Total chlorogenic acid; [b]) total quercetin; [c]) total kaempferol.

Firstly, we observed that most of the individual compounds within each class followed the same distribution pattern within the analyzed organs. The exceptions were chlorogenic acid isomer 1 and K-x1186 (Tables S1–S3, $p \leq 0.05$). Further, apart from a few cases, the distribution pattern of the compounds was not influenced by the irradiation regimes, neither at 2 WTA nor at 4 WTA (Table 2, Tables S1–S3, $p \leq 0.05$).

The highest concentrations of the chlorogenic acid isomers 1 and 2, quercetin derivatives, group 891, and group 1003 were generally assessed in leaves which emerged during the experiment (LT 2). Moreover, comparing just the leaf types, LT 2 also contained higher concentrations of kaempferol derivatives than leaves which had emerged before treatment initiation (LT 1) (Tables S1 and S2, $p \leq 0.05$). Contrary to that and in accordance with the PCA, chlorogenic acid isomer 1 and phenolic acid were most abundant in LT 1 (Table 2, Tables S1–S3, $p \leq 0.05$). The highest total concentrations of kaempferol derivatives and hinokinin were found in the stems (Table 2), although K-x1186 was most abundant in LT 2 at 2 WTA (Tables S1–S3, $p \leq 0.05$). The hinokinin concentrations in stems were about ten times higher than in leaves (Tables S1–S3, $p \leq 0.05$).

3.4 Effect of the PAR/UV-B combinations on the concentration of phenylpropanoids in leaves and stems

3.4.1 Phenolic acids

The total concentration of chlorogenic acid was not affected by the light regimes irrespective of the plant organ, the leaf type, and the harvesting date (Figs. 2A, 3A, and 4A). Moreover, the concentration remained at a constant level in LT 1 and in stems during the whole experimental period. In contrast, in LT 2 the concentration of total chlorogenic acid decreased from 2 until 4 WTA (Fig. 3A). This decrease was caused by concentration changes of 3-O-caffeoylquinic acid, which had concentrations up to 65-fold higher than those observed for chlorogenic acid isomers 1 and 2, and therefore overlaid the results of the latter compounds (Table S2). In contrast to 3-O-caffeoylquinic, the concentrations of chlorogenic acid isomer 1 significantly increased in both leaf types, LT 1 and LT 2, from 2 until 4 WTA (Tables S1 and S2). Examining the individual compounds we found that the accumulation of chlororgenic acid isomer 1 was favored either by high PAR in LT 2 (irrespective of the harvesting date) or by low PAR in stems (at 4 WTA) (Table S2 and S3). In contrast, the concentrations of chlorogenic acid isomer 2 were higher under low PAR in LT 1, but higher under high PAR in stems (at 4 WTA; Table S1 and S3). Although not statistically significant,
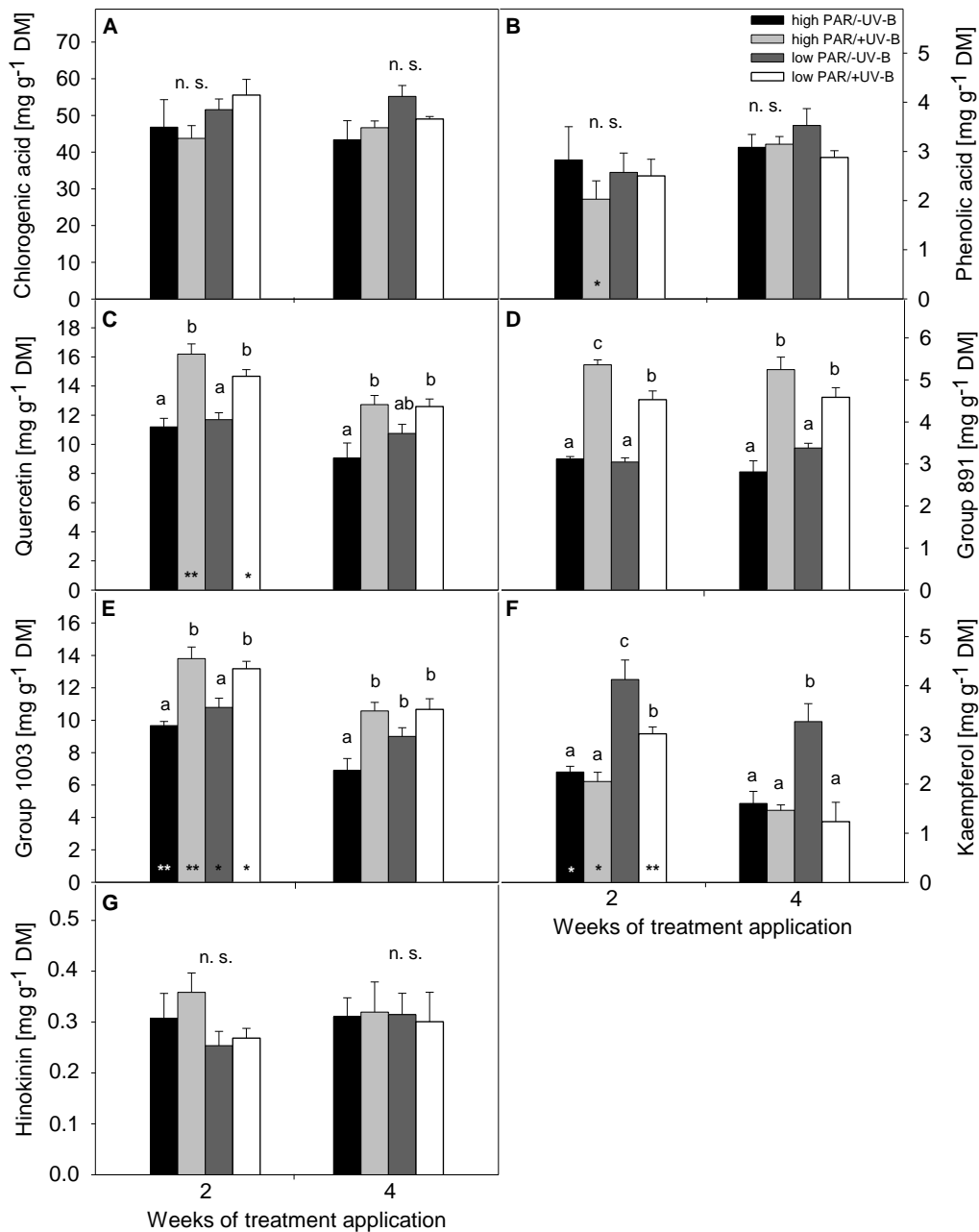
the accumulation of 3-O-caffeoylquinic acid in LT 1 was slightly promoted by low PAR as well as by UV-B radiation, irrespective of the PAR intensity (at 2 WTA; Table S1).

The concentration of the unidentified phenolic acid was influenced by the light regimes neither in LT 1 nor in stems, while in LT 2 the highest concentration was detected in the treatment groups high PAR/+UV-B and high PAR/–UV-B at 4 WTA (Fig. 3B).
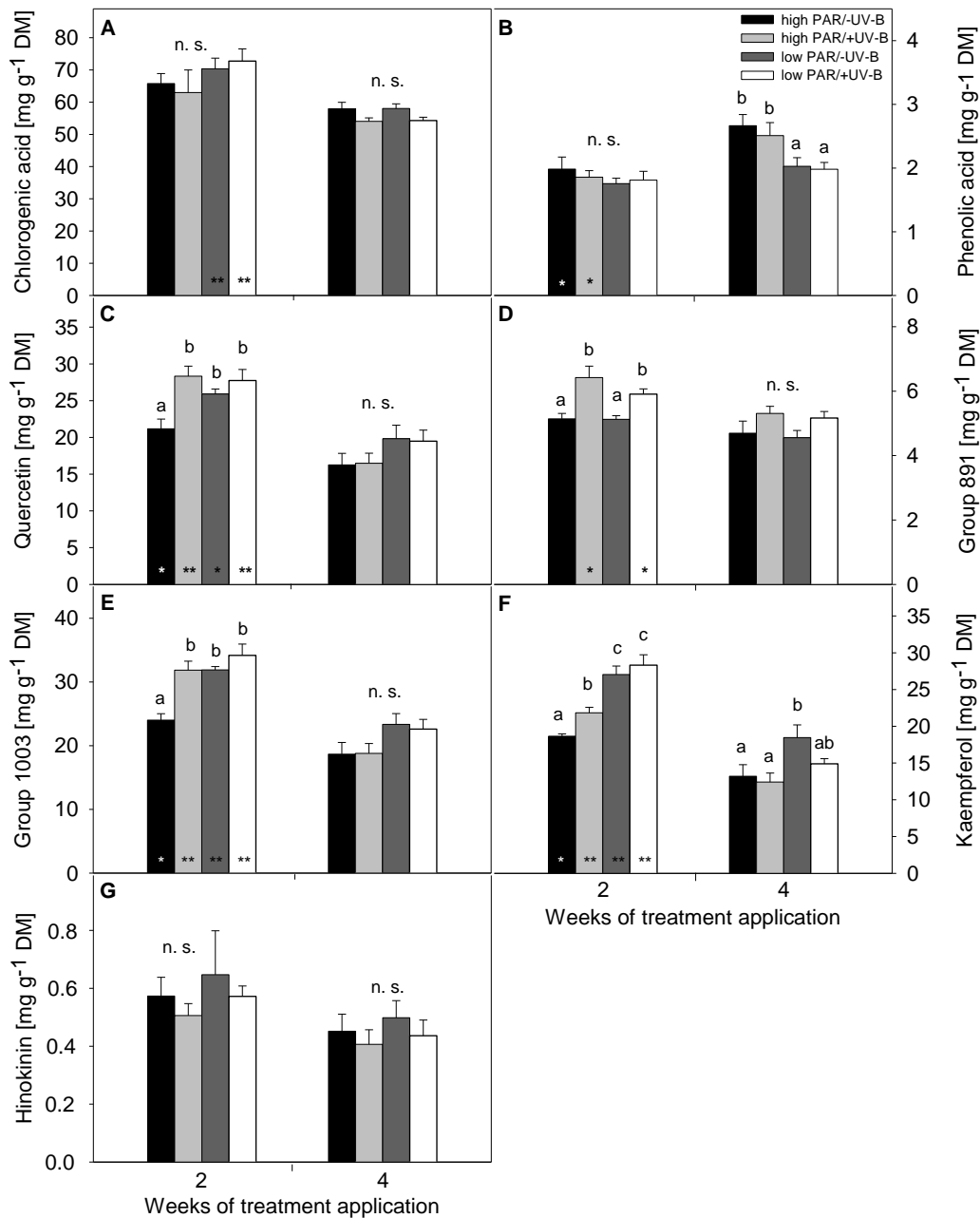
3.4.2 Quercetin

In LT 1, a significant enhancement of total quercetin was induced by UV-B radiation at 2 WTA, irrespective of the PAR level (Fig. 2C). Since the concentration significantly decreased in the same treatment groups (high PAR/+UV-B and low PAR/+UV-B) until 4 WTA, the +UV-B treatment groups and the treatment low PAR/–UV-B did not differ statistically any longer at the end of the experiment. Analogous to the total quercetin concentrations, also the accumulation of the majority of the individual derivatives, *e.g.*, Q-x680, Q-hexpen, Q-x878, and Q-hex, was promoted by UV-B radiation (Table S1). Moreover, in most cases the highest concentrations, at least by trend, were found when UV-B radiation was combined with high PAR. This was particularly noticed for Q-deshex, Q-ma-hex, and Q-x680 at 2 WTA (Table S1). In contrast, when UV-B radiation was absent, higher concentrations were mostly detected under low PAR (*e.g.*, in case of total quercetin concentrations, Q-hex, Q-x878, and Q-hexdeshex at 4 WTA; Fig. 2C, Table S1).
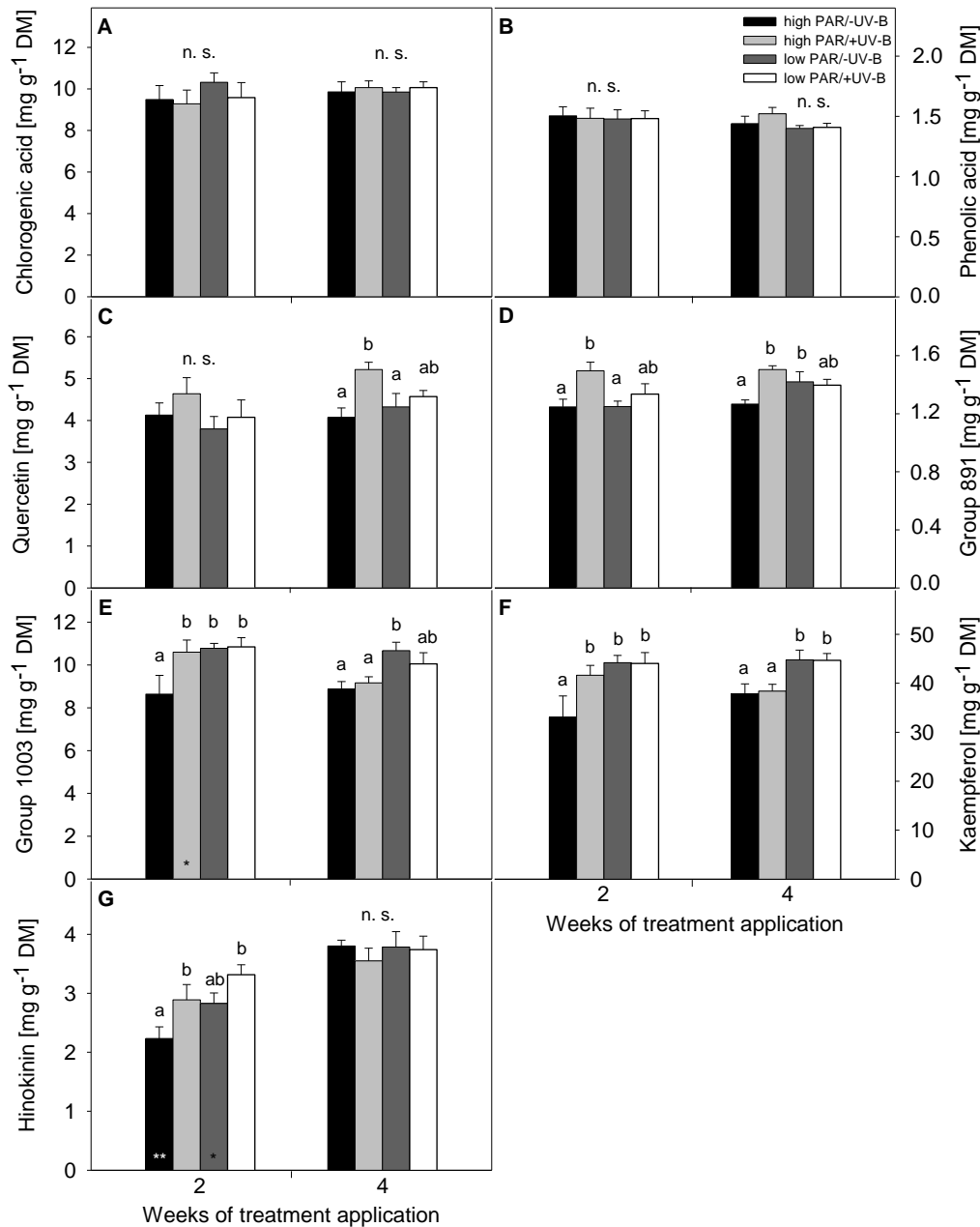
Similar to LT 1 at 4 WTA, in LT 2 the total concentrations of quercetin were higher in the treatment groups high PAR/+UV-B, low PAR/–UV-B, and low PAR/+UV-B at 2 WTA. The decreasing concentrations during the experimental period led to similar concentrations in all treatment groups at 4 WTA (Fig. 3C). These findings in turn were applicable to most of the individual derivatives, *i.e.*, to Q-deshex, Q-x680, Q-hex, Q-hexpen, and Q-x878 (Table S2). Thus, in both leaf types, LT 1 and LT 2, the differences among the treatment groups were greater at 2 WTA and faded during the time course. Moreover, the enhancement in quercetin concentrations in response to UV-B radiation was more pronounced in LT 1 than in LT 2. In stems, the total quercetin concentrations were promoted in the presence of UV-B radiation, especially at 4 WTA (Fig. 4C). According to LT 1, the highest concentration was induced by the treatment high PAR/+UV-B. Considering the individual quercetin derivatives, similar results were particularly observed for Q-x878 and Q-hex (Table S3). Q-dihex and Q-hexdeshex were completely absent in the stems, while Q-x680 could only be determined in plants when exposed to UV-B radiation.

Fig. 2. Effect of PAR and UV-B level on the accumulation of phenylpropanoids in *H. leucocephala* leaves which had emerged before treatment initiation. Plants were exposed to two PAR levels (high, low) including or excluding UV-B radiation (+UV-B, –UV-B). Concentrations of phenylpropanoid compounds (total chlorogenic acid (A), phenolic acid (B), total quercetin (C), group 891 (D), group 1003 (E), total kaempferol (F), hinokinin (G)) were determined after 2 and 4 weeks of treatment application (WTA). Mean ± standard error (*n* = 6). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, *p* ≤ 0.05); significant differences between WTA are indicated by asterisks (Student's *t*-test, * = *p* ≤ 0.05, ** = *p* ≤ 0.01). The scaling is individually adjusted to the concentration of the respective compound.

Fig. 3. Effect of PAR and UV-B level on the accumulation of phenylpropanoids in *H. leucocephala* leaves which emerged during the experiment. Plants were exposed to two PAR levels (high, low) including or excluding UV-B radiation (+UV-B, –UV-B). Concentrations of phenylpropanoid compounds (total chlorogenic acid (A), phenolic acid (B), total quercetin (C), group 891 (D), group 1003 (E), total kaempferol (F), hinokinin (G)) were determined after 2 and 4 weeks of treatment application (WTA). Mean ± standard error ($n = 6$). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, $p \leq 0.05$); significant differences between WTA are indicated by asterisks (Student's *t*-test, $* = p \leq 0.05$, $** = p \leq 0.01$). The scaling is individually adjusted to the concentration of the respective compound.

Fig. 4. Effect of PAR and UV-B level on the accumulation of phenylpropanoids in *H. leucocephala* stems. Plants were exposed to two PAR levels (high, low) including or excluding UV-B radiation (+UV-B, –UV-B). Concentrations of phenylpropanoid compounds (total chlorogenic acid (A), phenolic acid (B), total quercetin (C), group 891 (D), group 1003 (E), total kaempferol (F), hinokinin (G)) were determined after 2 and 4 weeks of treatment application (WTA). Mean ± standard error (*n* = 6). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, *p* ≤ 0.05); significant differences between WTA are indicated by asterisks (Student's *t*-test, * = *p* ≤ 0.05, ** = *p* ≤ 0.01). The scaling is individually adjusted to the concentration of the respective compound.

3.4.3 Kaempferol

Contrary to the quercetin derivatives, the total kaempferol concentrations in LT 1 were significantly higher under low PAR at 2 WTA (Fig. 2F). During the time course, a strong decrease was observed particularly in the treatment low PAR/+UV-B, which finally led to concentrations similar to those determined in the high PAR treatment groups. The highest concentrations were found in the treatment low PAR/–UV-B, irrespective of the harvesting date. Since K-x1186 was neither influenced by the irradiation regimes nor by the duration of exposure, the findings for the total kaempferol concentrations were related to K-x1172 (Table S1). Although the total kaempferol concentrations were notably higher in LT 2 than in LT 1, the trend was similar. Thus, analogous to LT 1 higher concentrations were observed in LT 2 under low PAR at 2 WTA (Fig. 3F). At 4 WTA higher concentrations were assessed in the treatment group low PAR/–UV-B, followed by low PAR/+UVB. These findings were applicable to K-x1172, while K-x1186 concentrations were significantly enhanced in the presence of UV-B radiation at 2 WTA; the highest concentrations were induced by low PAR/+UV-B (Table S2). Moreover, even in stems higher total kaempferol concentrations were determined in the treatment groups low PAR/–UV-B and low PAR/+UV-B at 2 WTA, although these groups did not differ statistically from the treatment high PAR/+UV-B (Fig. 4E). However, since the concentrations in the treatment group high PAR/+UV-B decreased from 2 until 4 WTA, higher concentrations were finally determined in the low PAR groups. Considering the individual kaempferol derivatives, the findings can especially be ascribed to K-x1172 (Table S3).

3.4.4 Quercetin/kaempferol ratio

The highest total quercetin/total kaempferol ratio was determined in the treatment high PAR/+UV-B, followed by high PAR/–UV-B and low PAR/+UV-B, irrespective of the leaf type; although the differences among the treatment groups were more pronounced in LT 1 (Table 3). The quercetin/kaempferol ratio in LT 2 was about 1, while it was notably higher in LT 1 (about 3 to 9, Table 3) and lower in stems (about 0.1).

3.4.5 Hinokinin

The hinokinin concentrations in both leaf types, LT 1 and LT 2, were generally low and statistically similar for all the treatment groups, irrespective of the harvesting date (Figs. 2G and 3G). Contrary to the leaves, the hinokinin accumulation in stems was promoted by UV-B radiation at 2 WTA, irrespective of the PAR level (Fig. 4G). By trend, higher concentrations

were induced at low PAR. Finally, due to the increase in the hinokinin concentrations in the absence of UV-B radiation, the treatment groups did not differ any longer at 4 WTA.

Table 3. Effect of PAR and UV-B level on the ratio of quercetin/kaempferol in LT 1, LT 2, and stems of *H. leucocephala*. Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation (+UV-B and –UV-B) and evaluated after 2 and 4 weeks of treatment application (WTA). LT 1: leaves which had emerged before treatment initiation; LT 2: leaves which emerged during the experiment.

			Quercetin/kaempferol ratio		
WTA	**Treatment**		**LT 1**	**LT 2**	**Stems**
2	**high PAR**	**–UV-B**	5.01 ± 0.17 b	1.13 ± 0.05 b	0.13 ± 0.01 b
		+UV-B	8.04 ± 0.33 c	1.30 ± 0.05 c	0.11 ± 0.01 ab
	low PAR	**–UV-B**	2.95 ± 0.20 a	0.96 ± 0.03 a	0.09 ± 0.01 a
		+UV-B	4.90 ± 0.19 b	0.98 ± 0.04 ab	0.09 ± 0.01 a
4	**high PAR**	**–UV-B**	5.94 ± 0.44 b	1.25 ± 0.03 b	0.11 ± 0.00 a
		+UV-B	8.78 ± 0.24 c	1.34 ± 0.03 b	0.14 ± 0.00 b
	low PAR	**–UV-B**	3.57 ± 0.43 a	1.08 ± 0.04 a	0.10 ± 0.01 a
		+UV-B	6.82 ± 0.20 b	1.30 ± 0.04 b	0.10 ± 0.00 a

Mean $\pm$ standard error ($n = 6$). Values followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$).

4   Discussion

We investigated the impact of four irradiation regimes, *i.e.*, two PAR levels including or excluding UV-B radiation, on the composition, amount, and distribution pattern of selected phenylpropanoids, including hinokinin, in leaves and stems of *H. leucocephala*.

4.1 Phenylpropanoid compounds in the H. leucocephala plants

In our work we tentatively identified 22 different phenylpropanoid compounds in the plant extracts, *i.e.*, three chlorogenic acid isomers, one unidentified phenolic acid, twelve quercetin derivatives, five kaempferol derivatives, and the dibenzylbutyrolactone lignan hinokinin (Table 1). The occurrence of two kaempferol glycosides, two quercetin glycosides, as well as hinokinin has been previously described in *H. leucocephala* (Ramos et al., 2006). However, as compared to Ramos et al. (2006) we found a greater number of phenylpropanoid compounds in the plant extracts, a result which can be attributed either to genetic variability,

developmental stage of the plants, and/or environmental divergences, or perhaps also to a more sensitive analytical method.

4.2 Distribution pattern and concentration of the phenylpropanoids as influenced by the light regimes

The qualitative composition of phenylpropanoids in plants is certainly genetically defined (Zimmermann and Glensa, 2007), while the quantity of the compounds is known to be influenced by environmental factors, and may also be affected by the tissue, the organ, and the developmental stage of the plant (*e.g.*, Bohm, 1987; Burchard et al., 2000; Laitinen et al., 2002; Schreiner et al., 2009). Correspondingly, the quantity of the phenylpropanoids in the *H. leucocephala* extracts was strongly dependent on the plant organ and the leaf type. Moreover, the single phenylpropanoids were affected by the irradiation regimes and the duration of exposure in a very specific manner (even within the same compound class), which explains the failure of the PCA of separating the treatment groups on basis of the chemical composition of the plant extracts. By contrast, the distribution pattern of the constituents within the analyzed organs (leaves and stems) was not influenced by the PAR/UV-B combinations (Table 2). Thus, irrespective of the light regimes and the harvesting dates, the stems stored higher amounts of hinokinin and kaempferols than the leaves, while LT 1 had higher concentrations of chlorogenic acid isomer 1 and phenolic acid than LT 2 (Fig. 1).

Nevertheless, with the exception of the latter compounds, younger leaves (LT 2) mostly contained higher phenylpropanoid concentrations than older leaves (LT 1) (Table 2). It has been proposed, that young, fully expanded leaves are photosynthetically more valuable to plants than older leaves, and should therefore be more protected against biotic and abiotic stress (Iwasa et al., 1996; Lambdon et al., 2003). On the other hand, the fact that chlorogenic acid isomer 1 and phenolic acid were most abundant in LT 1 (Table 2, Tables S1–S3) confirms that young leaves do not always possess higher concentrations, particularly if individual compounds are considered (Estiarte et al., 1999; Laitinen et al., 2002). Moreover, although the highest concentrations of chlorogenic acids, quercetin, and kaempferol derivatives most often were found in LT 2 (Table 2), a stronger influence of the light regimes and especially of UV-B radiation was observed in LT 1 (Figs. 2 and 3). Thus, the UV-B effect on phenylpropanoid synthesis was more pronounced in adult leaves, which were suddenly exposed to UV-B radiation, as compared to developing leaves, which were exposed to the UV-B wavelengths throughout their whole lifetime. Furthermore, the overall higher phenylpropanoid concentrations in LT 2 as compared to LT 1 indicate that the

phenylpropanoid synthesis in the younger leaves was not exclusively induced by UV-B radiation but also by other environmental factors, *e.g.*, by PAR (Table 2).

The most responsive compounds to the different light regimes were the quercetin derivatives. Their accumulation in leaves was strongly enhanced under UV-B exposure and occasionally promoted by high PAR intensity (Figs. 2C, 3C, and 4C, Tables S1–S3). The increased accumulation of quercetin in response to ambient UV-B levels is well documented (Kolb et al., 2001; Reifenrath and Müller, 2007; Morales et al., 2010; Agati et al., 2011). Moreover, our findings are in agreement with research on *Arabidopsis thaliana* reporting on higher concentrations of quercetin in leaves induced by high PAR/+UV-B in comparison to low PAR/+UV-B (Götz et al., 2010). Furthermore, studies on various plant species confirmed the promoting effect of high PAR on flavonol accumulation, including quercetin, even in the absence of UV-B radiation (Nitz et al., 2004; Agati et al., 2011; Müller et al., 2013). Interestingly, when our plants were not exposed to UV-B radiation, we found higher quercetin concentrations under low PAR (Fig. 2C, Table S1).

In contrast to quercetin, the concentrations of the kaempferol derivatives were not influenced by UV-B radiation (Figs. 2F, 3F, and 4F, Tables S1–S3). Earlier publications point out that keampferol derivatives are rather unaffected or only slightly affected by UV-B radiation, at least in comparison with quercetin derivatives (Olsson et al., 1998; Hofmann et al. 2003; Reifenrath and Müller 2007; Kuhlmann and Müller, 2009). Analogous to the results on other species (Olsson et al., 1998; Hofmann et al., 2003; Reifenrath and Müller, 2007; Kuhlmann and Müller, 2009), we observed a notable increase in the quercetin/keampferol ratio under UV-B as well as under high PAR exposure. The stronger increase in the accumulation of *ortho*-dihydroxylated flavonoids, such as quercetins, relative to *ortho*-monohydroxylated flavonoids, such as kaempferols, in response to UV-B radiation as well as high light intensity is generally explained by the higher antioxidant properties of the *ortho*-dihydroxylated flavonoids owing to the additional hydroxyl group in the B-ring of the flavonoid skeleton (Husain et al., 1987; Markham et al., 1998; Agati et al., 2011). Flavonoids have long been considered to primarily act as UV-screening compounds in the epidermis cells of plants (*e.g.*, Rozema et al., 1997 and references therein). However, it was shown that under UV-B exposure *ortho*-dihydroxylated flavonoids are accumulated to a greater extent than other, more effective UV attenuators, such as *ortho*-monohydroxylated flavonoids and hydroxycinnamic acids (HCAs) (Burchard et al., 2000; Tattini et al., 2000; Kotilainen et al., 2008). Moreover, the fact that flavonoids are not exclusively located in the epidermis but also in the mesophyll cells and in chloroplasts (Agati et al., 2007; Agati et al., 2009) put emphasis

on the important function of *ortho*-dihydroxylated flavonoids as scavengers of light induced reactive oxygen species (ROS) (Agati, et al. 2012; Agati et al., 2013). Therefore, we suggest that the quercetin derivatives were preferentially accumulated in the *H. leucocephala* leaves under UV-B and high PAR exposure to enhance the capacity for ROS detoxification.

Interestingly, the quercetin/kaempferol ratio decreased in the order LT 1, LT 2, and stems (Table 2). Besides its role in ROS scavenging, *ortho*-dihydroxylated flavonoids were reported to act as inhibitors of auxin transport and catabolism regulating the development of the whole plant and of individual shoot organs (Galston, 1969; Jansen, 2002; Lazar and Goodman, 2006; Beveridge et al, 2007; Buer and Djordjevic, 2009). While shade plants have long internodes and large leaves, sunny plants usually have short internodes and small, thick leaves. Thus, it is conceivable that the presence of the high quercetin concentrations in the group LT 1 (adult leaves) especially under high PAR regulated leaf growth by inhibition of auxin activity leading to adaptations of the leaf architecture to the light regimes. In contrast, in LT 2 (young leaves, which probably had not reached their final size at harvesting time) the quercetin/kaempferol ratio was lower than in LT 1 and auxin was possibly not inhibited by quercetin, or at least to a lesser extent. This hypothesis is strongly supported, *e.g.*, by the lower quercetin/kaempferol ratio (Table 3) accompanied by the larger leaf area in LT 2 at 4 WTA under low PAR/–UV-B as compared to the other treatment groups ($p \leq 0.05$, *data not shown*). Besides, the very high kaempferol concentrations in the stems (Fig. 4F) might indicate that the accumulation of the compounds in the leaves was accomplished not only by *de novo* synthesis but also by translocation processes through the stems (Del Baño et al., 2004).

Although chlorogenic acid derivatives made up the largest part of the phenylpropanoid compounds in both leaf types, LT 1 and LT 2, the concentrations were only slightly affected by the light regimes (Figs. 2A and 3A). In dependence on the organ and the leaf type, a certain effect was observed under the different PAR intensities, while UV-B radiation had a negligible impact on chlorogenic acid accumulation (Figs. 2A, 3A, and 4A, Tables S1–S3). Accordingly, it has been observed earlier that in contrast to flavonoids, HCAs, such as chlorogenic acid, are rather unaffected by different light conditions (Reuber et al., 1996; Burchard et al., 2000). The authors proposed that HCAs are constitutively present in plants and that they are increasingly replaced by antioxidant flavonoids under UV and high light exposure during leaf development (Burchard et al., 2000; Agati and Tattini, 2010). Chlorogenic acids are esters of caffeic acid and quinic acid, which are the precursors of a variety of phenolic compounds, including flavonoids. In this context, the competition between

the HCA and the flavonoid biosynthetic pathways has frequently been reported (Li et al., 1993; Kolb et al., 2001; Schoch et al., 2001; Agati and Tattini, 2010). Occasionally, an inverse relationship between the chlorogenic acid isomers and the quercetin derivatives was even observed in our experiment (*e.g.*, between chlorogenic acid isomer 1 and some quercetin derivatives in LT 2, Table S2 and S3). Moreover, although the concentrations of chlorogenic acid isomers were not steadily lower in the +UV-B treatments, there must have been a higher allocation of precursors towards the synthesis of quercetin derivatives in the +UV-B treatment groups, probably at the expense of HCA and kaempferol formation. Correspondingly, in LT 2 the concentration of 3-O-caffeoylquinic acid significantly decreased from 2 until 4 WTA (Fig. 3A). Thus, 3-O-caffeoylquinic acid was presumably degraded in the early leaf development in favor of the synthesis of other phenylpropanoid compounds, *e.g.*, quercetin derivatives (Table S2). However, in contrast to 3-O-caffeoylquinic acid the concentrations of chlorogenic acid isomer 1 significantly increased in both leaf types, LT 1 and LT 2, from 2 until 4 WTA (Tables S1 and S2). Thus, the individual isomers responded very differently to the light regimes, which indicates that not all HCAs were likewise replaced by flavonoids or accumulated to a lesser extend due to competition between the two biosynthetic pathways.

As shown, the formation of the chlorogenic acid isomers in the *H. leucocephala* plants was influenced rather by PAR than by UV-B radiation (Figs. 2A, 3A, and 4A, Tables S1–S3). Contrary to that, a number of studies report on enhancing effects of UV-B radiation on the accumulation of individual HCA esters (Lancaster et al., 2000; Tegelberg et al., 2004; Huyskens-Keil et al., 2007; Manukyan, 2013). The divergent findings might be explained by differences in the experimental setups. For instance, those studies designed to investigate the UV-B effects were not suited to recognize the impact of PAR; other experiments were confined to a single one PAR intensity. Moreover, variations in the temperature (Harbaum-Piayda et al., 2010) and in the applied UV-B doses (Huyskens-Keil et al., 2007) may have influenced the respective results. However, in accordance with our findings, the importance of PAR for HCA induction was likewise demonstrated in experiments on grape leaves (*Vitis vinifera* cv. *Silvaner*), which contained higher HCA concentrations under visible light exposure in the absence of UV radiation (Kolb et al., 2001).

Finally, our key findings are related to hinokinin. Since the expression of important enzymes of the phenylpropanoid pathway is known to be up-regulated by UV-B radiation, we hypothesized that the accumulation of hinokinin is enhanced under UV-B and high light exposure. Based on our results this hypothesis could not be confirmed. Thus, we did not find any effects of the irradiation regimes on the hinokinin concentrations in leaves, while its

accumulation in stems was slightly favored in the presence of UV-B radiation. Lignans, including hinokinin, were shown to occur in various plant organs, *e.g.*, in leaves, stems, and fruits (Row, 1978; Massanet et al., 1989; Ayres and Loike, 1990; Elfahmi et al., 2007). Nevertheless, the very high concentrations of hinokinin in the *H. leucocephala* stems as compared to the leaves in our experiment suggest that hinokinin does not play a major role in plant protection from irradiation in leaves. Moreover, considering the chemical structure, it can be excluded that hinokinin serves as ROS-scavenging agent (Eklund et al., 2005), although factual studies are still missing. Reports further put emphasis on the accumulation of lignans in the heartwood region of trees, where they are supposed to encrust the cell walls and to protect the heartwood from pathogen attack (Erdtman and Harmatha, 1979; Takaku et al., 2001; Suzuki and Umezawa, 2007). Therefore, we propose that hinokinin might rather contribute to cell wall stabilization in the *H. leucocephala* stems improving the structural stability and the resistance of the plants to environmental factors, including UV-B radiation (Greenberg et al., 1997; Shetty et al., 2002).

Conclusively, our study contributes to a better understanding of the inducibility, function, and interplay of phenylpropanoids, and in particular lignans, in different plant organs faced with different PAR/UV-B combinations. Here we demonstrate that the accumulation of flavonols is particularly determined by its ability to scavenge ROS, while the generation of distinct phenolic acids is affected by the demand of substrate for the formation of antioxidative flavonoids. Our results further indicate that lignans might contribute to the acclimation of the plant, *e.g.*, to UV-B radiation by cell wall encrustation in the stems. Nevertheless, as indicated the single phenylpropanoids were affected by the irradiation regimes in a very specific manner. In addition, the influence of the plant organ and tissue on the accumulation of the compounds complicates the interpretation and calls for further experiments on the photoprotective and complementary function of phenylpropanoids. Moreover, particular research is needed to elucidate in depth the role of lignans in different organs of herbaceous species and its accumulation in response to environmental factors.

The following pages of this chapter display the supplementary Tables S1, S2, and S3 and Figures S1 and S2.

Table S1. Effect of PAR and UV-B level on the concentration of chlorogenic acid isomers, quercetin, and kaempferol derivatives in *H. leucocephala* leaves which had emerged before treatment initiation (LT 1).

Comp. [a] [mg g^-1]	WTA	high PAR −UV-B			high PAR +UV-B			low PAR −UV-B			low PAR +UV-B		
1	2	2.51 ± 0.12	n. s.		2.57 ± 0.12			2.25 ± 0.12			2.52 ± 0.07		
	4	3.39 ± 0.25	n. s.	**	3.52 ± 0.22		**	3.93 ± 0.16		**	3.91 ± 0.21		**
2	2	6.38 ± 0.28	n. s.		7.02 ± 0.43			7.40 ± 0.54			7.61 ± 0.46		
	4	5.15 ± 0.36	A	*	5.41 ± 0.32	a	*	6.99 ± 0.37	b		6.71 ± 0.20	b	
3	2	30.93 ± 3.32	A		34.16 ± 3.52	ab		41.95 ± 3.11	ab		45.43 ± 4.11	b	
	4	34.84 ± 4.69	n. s.		37.73 ± 1.68			44.28 ± 2.98			38.41 ± 0.93		
4	2	0.37 ± 0.03	n. s.		0.40 ± 0.04			0.30 ± 0.04			0.33 ± 0.02		
	4	0.31 ± 0.04	n. s.		0.34 ± 0.03			0.27 ± 0.03			0.32 ± 0.02		
5	2	0.35 ± 0.02	A		0.48 ± 0.02	c		0.36 ± 0.02	a		0.43 ± 0.01	b	
	4	0.28 ± 0.04	n. s.		0.36 ± 0.02		**	0.35 ± 0.02			0.35 ± 0.01		**
6	2	4.59 ± 0.24	A		6.15 ± 0.29	b		4.77 ± 0.21	a		5.60 ± 0.17	b	
	4	4.10 ± 0.49	n. s.		4.92 ± 0.24		**	4.81 ± 0.32			4.91 ± 0.11		*
7	2	0.26 ± 0.01	n. s.		0.31 ± 0.02			0.26 ± 0.02			0.29 ± 0.01		
	4	0.20 ± 0.02	A		0.31 ± 0.01	c		0.24 ± 0.01	ab		0.28 ± 0.01	bc	
8	2	1.89 ± 0.16	A		2.79 ± 0.24	c		2.10 ± 0.13	ab		2.53 ± 0.15	bc	
	4	1.33 ± 0.17	A	*	1.84 ± 0.07	b	**	1.52 ± 0.12	ab	**	1.78 ± 0.13	b	**
9	2	2.06 ± 0.14	A		2.86 ± 0.11	b		2.15 ± 0.09	a		2.68 ± 0.09	b	
	4	1.47 ± 0.16	A	*	1.94 ± 0.11	b	**	1.86 ± 0.13	b		2.08 ± 0.06	b	**
10	2	0.29 ± 0.01	A		0.59 ± 0.03	c		0.30 ± 0.01	a		0.46 ± 0.01	b	
	4	0.19 ± 0.02	A	**	0.53 ± 0.04	c		0.24 ± 0.01	a	**	0.41 ± 0.14	b	
11	2	1.39 ± 0.05	A		2.60 ± 0.10	c		1.45 ± 0.06	a		2.35 ± 0.07	b	
	4	1.18 ± 0.09	A		2.48 ± 0.16	b		1.46 ± 0.05	a		2.46 ± 0.18	b	
12	2	2.14 ± 0.13	A		1.98 ± 0.18	a		4.03 ± 0.40	c		2.91 ± 0.14	b	
	4	1.49 ± 0.24	A	*	1.30 ± 0.09	a	**	3.00 ± 0.36	b		1.78 ± 0.08	a	**
13	2	0.10 ± 0.02	n. s.		0.07 ± 0.01			0.10 ± 0.02			0.11 ± 0.01		
	4	0.11 ± 0.02	n. s.		0.07 ± 0.01			0.11 ± 0.02			0.07 ± 0.00		**

[a]) Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation (+UV-B and −UV-B). Evaluation of phenylpropanoid compounds (Chlorogenic acid isomer 1 (1), Chlorogenic acid isomer 2 (2), 3-O-Caffeoylquinic acid (3), Q-dihex (4), Q-x680 (5), Q-hexpen (6), Q-hexdeshex (7), Q-hex (8), Q-x878 (9), Q-deshex (10), Q-ma-hex (11), K-x1172 (12), K-x1186 (13)) was done after 2 and 4 weeks of treatment application (WTA). Mean ± standard error (n = 6). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, $p \leq 0.05$); significant differences between WTA are indicated by asterisks (Student's *t*-test, * = $p \leq 0.05$, ** = $p \leq 0.01$).

Table S2. Effect of PAR and UV-B level on the concentration of chlorogenic acid isomers, quercetin, and kaempferol derivatives in *H. leucocephala* leaves which emerged during the experiment (LT 2).
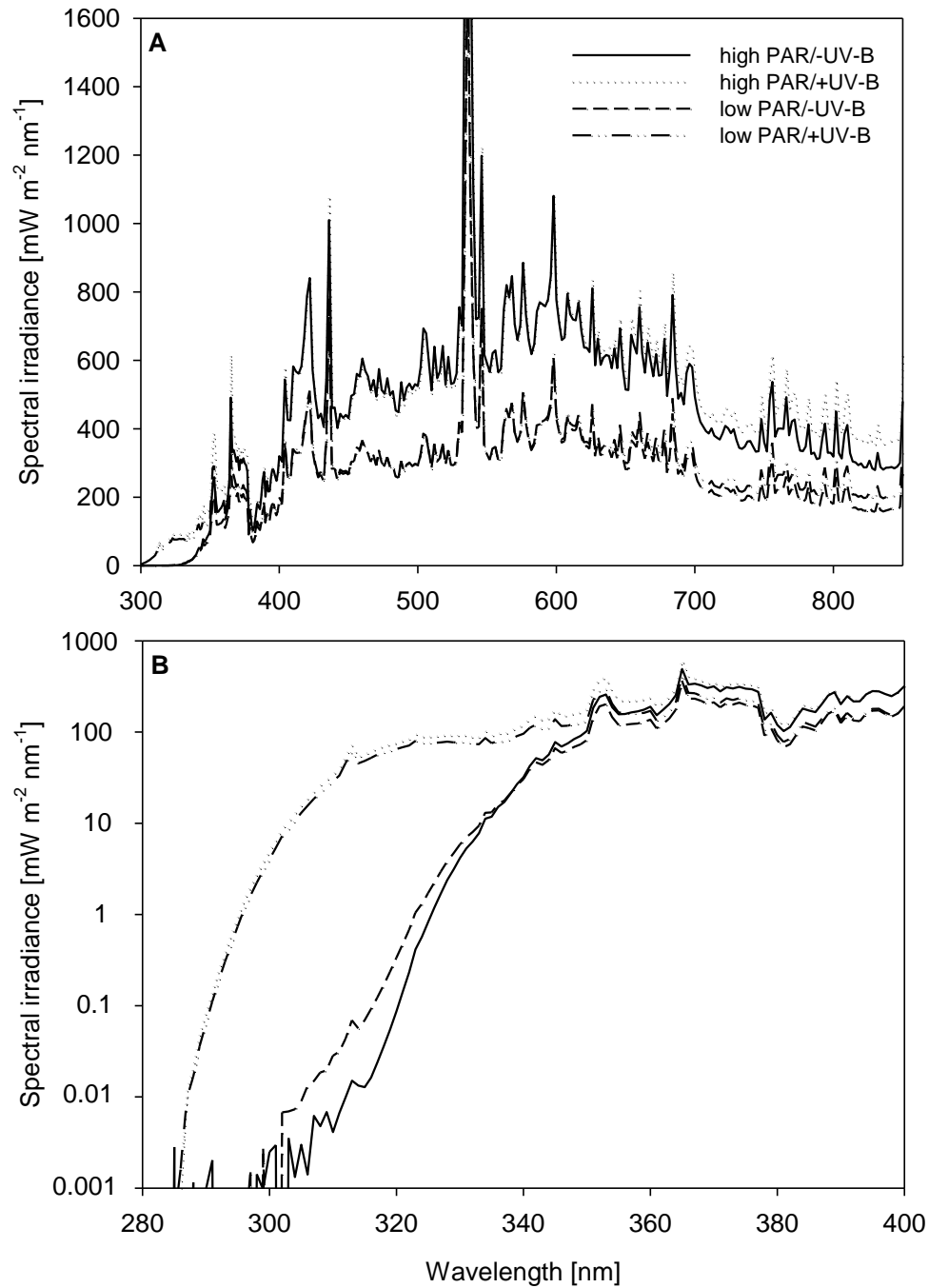
Comp. [a] [mg g^{-1}]	WTA	high PAR −UV-B			high PAR +UV-B			low PAR −UV-B			low PAR +UV-B		
1	2	1.19 ± 0.07	b		1.09 ± 0.08	ab		0.93 ± 0.04	a		0.96 ± 0.02	a	
	4	2.57 ± 0.12	b	**	2.52 ± 0.08	b	**	2.25 ± 0.06	a	**	2.05 ± 0.05	a	**
2	2	8.56 ± 0.36	n. s.		9.33 ± 0.60			8.12 ± 0.43			9.16 ± 0.44		
	4	9.26 ± 0.56	n. s.		8.98 ± 0.30			9.84 ± 0.30		**	9.40 ± 0.36		
3	2	56.01 ± 2.84	n. s.		57.45 ± 5.42			61.30 ± 2.97			62.65 ± 3.55		
	4	46.12 ± 1.56	n. s.	*	42.55 ± 0.95		*	45.94 ± 1.31		**	42.82 ± 1.01		**
4	2	0.70 ± 0.12	n. s.		0.54 ± 0.16			0.39 ± 0.08			0.35 ± 0.05		
	4	0.65 ± 0.07	b		0.52 ± 0.05	b		0.38 ± 0.03	a		0.36 ± 0.03	a	
5	2	0.66 ± 0.02	a		0.89 ± 0.02	b		0.86 ± 0.03	b		0.89 ± 0.06	b	
	4	0.49 ± 0.06	n. s.	*	0.51 ± 0.05		**	0.65 ± 0.07		*	0.63 ± 0.04		**
6	2	6.78 ± 0.40	a		8.82 ± 0.47	b		8.39 ± 0.34	b		8.84 ± 0.45	b	
	4	5.45 ± 0.52	n. s.		5.22 ± 0.47		**	6.91 ± 0.66			6.40 ± 0.51		**
7	2	0.48 ± 0.03	n. s.		0.55 ± 0.03			0.51 ± 0.02			0.53 ± 0.04		
	4	0.44 ± 0.04	n. s.		0.42 ± 0.01		**	0.44 ± 0.02			0.42 ± 0.02		*
8	2	5.00 ± 0.48	a		7.30 ± 0.49	b		6.82 ± 0.26	b		7.42 ± 0.57	b	
	4	3.17 ± 0.42	n. s.	*	3.01 ± 0.40		**	4.37 ± 0.60		**	4.07 ± 0.54		**
9	2	3.92 ± 0.23	a		5.39 ± 0.23	b		4.82 ± 0.14	b		5.14 ± 0.32	b	
	4	3.03 ± 0.02	n. s.	*	3.21 ± 0.28		**	3.96 ± 0.33		*	3.86 ± 0.25		*
10	2	1.34 ± 0.08	a		1.73 ± 0.15	b		1.42 ± 0.04	ab		1.62 ± 0.08	b	
	4	0.77 ± 0.13	n. s.		0.84 ± 0.07		**	0.80 ± 0.09		**	0.96 ± 0.11		**
11	2	2.45 ± 0.06	a		3.13 ± 0.09	c		2.68 ± 0.03	b		2.94 ± 0.07	c	
	4	2.26 ± 0.18	a		2.76 ± 0.15	b	*	2.30 ± 0.12	a		2.79 ± 0.12	b	
12	2	12.67 ± 0.30	a		15.04 ± 0.62	a		19.73 ± 1.00	b		20.65 ± 1.16	b	
	4	9.61 ± 1.12	a	*	9.06 ± 1.02	a	**	14.20 ± 1.38	b	**	11.44 ± 0.56	ab	**
13	2	5.98 ± 0.21	a		6.80 ± 0.24	b		7.32 ± 0.19	bc		7.74 ± 0.34	c	
	4	3.59 ± 0.47	n. s.	**	3.35 ± 0.25		**	4.25 ± 0.37		**	3.46 ± 0.17		**

[a]) Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation (+UV-B and −UV-B). Evaluation of phenylpropanoid compounds (Chlorogenic acid isomer 1 (1), Chlorogenic acid isomer 2 (2), 3-O-Caffeoylquinic acid (3), Q-dihex (4), Q-x680 (5), Q-hexpen (6), Q-hexdeshex (7), Q-hex (8), Q-x878 (9), Q-deshex (10), Q-ma-hex (11), K-x1172 (12), K-x1186 (13)) was done after 2 and 4 weeks of treatment application (WTA). Mean ± standard error ($n = 6$). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, p ≤ 0.05); significant differences between WTA are indicated by asterisks (Student's *t*-test, * = p ≤ 0.05, ** = p ≤ 0.01).

Table S3. Effect of PAR and UV-B level on the concentration of chlorogenic acid isomers, quercetin, and kaempferol derivatives in *H. leucocephala* stems.

Comp.[a] [mg g⁻¹]	WTA	high PAR −UV-B	high PAR +UV-B		low PAR −UV-B	low PAR +UV-B
1	2	0.41 ± 0.03 n. s.	0.45 ± 0.01		0.47 ± 0.02	0.46 ± 0.02
	4	0.44 ± 0.01 a	0.44 ± 0.01 a		0.50 ± 0.02 b	0.48 ± 0.02 ab
2	2	0.21 ± 0.01 n. s.	0.21 ± 0.01		0.21 ± 0.01	0.20 ± 0.01
	4	0.22 ± 0.01 ab	0.24 ± 0.01 b	*	0.21 ± 0.00 a	0.21 ± 0.01 a
3	2	8.88 ± 0.65 n. s.	8.63 ± 0.65		9.63 ± 0.44	8.92 ± 0.72
	4	9.20 ± 0.47 n. s.	9.38 ± 0.31		9.14 ± 0.21	9.37 ± 0.26
4	2	0	0		0	0
	4	0	0		0	0
5	2	0 a	0.03 ± 0.01 ab		0 a	0.02 ± 0.01 a
	4	0 a	0.04 ± 0.01 b		0 a	0.01 ± 0.01 a
6	2	2.08 ± 0.16 n. s.	2.47 ± 0.15		2.00 ± 0.10	2.10 ± 0.15
	4	1.90 ± 0.07 a	2.24 ± 0.06 b		2.20 ± 0.09 b	2.22 ± 0.08 b
7	2	0	0		0	0
	4	0	0		0	0
8	2	0.67 ± 0.04 n. s.	0.82 ± 0.04		0.65 ± 0.03	0.74 ± 0.05
	4	0.60 ± 0.03 a	0.73 ± 0.04 b		0.63 ± 0.03 a	0.67 ± 0.03 ab
9	2	0.11 ± 0.02 a	0.25 ± 0.02 c		0.10 ± 0.01 a	0.18 ± 0.02 b
	4	0.11 ± 0.01 a	0.26 ± 0.03 c		0.09 ± 0.01 a	0.19 ± 0.01 b
10	2	0.34 ± 0.01 a	0.47 ± 0.04 b		0.35 ± 0.02 a	0.37 ± 0.02 a
	4	0.29 ± 0.04 n. s.	0.36 ± 0.05		0.30 ± 0.03	0.25 ± 0.05
11	2	0.93 ± 0.14 n. s.	0.60 ± 0.24		0.69 ± 0.19	0.67 ± 0.31
	4	1.17 ± 0.15 n. s.	1.58 ± 0.10	**	1.11 ± 0.25	1.23 ± 0.13
12	2	29.03 ± 3.84 a	36.65 ± 1.82 b		39.56 ± 1.34 b	39.42 ± 1.99 b
	4	32.96 ± 1.83 a	33.16 ± 1.23 a		39.92 ± 1.84 b	39.61 ± 1.23 b
13	2	4.01 ± 0.55 n. s.	4.99 ± 0.27		4.65 ± 0.14	4.63 ± 0.27
	4	4.86 ± 0.28 n. s.	5.24 ± 0.23		4.86 ± 0.18	5.08 ± 0.22

[a]) Plants were exposed to two PAR levels (high and low) including or excluding UV-B radiation (+UV-B and −UV-B). Evaluation of phenylpropanoid compounds (Chlorogenic acid isomer 1 (1), Chlorogenic acid isomer 2 (2), 3-O-Caffeoylquinic acid (3), Q-dihex (4), Q-x680 (5), Q-hexpen (6), Q-hexdeshex (7), Q-hex (8), Q-x878 (9), Q-deshex (10), Q-ma-hex (11), K-x1172 (12), K-x1186 (13)) was done after 2 and 4 weeks of treatment application (WTA). Mean ± standard error (n = 6). Significant differences among the treatments are indicated by different letters (Duncan's multiple range test, $p \leq 0.05$); significant differences between WTA are indicated by asterisks (Student's *t*-test, * = $p \leq 0.05$, ** = $p \leq 0.01$).

Fig. S1. Simulated irradiance spectra of the four light regimes on a linear scale from 300 to 850 nm (A) and on a logarithmic scale showing the UV range from 280 to 400 nm (B).
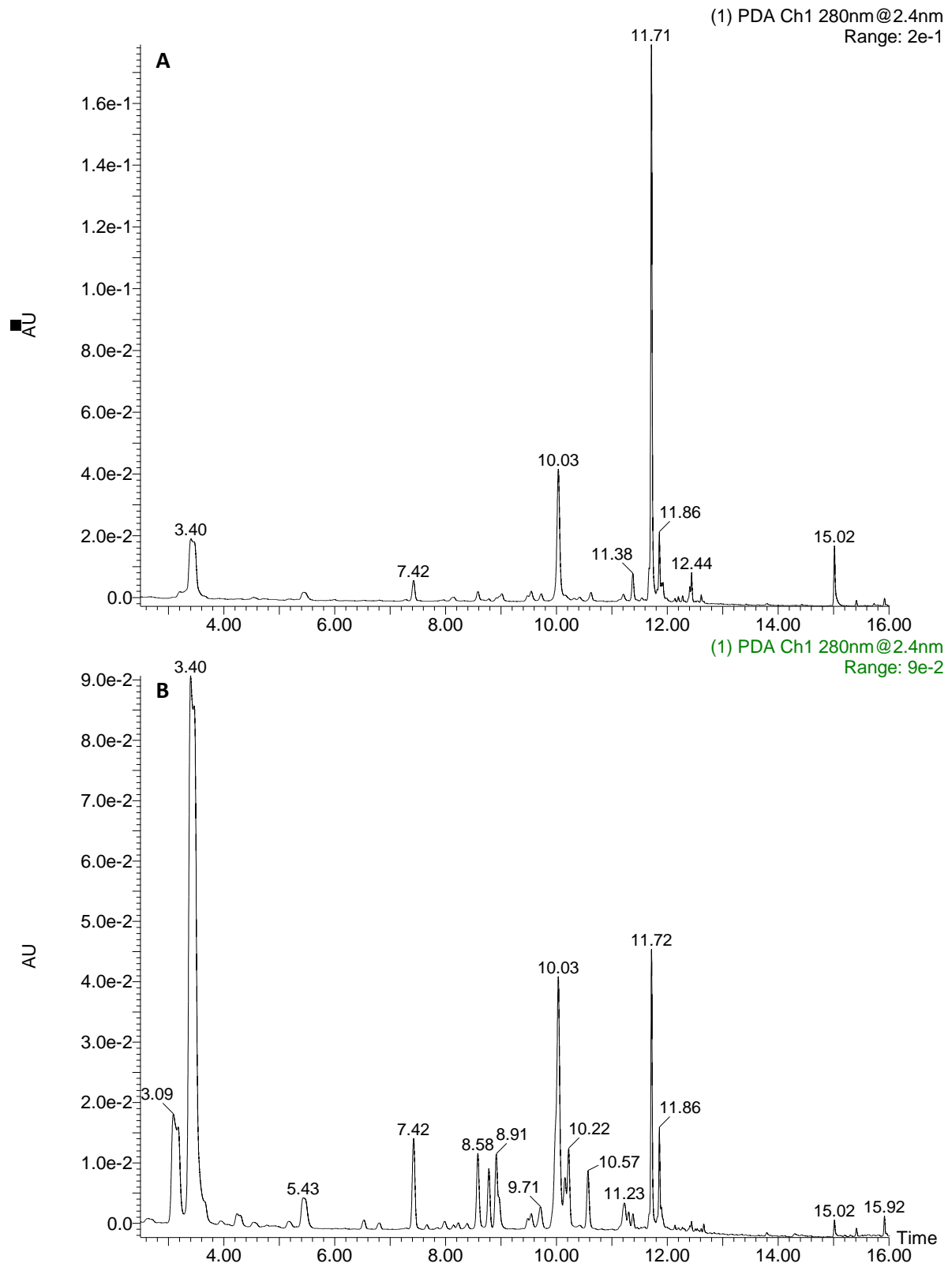
Fig. S2. UV chromatograms of a stem (A) and a leaf (B) extract of the *H. leucocephala* plants.

5   References

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F   Summary and conclusion

The key objective of the present work was to acquire fundamental knowledge on the impact of nutrient supply and light quality on the accumulation of pharmaceutically relevant secondary metabolites, particularly saponins and lignans, using *Centella asiatica* and *Hydrocotyle leucocephala* as examples. Experiments on the impact of N, P, and K supply on saponin and sapogenin (centelloside) accumulation in leaves of *C. asiatica* were conducted in the greenhouse using soilless culture. Thereby, the relationship among net photosynthesis, leaf N, P, and K concentrations, herb and leaf production, and centelloside accumulation as influenced by nutrient supply was investigated. Furthermore, the suitability of fluorescence-based indices for non-destructive tracking of centelloside accumulation *in vivo*, using the epidermal flavonols and anthocyanins as reference, was examined. For this purpose, different levels of N, P, and K supply were selected as experimental factors. In order to investigate the effects of light quality on saponin and lignan accumulation, experiments were conducted in technically complex sun simulators providing almost natural irradiance. Here, we postulated that high intensity of photosynthetic active radiation (PAR) and ambient level of ultraviolet (UV)-B radiation additively promote the accumulation of centellosides in leaves of *C. asiatica*. The specific UV-B response in terms of flavonoid accumulation was monitored *in vivo* by fluorescence recordings. Finally, the impact of different PAR/UV-B combinations on the concentration and distribution pattern of selected phenylpropanoids, *i.e.*, phenolic acids, flavonols, and in particular the lignan hinokinin, was examined in leaves and stems of *H. leucocephala*. The results ascertained in the single chapters can be summarized as follows:

1. The higher levels of N, P, or K supply (in the range from 0 to 150% of the amount in a standard Hoagland solution) enhanced net photosynthesis and herb and leaf yield of *C. asiatica*. However, exceeding nutrient-specific thresholds, the high availability of one single nutrient caused lower leaf N concentrations and a decline in net photosynthesis and plant growth. Irrespective of N, P, and K supply, the leaf centelloside concentrations were negatively associated with herb and leaf yield. Moreover, strong negative correlations were found between saponins and leaf N concentrations, while the respective sapogenins were negatively correlated with leaf K concentrations.

2. The accumulation of both flavonoids and anthocyanins was affected by N, P, and K fertigation in the same way as the centelloside accumulation, indicating that limitations in plant growth were generally accompanied by higher secondary metabolite concentrations.

Correspondingly, a close relationship was observed between the centellosides and the flavonoids and anthocyanins. Beyond, the fluorescence-based FLAV (flavonol) and ANTH_RG (anthocyanin) indices correlated fairly with flavonoid and particularly with anthocyanin concentrations. Moreover, the 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, the indices FLAV, ANTH_RG, as well as BFRR_UV enabled the *in situ* monitoring of flavonoid and centelloside concentrations in leaves of *C. asiatica*.

3. UV-B radiation favored herb and leaf production of *C. asiatica* as well as the content of epidermal flavonols, which was monitored *in vivo* by measurements of the fluorescence-based FLAV index. Accordingly, recordings of the ANTH_RG index indicate an increase in the content of anthocyanins under high PAR; this increase was likewise observed for the saponin concentrations. In contrast, UV-B radiation had no distinct effects on saponin and sapogenin concentrations. In general, younger leaves contained higher amounts of saponins, while in older leaves the sapogenins were the most abundant constituents.

4. The concentration of the selected phenylpropanoids, *i.e.*, phenolic acids, flavonols, and the lignan hinokinin, in *H. leucocephala* depended on the plant organ, the leaf age, the evaluated irradiation regimes, and the duration of exposure. By contrast, the distribution pattern of the compounds within the analyzed plant organs (leaves and stems) was not influenced by the light conditions. Based on the chemical composition of the extracts a principal component analysis enabled a clear separation of the plant organs and harvesting dates. In general, younger leaves mostly contained higher phenylpropanoid concentrations than older leaves. Nevertheless, more pronounced effects of the light regimes were detected in older leaves. As assessed, the individual compounds responded very differently to the PAR/UV-B combinations. Hinokinin was most abundant in the stems, where its accumulation was slightly enhanced under UV-B exposure.

Summarizing, the findings presented in the four chapters corroborate the feasibility to manipulate the accumulation of secondary metabolites in medicinal plants by modulation of the growing conditions. It was shown here, that nutrient and light supply influence the accumulation of centellosides in leaves of *C. asiatica*. Thus, a well-directed cultivation of this species, and analogously the domestication of other wild medicinal plants, requires a carefully

optimized and controlled fertilization as well as adequate light availability to steer the biosynthesis of pharmaceutically valuable saponins. Furthermore, our results raise a number of questions as related to the induction of the biosynthesis, location, and function of centellosides. Thus, further studies involving also molecular approaches are needed to close these information gaps and to elucidate the role of saponins in plant protection against abiotic factors, in particular against high PAR intensity. In the same line, the distinct accumulation of phenylpropanoid compounds in *H. leucocephala* as influenced by light regime, plant organ, and age of the tissue underlines the need of a more precise knowledge on the inducibility, the location, and the time of maximum concentration during plant development, as a prerequisite for targeting the enhancement of the desired compounds. Similar to the saponins, there are still a number of open questions concerning the regulation of biosynthesis and function of phenylpropanoids, especially lignans. In this scope, particular research is needed to elucidate their role and accumulation in different organs of herbaceous species. Finally, our experiments demonstrated for the first time that multiparametric fluorescence measurements enable the *in vivo* estimation of flavonoid and centelloside concentrations in *C. asiatica* leaves, substantiating the great potential of this technique for application in research and cultivation of medicinal plants. However, in view of this great potential, further studies are encouraged to proof the suitability of the technique and the identified indices for the prediction of secondary compounds beyond flavonoids and centellosides, and also in a broader range of plant species.