Epicuticular Waxes and Flavonol Aglycones of the European Mistletoe, *Viscum album* L.

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Cuticular waxes of *Viscum album* ssp. *album* contain oleanolic acid as main constituent, accompanied by aliphatic compounds like alkanes, esters and primary alcohols. A number of flavonol aglycones (methyl ethers of quercetin and kaempferol) have also been identified. Seasonal changes in amount and composition of cuticular waxes and the presence of flavonol aglycones are described and the ecophysiological significance of flavonoids on the surface of the mistletoe is briefly discussed.

Introduction

Micromorphological studies on the epicuticular waxes of Viscaceae have revealed a remarkable diversity of crystalloid structures (Weber, 1981; Ditsch and Barthlott, 1997). Since chemical data on cuticular waxes (CW) of Viscaceae are almost lacking, no correlations can be made between the crystalloid structures and their chemical composition. The occurrence of appreciable quantities of oleanolic acid in leaves of *Viscum album* is long known (Steiner and Holtzem, 1955), and the same triterpenoid was recently identified as main constituent in CW of *Phoradendron juniperinum* (Wollenweber *et al.*, 1999). It might, therefore, be a substantial constituent in the CW of Viscaceae leaves in general. In our studies on cuticular waxes from leaves of *Viscum album*, a distinct yellow coloration of the wax solutions was observed which might indicate the presence of externally accumulated flavonoids. As a matter of fact, quercetin and seven of its methyl derivatives were reported for *Viscum album* (Becker and Exner, 1977, 1980). These flavonols, however, were identified from hydrolyzed methanolic extracts, their localization was thus not considered. Three flavanones, namely naringenin-7-methyl ether (sakuranetin), nar-5,7-dimethyl ether and eriodictyol-3′-methyl ether (homoeriodictyol), were also found in hydrolyzed extracts, along with quercetin-7,3′-dimethyl ether and qu-3-methyl ether (Lorch, 1993). Three chalcones and two flavanones had been reported earlier as glycosides (Fukunaga *et al.*, 1987) (One of these chalcones was found before, by Becker and Exner, 1978). Recently a flavanone glycoside and a flavonol glycoside were reported from *Viscum alniformosanae* (Chou *et al.*, 1999).

When flavonoid aglycones are encountered in extracts of aerial plant parts, a closer look often reveals that these are accumulated on the leaf and stem surfaces. They can be obtained when the plant material is rinsed with organic solvents like acetone or chloroform (Wollenweber 1994, 1996). The quercetin methyl ethers reported earlier to occur IN mistletoe leaves were likely to be, in fact, components of the epicuticular material ON mistletoe leaves. We therefore carried out a study on the qualitative composition of the cuticular wax of *Viscum album*, considering also its quantitative change during the vegetation period.

Material and Methods

Plants of *Viscum album* ssp. *album* were collected either in the Botanical Garden at Darmstadt (growing on *Crataegus prunifolia*) or in an experimental garden designed for mistletoe growing (with *Malus domestica* as host) at Stuttgart-Hohenheim. Twigs were briefly rinsed with chloroform to dissolve the epicuticular wax material. The solutions were taken to dryness, "defatted"

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(MeOH, −10°, centrifugation) and passed over Sephadex LH-20, eluted with MeOH, to separate the flavonoids from the predominant terpenoids. The flavonoids were identified by comparative TLC of Sephadex fractions with markers (cf. Wollenweber *et al.*, 2000).

Quantitative determinations of flavonoid contents in CW were made using solutions obtained from leaves rinsed for 30 sec with $CHCl_3$ and made up to a defined volume. Absorption was measured with a 500 SL Spectrophotometer (Perkin Elmer) at 350 nm in $CHCl_3$ with quercetin-3,7,3'-trimethylether as standard.

Analyses of CW by prep. TLC and GC were carried out essentially as described before (Welker and Haas, 1999). The identification of oleanolic acid as trimethylsilyl ester / trimethylsilyl ether after silylation with BSA / pyridine (1:1, v:v) was achieved by GC-comparison with the authentic compound (Roth), using a CP-Sil 8 CB capillary column (25m × 0,32 mm i.d., Chrompack) with Helium as carrier gas. Temperature was programmed from 150–340° (rate 5°/min).

Leaf areas were determined using a Mini MOP area analyzer (Kontron), data of quantitative determinations are on the basis of cuticle area.

Results and Discussion

The total yield of CW from *Viscum* leaves in the first year is in the range of 120–160 μg cm^{-2} and increases up to ca. 200 μg cm^{-2} in the second year of leaf development before leaf abscission of the host plant occurs. The by far predominating component in the CW is oleanolic acid. Other triterpenoids like betulinic acid, β-amyrin and lupeol occur only in small amounts. The CW of young leaves comprise ca. 70% of oleanolic acid and it may be present up to 80% in the second vegetation period.

The portion of aliphatic compounds is correspondingly less prominent, the relative amounts decrease from ca. 30% to 20% of CW. The aliphatics comprise alkanes and primary alcohols as main components, whereas aldehydes, free fatty acids and alkyl esters are present in much smaller amounts. The chain length distributions of the aliphatic compound classes are in the range usually found in plant waxes (Bianchi, 1995). Some peculiarities are noticeable especially in the esters, where the comparatively short-chain homologues C_{38}–C_{42} predominate. The alkanes are composed mainly of C_{29} (ca. 80% of the fraction) with little variation during leaf development. The profiles of primary alcohols, aldehydes and free fatty acids range from ca. C_{22}–C_{30} with C_{26} as main constituent.

These components of CW are always accompanied by flavonol aglycones in variable amounts. Our TLC analysis revealed the presence, in the leaf wash, of quercetin-3,7,3′-trimethyl ether, qu-3,7 and qu-3,3′-dimethyl ethers, qu-3-methyl ether, kaempferol-3,7-dimethyl ether and kae-3-methyl ether. The highest content of these compounds in the CW is found in young developing leaves and may slightly exceed 10 μg cm^{-2} in spring of the first vegetation period. The phase of intense leaf expansion causes a decrease to 2 μg cm^{-2} in summer. During autumn, enhanced secretion of the flavonol aglycones seems to take place, since the contents increases up to 8 μg cm^{-2} in November. In the second vegetation period, flavonol aglycones are found only in the range of 1–2 μg cm^{-2}, until leaf abscission of the mistletoe occurs. It should also be mentioned that, according to TLC studies, male plants exhibited considerably lesser amounts of flavonoids than female plants.

In the epicuticular material of *Viscum album*, we identified four of the seven quercetin methyl derivatives reported earlier (Becker and Exner, 1977). Rhamnazin, isorhamnetin and rhamnetin (qu-7,3′-diMe/ qu-3′-Me/ qu-7-Me) as well as quercetin proper were not detected. These authors had deemed it unlikely that "higher amounts of other flavonoids" would be present, while they suggested the presence of 5-methyl ethers without giving any reason for this assumption. In the present study we found kaempferol-3,7-dimethyl ether and kae-3-methyl ether as additional flavonols, but no hint to 5-methylated flavonoids. The different results are probably due to the analysis of mistletoe material collected from different host trees (c.f. Becker and Exner, 1980). We did not detect any chalcones or flavanones in the leaf washes, these compounds undoubtedly being glyosidic tissue constituents.

Epicuticular methylated flavonoids are known to have antifungal and antibacterial properties (Tomá-Barberán *et al.*, 1988; 1990; Proksch and Rodriguez, 1985). Very young developing *Viscum*

leaves are probably most susceptible to fungal attack and may therefore produce epicuticular flavonols in a comparatively high concentration until thickening of the cuticle is sufficient to provide additional resistance. Exner (1978) had already stated, though not determined quantitatively, that highest flavonoid content was observed at flowering-time of mistletoe in early spring. Flavonoids in the CW are further capable to reduce ultraviolet irradiation (Rhoades, 1977; Robberecht and Caldwell, 1978; Proksch and Rodriguez, 1985). This function as an ultraviolet screen might be the reason for the increase of the flavonoids in mistletoe CW at the end of the first growing season when leaf abscission of the host takes place. This, at first sight, is in agreement with the yellowish appearance of *Viscum album* plants during winter. However, alterations in chlorophyll metabolism are probably of more importance (Tuquet and Sallé, 1996). The slight quantitative variation in the generally minute amount of flavonoid aglycones in the CW is certainly not the cause of the often observed more yellow coloration of male plants as compared to females. We further observed that isolated cuticles of *Viscum* leaves also have a distinct yellow colour which cannot be removed even after prolonged extraction with solvents like $CHCl_3$, acetone or methanol. The flavonoids of the hemiparasitic mistletoe are probably involved in a number of physiological adaptations. They might thus provide promising links between phytochemistry and ecophysiology.

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