UV Radiation Dependent Flavonoid Accumulation of *Cistus laurifolius* L.

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Epicuticular and intracellular flavonoids of *Cistus laurifolius* grown with and without UV radiation in a phytotron as well as under natural garden conditions in the field were studied. The amount of intracellular flavonoid glycosides of leaves receiving UV-A radiation was two fold higher than that measured in the absence of UV-A radiation, whether grown in the phytotron or in the field. Exposure of previously protected leaves to UV-A radiation increased the intracellular flavonoid glycoside content to that of unprotected leaves. The qualitative composition of intracellular flavonoid glycosides showed a reduced amount of quercetin-3-galactoside to the myricetin monosides when the leaves were grown without UV-A radiation in the field and in the phytotron. Epicuticular flavonoid aglycones were not influenced by UV radiation significantly.

Introduction

Light is a very important factor in the regulation of metabolism and during the differentiation processes of plants [1]. Leaf and floral pigments may contribute to the protection of plant tissues from damaging UV radiation. Due to their characteristic absorption maxima in the UV range, especially flavonoids, which have subsequently been found in all higher plants [2], are considered as an important barrier for the damaging UV radiation. Their location mainly in the epidermal vacuoles additionally favours this hypothesis. Experiments have shown that the key enzymes of flavonoid biosynthesis are induced by UV radiation, for example PAL [3], 4-coumarate-CoA-ligase [4], chalcone synthase [5] and chalcone-flavanone isomerase [3].

Plant cell cultures [6] or at least phytotron experiments, are commonly used to demonstrate the effects of UV radiation under defined conditions. Comparisons between phytotron and field experiments, under identical light conditions, are desirable to demonstrate how well the phytotron mimics natural sun light [7]. The genus *Cistus* comprises woody evergreen shrubs of the mediterranean macchies and garigues. During our study of its epicuticular pattern of flavonoid aglycones [8, 9] we recently focused our attention to seasonal and ontogenetic variations in the epicuticular and intracellular flavonoid pattern of *C. laurifolius* [10]. In this report we show that UV-A radiation is an important factor in determining the quantitative and the qualitative pattern of the intracellular flavonoid glycosides in *C. laurifolius*.

Abbreviations: kae, kaempferol; que, quercetin; myr, myricetin; glu, glucose; gal, galactose; ara, arabinose; rham, rhamnose; CC, column chromatography; UV, ultraviolet; RP, reversed phase; HPLC, high performance liquid chromatography; LPLC, low pressure liquid chromatography.

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Materials and Methods

C. laurifolius L. plants were grown from seeds collected at their natural habitats and cultivated in the experimental fields of the Botanical Institute of the University of Cologne. UV radiation was excluded from leaves and twigs of the analyzed plants by a box of plexiglass (total UV absorption below 380 nm). The open bottom of the box was covered with black gauze to insure adequate air circulation and preventing the leaves and twigs inside the box from higher temperatures than the control branches, while reducing the reflectance and scattering of UV radiation as much as possible.

Shoots from seeds of cultivated plants were grown in a phytotron under the following conditions: 16 h light/8 h dark; white light (irradiance 2 mWatt/cm^2; temperature: day 25° +/− 1 °C, night 23° +/− 1 °C; humidity 65 +/− 5%. Additional UV radiation: Philips TL/09 R-UV-A, emission maximum at 350 nm. Shoots were directly exposed to a mixture of white light/UV-A radiation lamps at a distance of 50 cm without any filter.

Total light intensity was about 1.65 mWatt/cm^2; white light irradiance 1.5 mWatt/cm^2; UV radiation 0.15 mWatt/cm^2). Irradiance of natural day light was estimated up to 80 mWatt/cm^2; with the plexiglass filter up to 70 mWatt/cm^2. Data were measured with the Li-Cor-185A Quantum/Radiometer.

In the phytotron experiments elliptical tertiary leaves of the plantlets of the same size (length 2.5–3.1 cm, width 1.1–1.3 cm) were harvested 40 days after germination.

Separation of intracellular and epicuticular flavonoids was achieved by washing the leaves with chloroform three times to remove the epicuticular resin-flavonoid mixture. From this mixture the waxes were removed by precipitation in ice cold MeOH at −20 °C for 12 h. For preparative isolation of individual flavonoids the supernatant was concentrated and individual compounds separated by CC on polyamide SC-6 followed by RP-8/18 LPLC. Final purification was achieved by CC on Sephadex LH-20. Individual compounds were identified by TLC on cellulose and polyamide, UV spectroscopy and by comparison with authentic samples (9, 10). For analytical purposes the chloroform extract, after precipitation of the waxes, was dried under N_2, dissolved in 1 ml MeOH/10 mg resin and analyzed by HPLC according to [11].

The washed leaves were ground in liquid nitrogen and extracted with 80% methanol for analysis of the intracellular flavonoids. Preparative isolation of the intracellular flavonoids was achieved by CC on polyamide SC-6, followed by RP-8/18 LPLC and finally purified by CC on Sephadex LH-20. Analysis of the sugar moieties was performed by acid hydrolysis and identification of individual sugars by TLC on cellulose and gas chromatography of the trimethylsilyl derivatives. Aglycones were detected by TLC on polyamide DC-6. The structures of myr-3-galactoside, myr-3-arabinoside, myr-3-rhamnosido-glucoside and que-3-arabino-glucoside were verified by FAB-MS as well as NMR analysis.

For quantitative analysis by HPLC the flavonoid glycosides were dissolved in water/methanol/acetonitrile/tetrahydrofuran (65:10:5:25) prior to injection. Isolated crystallized flavonoids were used as external and in some cases internal standards.

HPLC analysis was performed on a Spectra Physics liquid chromatograph (Santa Barbara, Calif. U.S.A.); equipped with a Shimadzu SPD-2A UV detector; Shimadzu C-R6 A integrator and Hypersil MOS-RP-C-8 column (125 mm × 4.6 mm, particle size 5 µm). Isocratic solvent system I for flavonoid glycosides: water (0.5% phosphoric acid)/methanol/acetonitrile/tetrahydrofuran (81:6.5:5.5:7), flow rate 1.2 ml/min; detection at 350 nm. Isocratic solvent system II for the epicuticular flavonoids: water (0.5% phosphoric acid)/methanol/acetonitrile/tetrahydrofuran (68:6:6:20). Detailed analytical and preparative chromatography and purification of the flavonoids is described in detail recently [11].

Results

The epicuticular flavonoid aglycones and intracellular flavonoid glycosides of *C. laurifolius* identified in this study are listed in Tables I and II, respectively. Epicuticular flavonoids consist mainly of flavonols, and especially a variety of different quercetin methylethers. The intracellular glycosides are comprised of myricetin-, quercetin- and to a minor degree kaempferol-O-mono- and O-biosides.

Each year the evergreen *C. laurifolius* looses the leaves of the previous season, while new leaves develop out of winterbuds in spring. Buds of *C. laurifolius*, that had been covered with a plexiglass box at the beginning of the vegetation period in June, showed a marked decrease in intracellular flavon-

Table I. Identified flavonoid glycosides of *C. laurifolius* and their relative retention times (rrt), HPLC on Hypersil RP-C-8 (125 mm × 4.6 mm, particle size 5 µm, solvent system I, flow rate 1.2 ml/min).

Flavonol glycoside	rrt
Kae-3-glc	2.18
Kae-3-rham-glc	1.80
Que-3-ara	2.04
Que-3-gal	1.50
Que-3-rham	2.67
Que-3-rham-glc	0.85
Que-3-glc-ara	1.21
Myr-3-ara	1.31
Myr-3-gal	1.00
Myr-3-rham	1.65
Myr-3-glc-rham	0.85

Table II. Identified flavonoid aglycones of *C. laurifolius* and their relative retention times (rrt), HPLC on Hypersil RP-C-8 (125 mm × 4.6 mm, particle size 5 µm, solvent system II, flow rate 1.2 ml/min).

Flavonol aglycone	rrt	Flavon/flavanone aglycone	rrt
Kae-3-OMe	1.50	Apigenin	1.22
Kae-7-OMe	3.42	Ap-7-OMe	2.20
Kae-4′-OMe	3.42	Ap-7,4′-diOMe	3.13
Kae-3,7-diOMe	2.65	Luteolin	0.89
Kae-3,4′-diOMe	2.65	Lut-7-OMe	1.58
Quercetin	1.17	Lut-3′-OMe	1.03
Que-3-OMe	1.00	Lut-7,3′-diOMe	1.78
Que-7-OMe	1.92	Naringenin	1.30
Que-3′-OMe	1.44	Nar-7-OMe	2.69
Que-3,7-diOMe	1.74	Pinocembrin	2.50
Que-3,3′-diOMe	1.19		
Que-3,4′-diOMe	1.07		
Que-5,3′-diOMe	0.43		
Que-7,3′-diOMe	2.56		
Que-3,5,3′-triOMe	0.31		
Que-3,7,3′-triOMe	2.06		
Que-3,7,3′,4′-tetraOMe	1.92		

oid content in the first completely unfolded leaves compared to leaves exposed to sun light (Table III A). The flavonoid glycosides obtained from leaves of plants grown in the phytotron yielded similar results. The average of four samples (each value representing ten unfolded leaves of the same size) is shown in Table III B. The leaves excluded from UV radiation in both cases show significantly lower amounts of flavonoid glycosides (about 50%) than the sun light exposed leaves. Removal of the plexiglass-box at the end of June of several leaves from plants grown in the field, resulted in an increase in intracellular flavonoid content of these leaves within 21 days to the same level as leaves continuously exposed to natural sun light (14.4 +/− 1.4 nmol/mg dry weight). The same results were obtained by exposion of the phytotron plants with UV-A radiation. Although the amount of total radiation (Watt) in the phytotron is much less than field levels, the intracellular flavonoid amount whether taken from plants in the field or in the phytotron is comparable. This indicates a dependency of intracellular flavonoid accumulation on light-quality rather than light-quantity.

Table III. Intracellular flavonoid glycosides of leaves of *C. laurifolius* in field (A) and phytotron (B) studies.

Date	Samples	Without UV radiation (nmol/mg dry weight)	Control exposed to sun light (nmol/mg dry weight)
A. Field studies			
8.6	3	6.2 +/− 0.3	14.2 +/− 2.5
29.6	3	9.4 +/− 4.1	17.3 +/− 2.8
20.7	3	7.2 +/− 2.9	15.6 +/− 3.7
B. Phytotron studies			
	Samples	White light only (nmol/mg dry weight)	With additional UV-A radiation (nmol/mg dry weight)
	4	8.4 +/− 1.0	15.8 +/− 1.1

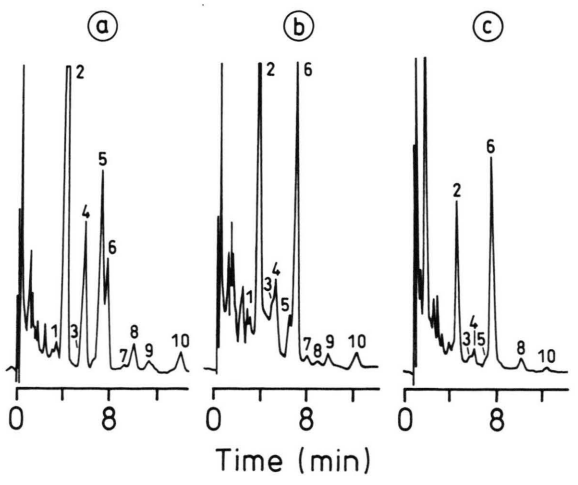
The influence of UV radiation on the composition of intracellular glycosides is illustrated in Fig. 1. The pattern of Fig. 1 b (field studies, UV radiation excluded) and 1 c (phytotron, UV radiation excluded) indicate that the overall dominant compound myr-3-gal (compound **2**) is accompanied by myr-3-rham (compound **6**) in both cases. In the phytotron myr-3-rham (compound **6**) is the dominating flavonoid. UV irradiated leaves accumulate que-3-gal (compound **5**) to about 14% of the total intracellular flavonoids (Fig. 1 a). In leaves without UV radiation this compound only reaches 4% (Fig. 1 b) and 1% (Fig. 1 c) respectively.

In contrast to the changes observed in flavonoid glycoside pattern the amount and the composition of epicuticular flavonoid aglycones were not influenced significantly by the UV radiation. Approximately 50 mg of epicuticular flavonoids per mg resin were obtained in all cases and the amount of resin per leaf or per mg dry weight respectively did not change significantly. Analysis of the flavonoid composition revealed lower values for the main compound que-3-methylether and a slight increase of que-3,4′-O-methylether, when the leaves were excluded from UV radiation (phytotron and field).

Discussion

Plant action spectra in response to UV radiation show a maximum at about 290–310 nm [12, 13], which is per definition within the UV-B range. Excluding the extremely damaging UV-C and short wave UV-B radiation with special filters, experimentors usually work within this UV-B region. So most if not all reported data of plant responses to UV radiation focuse on wavelenghts below 320 nm, like morphological effects [14], influence on growth and photosynthesis [15] or general biosynthetic pathways [16]. Effects of UV radiation on plant pigments are considered as non-damaging. Reports exist for the stimulating effect of UV-B radiation on either the synthesis of anthocyanins [17] or the increase of flavonoids in plant tissues. A linear relation of flavonoid content to increased UV-B radiation has been demonstrated [18]. This effect may be due to a demonstrated higher activity of the enzymes involved in flavonoid biosynthesis [19]. Detailed analysis of the key enzyme chalcone synthase gene in *Petroselinum hortense* revealed a UV-B radiation responsive sequence within its upstream promotor [20]. On the other hand effects of UV-A radiation on plant flavonoid content was only demonstrated synergistic to UV-B radiation, both applied at very high fluence rates increasing the anthocyanin content in *Sorghum vulgare* [21]. Effects of visible light on either flavonoid amount or composition are not documented.

More than 90% of the emitted ultraviolet emission of the sun reaching the ground is in the UV-A region (315–350 nm), whereas less than 10% is made up of UV-B radiation (280–315 nm) [22]. Therefore it seems unlikely that the reported high dosis of UV-B radiation actually reach the cell plasma or the vacuoles of plants, especially those



Flavonoid glycoside

1. Que-3-glc-ara
   Myr-3-glc-rham
2. Myr-3-gal
3. Que-3-glc-rham
4. Myr-3-ara
5. Que-3-gal
6. Myr-3-rham
7. Kae-3-rham-glc
8. Que-3-ara
9. Kae-3-glc
10. Que-3-rham

Fig. 1. Influence of UV-A radiation on the composition of flavonoid glycosides in leaves of *C. laurifolius*. (Conditions: HPLC on Hypersil MOS RP-C-8, 125 mm × 4.6 mm, 5 µm particle size, flow rate 1.2 ml/min, solvent system I, detection at 350 nm). a, Field studies with sun light; b, field studies, without UV radiation; c, phytotron studies, without UV radiation.

covered with a thick epicuticular wax-resin mixture [23] like *C. laurifolius*. Highly diluted crude resin extracts of *C. laurifolius*, dissolved in methanol (10 µg per ml methanol) show a complete UV-Cut-off below 320 nm, after separation of wax and flavonoids.

Our results demonstrate that even without the UV-B part of the sun spectrum UV acclimation of leaves of *C. laurifolius* correlates with an increase in the intracellular flavonoid content of about two fold over the levels measured in leaves, which are excluded from natural UV radiation. This indicates that wavelengths above 320 nm in the UV-A band of the sun UV spectra are mainly responsible for accumulation of intracellular flavonols in *C. laurifolius* under natural light conditions. The UV radiation we used in the phytotron consists of 99.3% of UV-A, which closely mimics the natural sun light conditions measured by different authors [24]. Our results show that the described relation of UV-B to UV-A radiation, even when applied at much lower levels than that found in natural light, induced an increase in the intracellular flavonoid content in *C. laurifolius* leaves up to the same level (15 nmol/mg dry wt.) measured in the leaves exposed to natural sun light. This level was also attained in leaves exposed to natural sun light after they had developed for several weeks in the absence of UV radiation. In *C. laurifolius* in the case of UV-A radiation light quality rather than light quantity determines the actual amount of flavonoids. Effects of UV-A radiation on individual flavonoids in *C. laurifolius* is also obvious. In the absence of UV radiation less que-3-gal and more myricetin glycosides accumulate than in UV irradiated leaves. Since the data indicate that the total biosynthesis of myr-3-rham is not influenced by UV-A radiation, a higher amount of this compound accumulates in relation to other intracellular flavonoids. Consequently it is the main flavonoid in UV radiation protected leaves in addition to myr-3-gal. Differences of intracellular flavonoid composition between phytotron and field experiments like reported for *Avena sativa* [25], may be due to the absence of UV-A radiation in the phytotron. How UV-A radiation may stimulate flavonoid synthesis remains unclear. Phytochrome plays a central role in UV-B induced pigment formation although it is not essentially in all cases [26]. Since in opposite to UV-B data we did not find any quantity effect of UV-A radiation, the mechanism of its action may be different too. A possible involvement of cryptochrome in UV-A mediated responses is discussed [27]. An interesting alternative has been demonstrated recently by Takahama, who showed a stimulation of flavonoid synthesis in *Vicia faba* by exogenously applying µM amounts of H_2O_2 [28]. The data suggest a direct involvement of increased levels H_2O_2 in the oxidation of flavonols to flavonols. If UV-A radiation enhances radical formation [29], the radical triggered stimulation of flavonoid synthesis may act very rapidly. Since flavonoids are potent quenchers of toxic radicals [30], increasing their intracellular levels after UV-A induced radical formation may provide a powerful tool for the plant to protect from effects of any damaging UV radiation.

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[1] H. Mohr and P. Schopfer, Lehrbuch der Pflanzenphysiologie, 3. Aufl., Springer, Berlin 1978.
[2] J. B. Harborne, The Flavonoids – Advances in Research, Academic Press, New York 1980.
[3] J. Chapell and K. Hahlbrock, Nature **311,** 76 (1984).
[4] C. Douglas, H. Hoffmann, W. Schulz, and K. Hahlbrock, EMBO **6,** 1189 (1987).
[5] A. J. van Tunen, R. E. Koes, C. E. Spelt, A. R. van der Krol, A. R. Stuitje, and J. M. N. Mol, EMBO **7,** 1257 (1988).
[6] B. Moehle, W. Heller, and E. Wellmann, Phytochemistry **24,** 465 (1985).
[7] M. M. Caldwell, R. Robberecht, and S. D. Flant, Physiol. Plant **58,** 445 (1983).
[8] T. Vogt, P. Proksch, P.-G. Gülz, and E. Wollenweber, Phytochemistry **26,** 1027 (1987).
[9] T. Vogt, P. Proksch, and P.-G. Gülz, J. Plant Physiol. **131,** 25 (1987).
[10] T. Vogt, P.-G. Gülz, and V. Wray, Phytochemistry **27,** 3712 (1988).
[11] T. Vogt and P.-G. Gülz, J. Chromatogr. **537,** 453 (1991).
[12] M. M. Caldwell, Plant response to solar ultraviolet radiation, in: Encyclopedia of Plant Physiology (O. L. Lange, P. S. Nobel, C. B. Osmond, and H. Ziegler, eds.), Vol. **12A,** p. 170, Springer, Berlin, Heidelberg, New York 1981.
[13] M. M. Caldwell, Photophysiology **VIII.,** 131 (1971).
[14] A. H. Teramura, Physiol. Plant. **58,** 415 (1983).
[15] M. Tevini and A. Teramura, Photochem. Photobiol. **50,** 479 (1989).
[16] R. M. Klein, Bot. Rev. **44,** 1 (1978).
[17] S. J. Lindoo and M. M. Caldwell, Plant. Physiol. **61,** 287 (1978).
[18] E. Wellmann, Ber. Dtsch. Bot. Ges. **98,** 99 (1985).
[19] K. Hahlbrock, K. H. Knobloch, F. Kreuzaler, J. R. M. Potts, and E. Wellmann, Eur. J. Biochem. **61,** 199 (1976).
[20] P. Schulze-Lefert, M. Becker-Andre, K. Hahlbrock, and W. Schulz, EMBO J. **8,** 651 (1989).
[21] H. Drumm-Herrel and H. Mohr, Photochem. Photobiol. **33,** 391 (1981).
[22] E. Wellmann, Planta **101,** 283 (1971).
[23] R. Robberecht and M. M. Caldwell, Oecologia **32,** 277 (1978).
[24] G. Volden and K. Henriksen, Photodermatology **3,** 106 (1986).
[25] G. Popovici and G. Weissenboeck, Ber. Dtsch. Bot. Ges. **89,** 483 (1976).
[26] E. Wellmann, UV radiation in Photomorphogenesis, in: Encyclopedia of Plant Physiology, New Series: Photomorphogenesis (W. Shropshire and H. Mohr, eds.), Vol. **16B,** p. 745, Springer, Berlin 1983.
[27] N. Duell-Pfaff and E. Wellmann, Planta **156,** 213 (1982).
[28] U. Takahama, T. Egashira, and K. Wakumatsu, Plant Cell Physiol. **30,** 951 (1989).
[29] M. J. Peak and J. G. Peak, Photochem. Photobiol. **6,** 649 (1990).
[30] R. A. Larson, Phytochemistry **27,** 969 (1988).